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Στους γονείς μου, Γιώργο και Χάρι
Στις θείες μου, Ρίτσα και Ιάσμη

TABLE OF CONTENTS

ΠΕΡΙΛΗΨΗ

Το εντεροενδοκρινές σύστημα συντονίζει τη φυσιολογική απάντηση του οργανισμού που ακολουθεί την λήψη τροφής. Τα εντεροενδοκρινή κύτταρα αισθάνονται/αναγνωρίζουν τα θρεπτικά συστατικά που βρίσκονται στον εντερικό αυλό και απαντούν σε αυτά τα ερεθίσματα εκκρίνοντας ορμόνες. Υπάρχουν πάνω από 15 τύποι εντεροενδοκρινών κυττάρων που εκκρίνουν μια σειρά από εντερικές ορμόνες, οι οποίες επιτελούν κυριάρχο ρόλο στο συντονισμό της πέψης του φαγητού, στον έλεγχο της όρεξης και στη ρύθμιση των επιπέδων γλυκόζης στο αίμα. Τα I-κύτταρα αποτελούν ένα τύπο εντεροενδοκρινών κυττάρων που κυρίως εντοπίζονται στο εγγύς λεπτό έντερο (δωδεκαδάκτυλο) και εκκρίνουν την ορμόνη χολοκυστοκινίνη όταν διεγείρονται από θρεπτικά συστατικά (κυρίως λίπη). Η χολοκυστοκινίνη θεωρείται ως η αρχετυπική ορμόνη που δημιουργεί αίσθημα κορεσμού μετά το φαγητό και διαμεσολαβεί ανορεξικά σήματα από το έντερο προς τον εγκέφαλο, μέσω του πνευμονογαστρικού νεύρου. Επιπλέον, η χολοκυστοκινίνη διευκολύνει την πέψη παρατείνοντας το χρόνο παραμονής της τροφής στο στόμαχο ενώ παράλληλα διεγείρει την απελευθέρωση της χολής από τη χοληδόχο κύστη και την έκκριση των παγκρεατικών ενζύμων.

Μέχρι πρόσφατα, οι προσπάθειες για το χαρακτηρισμό των εντεροενδοκρινών κυττάρων περιορίζονταν στην μελέτη κυτταρικών σειρών-μοντέλων. Η ανάπτυξη διαγονιδιακών μοντέλων ζώων με γενετικά σημασμένα εντεροενδοκρινή κύτταρα μας δίνει τη δυνατότητα μελέτης των φυσιολογικών εντεροενδοκρινών κυττάρων. Για την έρευνά μας, χρησιμοποιήσαμε ένα διαγονιδιακό μοντέλο μυός/ποντικού το οποίο εκφράζει την πράσινη φθορίζουσα πρωτείνη eGFP κάτω από τον έλεγχο του υποκινητή του γονιδίου της χολοκυστοκινίνης με σκοπό να μελετήσουμε τα I-κύτταρα.

Αρχικά, αναπτύξαμε ένα αξιόπιστο πρωτόκολλο για την απομόνωση των κυττάρων χολοκυστοκινίνης του δωδεκαδακτύλου που αποτελούν τον πληθυσμό των τυπικών I-κυττάρων. Χρησιμοποιώντας την ημι-ποσοτική μέθοδο RT-PCR, δείξαμε πως τα δωδεκαδακτυλικά I-κύτταρα περιέχουν mRNA μετάγραφα τα οποία κωδικοποιούν την έκφραση πρωτεϊνών υποδοχέων των μακράς αλύσου λιπαρών οξέων, κοντής αλύσου λιπαρών οξέων καθώς και ενδοκανναβινοειδών πεπτιδίων. Παράλληλα, αναλύσαμε το ορμονικό περιεχόμενο των δωδεκαδακτυλικών I-κυττάρων ανακαλύπτοντας ότι ένας υποπληθυσμός κυττάρων χολοκυστοκινίνης συν-εκφράζει μαζί με τη χολοκυστοκινίνη και άλλες εντερικές ορμόνες όπως η προγλουκαγόνη, το εξαρτώμενο από τη γλυκόζη ινσουλινοτροπικό πεπτίδιο, το πεπτίδιο YY, η νευροτενσίνη, η εκκριτίνη/σεκριτίνη και

παραδόξως η ορμόνη γκρελίνη- η μοναδική από τις εντερικές ορμόνες που διεγείρει την όρεξη για τη λήψη τροφής.

Τα ευρήματά μας συνηγορούν στη δυνατότητα των κυττάρων χολοκυστοκινίνης του δωδεκαδακτύλου να αναγνωρίζουν λιπαρά οξέα μακράς και κοντής αλύσου, προιοντα μεταβολισμού των λιπαρών οξέων καθώς και εντερικά ενδοκανναβινοειδή πεπτίδια. Επιπλέον, υποστηρίζουν την θεώρηση πως υπάρχει αλληλοεπικάλυψη ανάμεσα στους διάφορους τύπους των εντεροενδοκρινών κυττάρων και ότι είναι πιθανό ένας υποπληθυσμός των I-κυττάρων να εκκρίνουν χολοκυστοκινίνη σύγχρονα με άλλες εντερικές ορμόνες.

ABSTRACT

The enteroendocrine system orchestrates the physiological responses to food intake. Enteroendocrine (EEC) cells sense nutrients and secrete hormones in response to them. There are more than fifteen subtypes of EEC cells that secrete a range of gut hormones, which play a pivotal role in the co-ordination of food digestion, control of appetite and the regulation of glucose homeostasis. I-cells represent a subset of enteroendocrine cells that are mainly localized in the proximal small intestine (duodenum) and release cholecystokinin (CCK) in response to nutrients (mainly fat). CCK is the archetypal satiety hormone that transmits anorectic signals to the brain via a gut-to-brain signalling pathway, mediated by vagal afferent neurons. Additionally, CCK has a key pro-digestive function by inhibiting gastric emptying and stimulating the release of bile from the gallbladder and the secretion of pancreatic enzymes.

Until recently, the characterization of enteroendocrine cells has been restricted to cell line models. The development of transgenic animal models with genetically tagged enteroendocrine cells enabled us to study native enteroendocrine cells. We used a transgenic mouse model that express enhanced Green Fluorescence Protein (eGFP) under the control of Cck gene promoter, in order to study native I-cells.

Initially, we developed a robust protocol for the isolation of duodenal CCK-containing cells that represent the typical I-cells. By using semi-quantitative RT-PCR, we revealed that duodenal I-cells contain mRNA transcripts encoding key long chain fatty acid (LCFA), short chain fatty acid (SCFA) and endocannabinoid receptors. We also analysed the gut hormone content of duodenal I-cells and found that a subpopulation of CCK-containing cells co-express CCK with proglucagon, glucose-dependent insulinotropic peptide (GIP), Peptide YY (PYY), neurotensin, secretin and surprisingly the orexigenic hormone ghrelin. Our findings suggest that duodenal I-cells have the capacity to sense LCFA, SCFA, fatty acid lipid amides and intestinal endocannabinoid peptides. They also indicate that there is a significant overlap between I-cells and other subsets of EEC cells and that a subset of I-cells may co-release CCK with other gut hormones.

CHAPTER 1

Introduction

SYNOPSIS

A general introduction to enteroendocrine cells and I-cells specifically:
-Why is their study important?
-What are their characteristics?
-How they can sense nutrients?
-What hormones do they contain/secrete?
-What are the functions of cholecystokinin?
-Main questions-Aims of the project

1.1 General Introduction

1.1.1 The pandemic of “diabesity”

The World Health Organization (WHO) estimates that more than 700 million people worldwide are obese (having a Body Mass Index BMI>30 kg/m^2) and around 2 billion people are overweight (having a Body Mass Index BMI>25 kg/m^2). In Greece, the prevalence of obesity is 30.3% in males and 26.4% in females, whereas the percentage of overweight and obese people rises up to 77.5% for males and 63.2% for females (Dataset from WHO Global Infobase, 2010 https://apps.who.int/infobase/). Obesity is now considered as an epidemic that affects many countries and has become a major health threat.

Excess of weight predisposes to the development of a cluster of metabolic disorders that are collectively known as metabolic syndrome (Whitlock *et al*., 2009). The uncontrolled rise in prevalence of obesity during the last 4 decades has led to a rapid increase in the prevalence of Type II Diabetes Mellitus (T2DM) that is caused by non-sufficient release of insulin from endocrine pancreas and peripheral insulin resistance. The co-existence of obesity and T2DM lead to the generation of the term “diabesity” to describe the metabolic profile of obese patients that have T2DM (Astrup and Finer, 2000). These patients usually have metabolic syndrome and they are at increased risk to develop cardiovascular disease (CVD) and other co-morbidities including cancer.

It is clear that obesity is a multi-factorial disease (Speakman and O'Rahilly, 2012). The discovery of the leptin gene and leptin signalling system (Considine *et al*., 1996, Zhang *et al*., 1994) demonstrated a genetic cause of obesity and destigmatized

patients who developed severe obesity due to the abnormal regulation of body energy balance control. Genetics and genome-wide association studies (Farooqi and O'Rahilly, 2008, Frayling *et al*., 2007) have revealed the role of genetic defects and polymorphisms (genetic variation) in obesity. In addition to genetic variation, epigenetic factors such as foetal overnutrition (Symonds *et al*., 2009) or low weight in birth (Stein *et al*., 2009) may contribute to the development of obesity and diabetes in adults, respectively. In parallel, environmental factors and stress may contribute to the development of obesity. All these factors may have a role but the principal explanation of why we become obese is the positive energy balance. The “energy balance” term represents the equation between energy uptake (food intake) and energy expenditure. If energy from food intake is higher than energy spent, this positive energy balance leads to excess storage of fat and the development of obesity.

1.1.2 Regulation of food intake: The role of gut hormones

A homeostatic mechanism regulates body weight by adjusting food intake according to the energy status of the organism. This control of food intake is orchestrated by the brain that integrates peripheral signals (from the intestine, the pancreas and the adipose tissue) and responds by regulating feeding behaviour. Appetite is controlled by hormonal and neuronal signals that are transmitted from the gut to the central nervous system (CNS). This gut-to-brain signalling axis involves a range of gut hormones that signal to the brain –via neuronal or humoral pathways- in response to energy intake (feeding or fasting) and regulate food intake and energy homeostasis. These gut hormones are mainly “short-term” regulators of food intake that control termination or initiation of meal consumption.

The majority of gut hormones are anorectic peptides that are released in response to sensed nutrients and inhibit appetite. This category includes cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), Gastric Inhibitory Peptide/Glucose Insulinotropic Peptide (GIP), oxydomontulin (OXM) and Peptide Tyrosine (PYY); all of which are secreted following a meal to induce satiety. Despite the many anorectic peptides, the only orexigenic gastrointestinal hormone is ghrelin. Ghrelin is considered to promote appetite and act as a meal initiation signal. It has the opposite secretion profile of the classic anorectic peptides and its circulating levels are suppressed postprandially and increased preprandially so as to stimulate feeding.

Short-term energy intake is dependent on the presence or absence of nutrients in the gut lumen. Shortly after a meal, levels of anorectic gut hormones increase and cause activation of CNS neurons that are localized in the brainstem and the hypothalamus. These neurons form a network by having reciprocal connections between them (Murphy and Bloom, 2006, Schwartz *et al*., 2000). The anorectic function of gut peptides is mainly mediated via binding to the gut hormone receptors localized in the vagal afferent neurons (neuronal pathway). Vagal-afferent mediated signals influence the activation of neurons in the nucleus tractus solitarius (NTS) and area postrema (AP) of the brainstem. Additionally, circulating gut hormones in plasma are considered to activate neurons directly in the AP of the brainstem and the median eminence (ME) of the hypothalamus; both areas having incomplete blood-brain-barrier (BBB) (Ermisch *et al*., 1992). In the arcuate nucleus (ARC) of the hypothalamus, there are two populations of neurons that are crucial for the regulation of food intake. One group of neurons co-express agouti-related peptide (AgRP) and neuropeptide Y (NPY) and are orexigenic whereas the other group of neurons that co-express pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) suppresses appetite. Activation of neurons by anorexigenic hormones indirectly leads

to the activation of POMC/CART neurons that are projecting to paraventricular nucleus (PVN) to inhibit appetite. In parallel, the modest rise of circulating leptin levels contributes to the suppression of food intake. Leptin is derived from adipocytes and transmit signals from long-term energy storage compartments to the brain. It activates POMC/CART neurons and inhibits AgRP/NPY neurons to suppress food intake and increase energy expenditure (Pelleymounter *et al*., 1995, Schwartz *et al*., 2000). This is a “long-term” effect implying that leptin signalling does not contribute to satiety, following the consumption of a meal.

Induction of satiety is not the only function of anorexigenic gut hormones. They also control digestion by regulating the flow rate of nutrients to the intestine, the availability of digestive enzymes and the gastrointestinal motility. Importantly, they also stimulate insulin release from endocrine pancreas to control glucose homeostasis, utilize nutrients/energy and modulate “long-term” regulation of energy balance.

During fasting, the levels of circulating CCK, GLP-1 and PYY are low, whereas the concentration of circulating ghrelin is high. Ghrelin activates neurons in the NTS and AP of the brainstem (Lawrence *et al*., 2002) and the orexigenic AgRP/NPY hypothalamic neurons (Chen *et al*., 1993, Wang *et al*., 2002) to stimulate food intake (Figure 1.1).

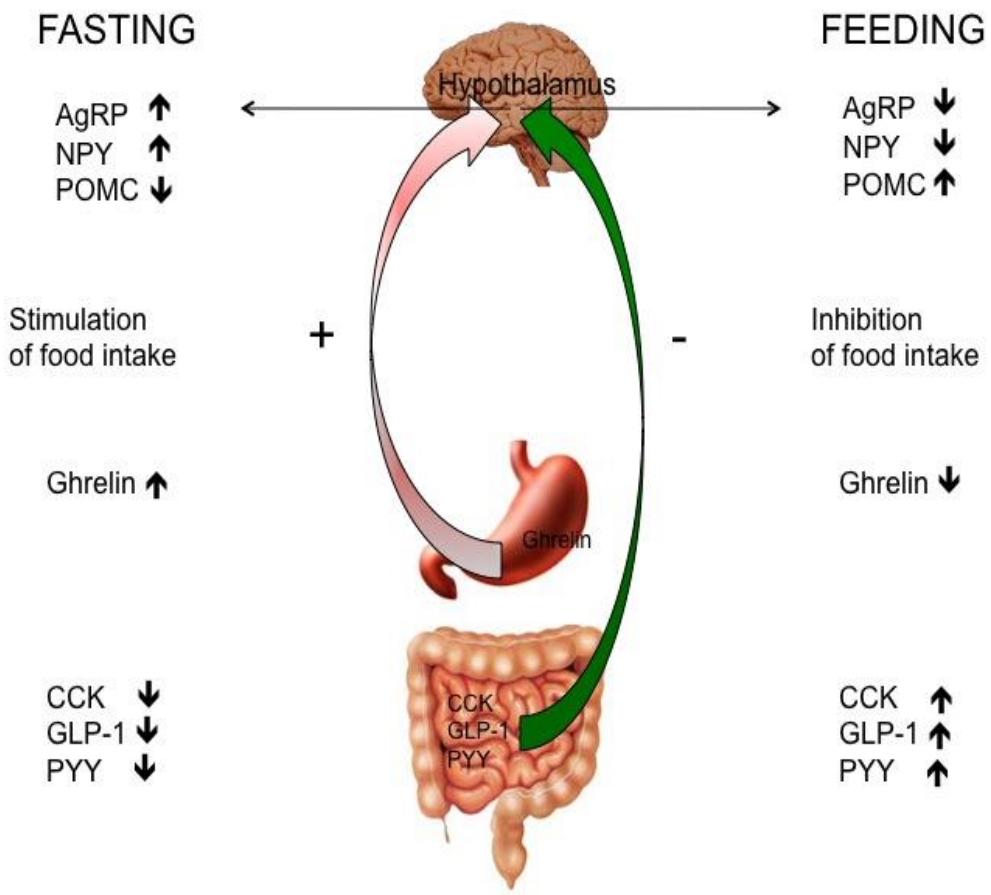

Figure 1.1 Regulation of appetite during feeding or fasting
A gut-to-brain signalling mechanism regulates appetite in response to energy intake. Postprandially, intestinal enteroendocrine cells release anorectic gut hormones (CCK, GLP-1, and PYY) that signal to the brainstem and the hypothalamus. Integration of peripheral anorexigenic signals leads to the activation of hypothalamic POMC neurons that suppress appetite and induce perceived satiety in order to stop caloric intake. In contrast, during fasting (energy restriction) ghrelin is released from stomach and small intestine whereas secretion of CCK, GLP-1 and PYY is inhibited. Ghrelin activates AgRP/NPY- expressing hypothalamic neurons and stimulates food intake. Adapted and modified from (Larder and O'Rahilly, 2012)

1.1.3 Enteroendocrine cells in gastrointestinal tract

Gut hormones are released from enteroendocrine (EEC) cells in response to nutrients. EEC cells occur at a low frequency scattered diffusely in the gastrointestinal tract between enterocytes. Although EEC cells represent around 1% of the intestinal mucosal cell population, they constitute the largest endocrine organ of the body- the enteroendocrine system. EEC cells have a characteristic conical “flask-shape” morphology with a narrow apical pole and a broad basolateral surface. The apical membrane contains microvilli projecting to the gut lumen and it is at this membrane that nutrient sensing is considered to happen. The majority of EEC cells in the small intestine have this direct contact with the gut lumen, so they are classified as “open-type” cells. EEC cells that do not possess an apical membrane projecting to the gut lumen are classified as “closed-type”. In the duodenum, very few EEC cells have “closed-type” morphology. These EEC cells contain serotonin or ghrelin and are considered to respond to mechanosensory stimuli but not to nutrients. The basolateral pole of “open-type” cells contains numerous secretory vesicles that store gut peptides ready for secretion. Another characteristic of EEC cells is the basal processes that have a dendritic morphology and are in contact with enterocytes. These processes may mediate paracrine action or have a role as sensors of intestinal mucosa environment (Bohórquez *et al*., 2014).

The EEC cell lineage is endoderm-derived (Barker *et al*., 2007, Barker *et al*., 2008) and is characterized by rapid renewal, as exhibited by intestinal epithelial cells. All intestinal mucosal cells are derived from a single population of Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-positive stem cells localized at the bottom of the intestinal crypts (Barker *et al*., 2007). There are 4-6 Lgr5+ stem cells in each crypt which can be differentiated into EEC cells or other epithelial lineages that include the regulatory Paneth cells, mucous releasing goblet cells and the

enterocytes, which represent the most abundant cell type in the intestinal epithelium. Stem cell-derived EEC cells migrate from the crypt towards the villi and a complex network of transcription factors regulates their differentiation towards a fully differentiated specific EEC cell subtype. Two critical transcription factors for the development of EEC cells are Mammalian Atoh Homolog 1 (Math1) and Neurogenin 3 (Ngn3). Math1 is crucial for the development of the secretory lineages (EEC, goblet and Paneth cells) (Yang *et al*., 2001), whereas Ngn3 , acting downstream of Math1, is critical for the development of all EEC cells (Jenny *et al*., 2002, Mellitzer *et al*., 2010).

The enteroendocrine system is very complex and consists of more than 15 subtypes of EEC cells that can secrete more than 20 gut peptides (Brubaker, 2012, Rindi *et al*., 2004). Classification of different EEC cell subtypes is traditionally referred as the "Wiesbaden classification" (Creutzfeldt, 1970) and is based on the ultrastructural characteristics (size, shape and density of secretory vesicles) and immunohistochemical profile of EEC cells. The traditional classification is based on the assumption that the size and shape of granules of each EEC cell subtype is unique and that each cell-specific granule contains only one hormone (Bordi *et al*., 2000, Solcia *et al*., 1975). This formed the "one cell-one hormone" hypothesis that became a dogma in the field of neuroendocrinology. However, the presence of 2 different hormones (GLP-1 and PYY) in the same granules of L-cells questioned the validity of this hypothesis. The continuous discovery of novel peptides and new co-localization patterns of several gut hormones in the same subtype may lead to a new classification in the future based on principal hormone content of EEC cells rather than their morphology (Helander and Fandriks, 2012). The following table shows the traditional classification of gastrointestinal EEC cells (Table 1.1).

Name	Principal Peptide	Localization
D cells	Somatostatin	Throughout GI tract Fewer in ileum and colon
EC cells	Serotonin (5-HT)	Throughout GI tract Concentrated in duodenum
ECL cells	Histamine	Stomach
G cells	Gastrin	Stomach Very few in duodenum
I cells	CCK	Small Intestine, mainly duodenum Fewer in jejunum and ileum
K cells	GIP	Small Intestine, mainly duodenum Fewer in jejunum and ileum
L cells	GLP-1/GLP-2 PYY/OXM	Few in duodenum/jejunum Concentrated in ileum and colon
M cells	Motilin	Mainly duodenum Fewer in jejunum and ileum
N cells	Neurotensin	Few in duodenum Concentrated in jejunum/ileum
S cells	Secretin	Duodenum and jejunum Concentrated in duodenum
X/A (P/D1) cells	Ghrelin/Obestatin	Throughout the intestine Concentrated in stomach

Table 1.1: Enteroendocrine cells of the gastrointestinal tract
There are more than 15 subtypes of EEC cells in the pancreas and in the intestine.
This table presents 12 different subtypes of EEC cells that are present in the gastrointestinal tract. It includes the name, the hormonal content and the localization of each subtype. Nesfatin-containing cells were identified recently and they don't have an official name. Table is adapted and modified from (Rindi *et al*., 2004)

Intestinal EEC cells can sense a wide range of nutrients that are present in the gut lumen. These can be categorized as carbohydrates, proteins or lipids. Lipids include Long Chain Fatty Acids (LCFA) that are products of breakdown of triglycerides and Short Chain Fatty Acids (SCFA) that are mainly generated by gut microbiota in the colon. EEC can also sense products of proteolytic cleavage including small peptides and amino acids. Additionally, EEC cells have the capacity to sense bile acids, that are key components of synthesis of released bile and fatty-acid amides that are products of metabolism of LCFA generated and released from neighbouring enterocytes. EEC cells express a panel of G-protein coupled receptors (GPCRs) that are assumed to be localized in the apical but also in basolateral membranes and act as chemosensors. These GPCRs can sense nutrients in the gut lumen, triggering release of hormones in response to stimuli (Psichas et al., 2015). Consequently, released hormones can act via an endocrine, neuronal and paracrine or autocrine way (Figure 1.2).

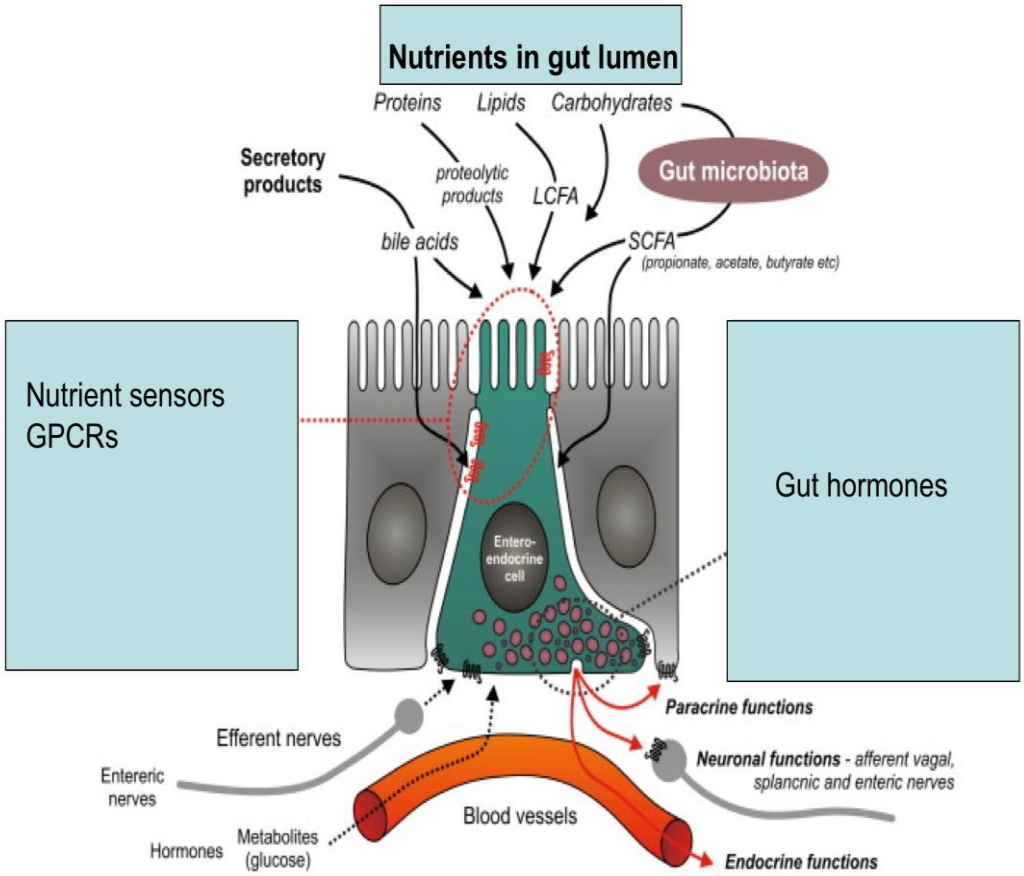

Figure 1.2 Overview of intestinal EEC cells
Intestinal EEC cells are embedded between enterocytes and have a conical morphology, with apical finger-like microvilli that project into the gut lumen and concentrated secretory hormone-containing vesicles in the broad basal membrane. EEC cells can sense LCFA, SCFA, proteolytic degradation products, bile acids and possibly carbohydrates. They contain a repertoire of GPCRs that function as nutrient sensors and mediate hormone release in response to ingested nutrients and their metabolites. EEC cells release bioactive peptides at their basolateral membrane. These gut hormones circulate in plasma and signal to receptors in remote organs (endocrine functions) or they transmit signals to the brain via vagal afferent neurons. Released gut hormones can also act as local peptides that signal via a paracrine manor to intestinal epithelial or enteroendocrine cells to regulate their function. It is unclear whether secreted peptides bind to receptors localized in the same EEC cells subtype (autocrine function), participating in a feedback regulation mechanism of EEC cells function. Red serpentine symbols indicate hypothesized localization of GPCRs chemosensors whereas black serpentine symbols indicate receptors that receive information from plasma metabolites, vagal efferent neurons and locally released gut peptides. Adapted and modified from (Engelstoft *et al*., 2008)

1.1.4 EEC cells: Formerly neglected cells- currently emerging players in pathogenesis of obesity and type II diabetes

Gut hormones were traditionally considered as regulators of appetite, but their primary role in the homeostatic pathways that regulate energy balance has probably been underestimated in the past. The reason for this neglect was partly the limited understanding of the contribution of the enteroendocrine system to the regulation of energy homeostasis. Characterization of EEC cells using electron microscopy techniques and studies employing surrogate enteroendocrine cell line models provided a basic, although limited, insight at the enteroendocrine system although it did not explain its multiple and complex systemic functions. The obscure nature and complexity of the enteroendocrine system -an organ formed by scattered single cells of different subtypes, localized throughout another organ- and the incapability to isolate and study native EEC cells are the main reasons that make the study of enteroendocrine system particularly difficult.

The crucial role of the appetite-regulating hormones was revealed during efforts to develop efficient treatments for “diabesity”. The initial breakthrough was the discovery that GLP-1 and GIP can induce glucose-stimulated insulin secretion (GSIS) from the pancreas, the so called “incretin effect”. The “incretin effect” can be defined as the significantly enhanced secretion of insulin after oral administration of glucose in comparison with *iv* (intravenous) infusion of isoglycemic glucose solution (Drucker, 2006, Drucker and Nauck, 2006). The characterization of the “incretin effect” led to the development of drugs that are currently used for the treatment of T2DM. These therapeutic molecules are mainly the “incretomimetic” GLP-1 receptor agonists (exenatide, liraglutide) that stimulate insulin release without causing weight gain. In fact, GLP-1 mimetics contribute to weight loss, because of the anorectic action of GLP-1 (Buse *et al*., 2009, Kolterman *et al*., 2005). Another class of anti-diabetic drugs

based on incretin hormones biology are inhibitors of dipeptidylo-peptidase-IV (DPP4), a peptidase that degrades circulating GLP-1 (Mentlein *et al*., 1993) resulting in a very short half-life (1-2 minutes) (Vilsboll *et al*., 2003). DPP4 inhibitors (sitagliptin, vildagliptin) stabilize GLP-1 levels and thereby prolong its effect on insulin release, but they do not cause weight loss (Raz *et al*., 2006). GLP-1 based drugs are extensively used in therapeutic treatment schemes for T2DM, highlighting the importance of gut-derived incretins. Unfortunately, these drugs cannot be considered as monotherapies against "diabesity" probably because they decrease energy expenditure in parallel with energy intake. Therefore, they induce only modest weight loss (Flint *et al*., 2000, Tharakan *et al*., 2011)

Currently, it is agreed that the only effective treatment for "diabesity" is bariatric surgery (Mingrone *et al*., 2012, Sjostrom *et al*., 2012). Bariatric surgery operations (bariatric procedures) induce a substantial weight loss that can be maintained for more than 10-15 years but (in the majority of patients) they also improve glycaemic control or achieve complete resolution of T2DM (Mingrone *et al*., 2012, Sjostrom *et al*., 2004, Sjostrom *et al*., 2007, Sjostrom *et al*., 2012). Strikingly, resolution of T2DM occurs very rapidly, even before significant weight loss has taken place.

The most effective bariatric procedures are the "gastric bypass" operations that combine restrictive and malabsorptive characteristics (mainly Roux-en-Y gastric bypass). After gastric bypass, the anatomy of gastrointestinal tract is changed and the remaining small stomach pouch (former by the removal of a larger stomach remnant) is anastomosed to the distal jejunum. In this way, there is an enhanced delivery of nutrients from stomach to distal small intestine, bypassing duodenum and proximal jejunum (Ashrafian *et al*., 2010, Dixon *et al*., 2012). Bariatric operations do not necessarily have to involve surgical rerouting of nutrients, they may simply restrict the size of stomach. Examples of restrictive procedures include gastric banding or sleeve

gastrectomy in which the size of stomach is reduced by ~75% but the anatomy of gastrointestinal tract remains the same. Sleeve gastrectomy leads to rapid gastric emptying and increased flow of nutrients in the duodenum.

Both types of bariatric procedures seem to achieve resolution of T2DM by alteration of circulating levels of gut hormones (Chandarana and Batterham, 2012, Schauer *et al*., 2012). Bariatric surgery induces a series of weight loss- independent changes (Mingrone *et al*., 2012, Schauer *et al*., 2012) in the secretion profile of both proximal and distal gut hormones. The combination of these changes leads to the beneficial effects of bariatric surgery in inhibition of appetite, weight loss and improved glucose tolerance. The mechanism that triggers the metabolic effects of bariatric surgery is unclear, possibly because of its multi-factorial nature and the poor understanding of the changes that occur to enteroendocrine system of humans who undergo metabolic surgery.

Different hypotheses have been developed about the critical role of gut-hormones derived from proximal ("foregut hypothesis") (Rubino *et al*., 2006) or distal small intestine ("hindgut hypothesis") (Ashrafian *et al*., 2010, Cummings *et al*., 2004). Bariatric procedures cause a change in the intestinal hormonal milieu and seem to result in decreased secretion of ghrelin from the gastrointestinal tract (le Roux *et al*., 2006, Scott and Batterham, 2011) in parallel with increased release of GLP-1 and PYY from distal intestine (Olivan *et al*., 2009, Peterli *et al*., 2009). It is, still, unclear if circulating CCK levels change but it is suggested that CCK release is increased after sleeve gastrectomy (Peterli *et al*., 2012) and the density of CCK-expressing cells is increased in the (bypassed) proximal intestine, after gastric bypass (Ockander *et al*., 2003).

The increased levels of anorectic (mainly PYY and GLP-1) and insulinotropic hormones (GLP-1) combined with decreased levels of the orexigenic peptide ghrelin promote inhibition of appetite and may represent a key factor that explain the maintenance of weight loss after bariatric procedures. In contrast with surgical approaches, long-term diet is characterized by a high relapse rate to obesity (Anderson *et al*., 2001). It seems that the changes in circulating levels of gut-derived hormones after prolonged diet is similar to changes induced by acute energy restriction, characterized by increase of ghrelin and decrease of CCK and PYY (Sumithran *et al*., 2011). These persistent changes in satiety hormones that follow weight loss, promote appetite and contribute to weight gain (Tschop and DiMarchi, 2012).

The failure to maintain weight loss following dietary intervention in comparison with the persistent weight loss and resolution of T2DM that follow after bariatric surgery, indicate the critical role of enteroendocrine system in the regulation of body weight and energy homeostasis. The rapid metabolic improvements induced by bariatric surgery raised the possibility to develop pharmacological therapies that could have a beneficial effect by replicating the changes in the intestinal hormonal milieu, without the need for surgery. Study of EEC cells progressively becomes the epicentre of anti-obesity research, aiming to develop a medicinal approach for the treatments of the global epidemic of “diabesity” (Tschop and DiMarchi, 2012).

1.2 CCK containing/releasing cells in the intestine (I-cells)

1.2.1 Morphology and function

CCK is released by a subtype of enteroendocrine cells that are traditionally called I-cells. This name was given to CCK-secreting cells, according to the Wiesbaden classification system which is based on the ultrastructural characteristics of the enteroendocrine cells (Creutzfeldt, 1970).

I-cells have the typical characteristics of enteroendocrine cells in that they are found in intestinal mucosa embedded between enterocytes and have a flask-shape morphology with a narrow apical membrane, projecting into the gut lumen, and a broad basolateral membrane. Secretory granules, containing bioactive peptides, are localised in close proximity to the basolateral membrane. The fact that I-cells have characteristic apical membrane “microvilli-like” processes that extend into the gut lumen classifies them as “open-type” and has led to the suggestion that I-cells have the capacity to sense directly luminal nutrients. In addition, I-cells have been reported to possess basal membrane pseudopod-like processes that project to the local enterocytes, suggesting that they may transmit signals to neighbouring epithelial cells (paracrine action) or receive information from intestinal mucosa (Chandra *et al*., 2010).

I-cells are localized in the small intestine and are not present in the stomach or the colon. They can be found throughout the small intestine, although they are most abundant in the proximal small intestine (duodenum) and their frequency decreases distally towards ileum.

I-cells release CCK in response to ingested nutrients, in particular fat, peptides and amino acids. Lipids in the form of triglycerides are not sensed by EEC cells because triglycerides are not absorbed by intestinal mucosa. However, EEC cells have the ability to sense fatty acids (FAs) that are generated after the break-down of triglycerides into FAs and glycerol by pancreatic lipase (Feinle *et al*., 2003, Matzinger *et al*., 2000). But not all produced FAs have the ability to trigger CCK release. Only LCFA that have a chain length of twelve (12) or more carbon atoms can stimulate CCK secretion in humans (McLaughlin *et al*., 1999) and in the enteroendocrine cell line model STC-1 cells (McLaughlin *et al*., 1998). On the contrary, medium chain fatty acids (MCFA) or SCFA (having a carbon chain length with less than 12 carbon atoms) do not act as secretagogues for CCK release (Feltrin *et al*., 2004, McLaughlin *et al*., 1999). Intact protein can stimulate secretion of CCK in humans and rodents (Hopman *et al*., 1985, Liddle, 1995) and neuropeptide bombesin can directly cause CCK release (Snow *et al*., 1994). Amino acids are more potent stimuli for CCK secretion than peptides/protein hydrolysates. The aromatic L-amino acids phenylalanine and tryptophan are potent stimuli that trigger CCK secretion. This response is mediated by CaSR (calcium-sensing receptor), a GPCR that is highly enriched in I-cells (Liou *et al*., 2011b, Wang *et al*., 2011). In contrast to fat and amino acids, there is no direct evidence that CCK can be secreted in response to carbohydrates. It is still unclear if native EEC cells (and specifically I-cells) express sweet taste receptors as has been reported for lingual taste cells (Reimann *et al*., 2012).

1.2.2 CCK bioactive forms-CCK receptors

Translation of Cck gene leads to the synthesis of a 115 amino acid precursor protein of circulating CCK that is called preproCCK (Figure 1.3). PreproCCK contains a N-terminal signal peptide of 20 amino acids. Cleavage of this signal peptide results in a 95 amino acid polypeptide (proCCK). ProCCK is characterized by a spacer peptide

(aa 1-25) in the aminoterminal region, four endoproteolytic monobasic sites (R_{25}, R_{50}, K_{61}, R_{75}) and an amidation site (aa 83-86, sequence Phe-Gly-Arg-Arg) that contains a dibasic (R-R) cleavage site. The tetrapeptide sequence (aa 83-86) is crucial for the generation of bioactive forms of circulating CCK. It undergoes proteolytic cleavage by prohormone convertases that generate a substrate which is further cleaved by carboxypeptidase E and amidated by peptidylglycine α-amidating monooxygenase (PAM) (Rehfeld *et al*., 2008). These modifications lead to the generation of the bioactive forms of CCK that have the same amidated carboxy-terminal heptapeptide sequence (-Tyr-Met-Gly-Trp-Met-Asp-PheNH_2), a sequence that contains the key epitope for binding to CCK receptors. Different circulating forms of CCK are generated from the processing of the largest bioactive form (CCK aa 1-83) by prohormone convertases. Cleavage of CCK 1-83 occurs at monobasic sites and produces bioactive CCK peptides of different length and activity (CCK-58, CCK-33, CCK-22, CCK-8) that are released to circulation (Figure 1.3). CCK-8 is biologically the most active of these peptides (Rehfeld, 1998). Processing of proCCK in I-cells is dominated by prohormone convertase 1/3 (PC1/3) and the cleavage of proCCK by this enzyme results in a mixture of all the bioactive forms of CCK (Rehfeld *et al*., 2008) . Plasma and intestinal extracts also contain a mixture of circulating CCK forms with CCK-33 being the most abundant (Rehfeld *et al*., 2001). This is contrary to the expression profile of CCK in CNS, where CCK-8 is the predominant form (Rehfeld and Hansen, 1986) .

Another important modification of proCCK is the sulfation of Tyr-77 (present in the conserved C-terminal heptapeptide sequence) in the trans-Golgi apparatus by sulfotransferases. Sulfation of CCK determines to which CCK receptor the released peptide will bind. CCK can bind to two subtypes of receptors, CCK-A receptor and CCK-B receptor. CCK-A receptor is expressed in the gastrointestinal tract, pancreas,

gallbladder and some regions of the brainstem (AP, NTS and DMV). The CCK-A receptor is mainly responsible for mediating CCK-induced satiety signals. In contrast, CCK-B receptor is mainly expressed in the CNS, where it localises to the brainstem, hypothalamus and cerebral cortex (Regard *et al*., 2008).

Both CCK receptors require the ligand to contain an α-amidated last carboxy-terminal amino acid. An important functional difference between the two receptors is that the CCK-A receptor can only recognize peptides with the characteristic CCK heptapeptide (-Tyr-Met-Gly-Trp-Met-Asp-PheNH_2) which contains a sulfated tyrosine residue; whereas CCK-B receptor recognize ligands with the α-amidated carboxy-terminal tetrapeptide (-Trp-Met-Asp-PheNH_2) (Miller and Gao, 2008). Consequently, sulfated CCK binds to CCK-A receptor and CCK-B receptor. In contrast, unsulfated CCK (that has a 500-1000 fold reduced binding affinity to CCK-A receptor, in comparison with sulphated CCK) binds to the CCK-B receptor (Rehfeld and Agersnap, 2012).

Therefore, sulfated CCK was traditionally considered as the biological active form since it induces the classic effects of the hormone in the gastrointestinal tract that are mediated by CCK-A receptors, such as the cholecystokinetic effect (gallbladder emptying). Nevertheless, it is not accurate that unsulfated CCK has no biological role. Unsulfated CCK is a regulatory peptide that acts as a neurotransmitter in CNS or as a local growth factor in intestine (Rehfeld and Agersnap, 2012) I-cells produce (release?) unsulfated forms of CCK along with sulfated/bioactive peptides (Bonetto *et al*., 1999) . Unsulfated CCK binds to CCK-B receptor with approximately the same affinity as gastrin (sulfated and non-sulfated gastrin). This is not surprising, because the C-terminal tetrapeptide recognized by CCK-B receptor is identical in CCK and gastrin. There is an extensive C-terminal domain homology between CCK and gastrin

(the last 5 amino acids of the carboxyterminal region are identical), a shared property that has hampered attempts to raise specific CCK antisera that detect anti-CCK8, but do not cross-react with gastrin.

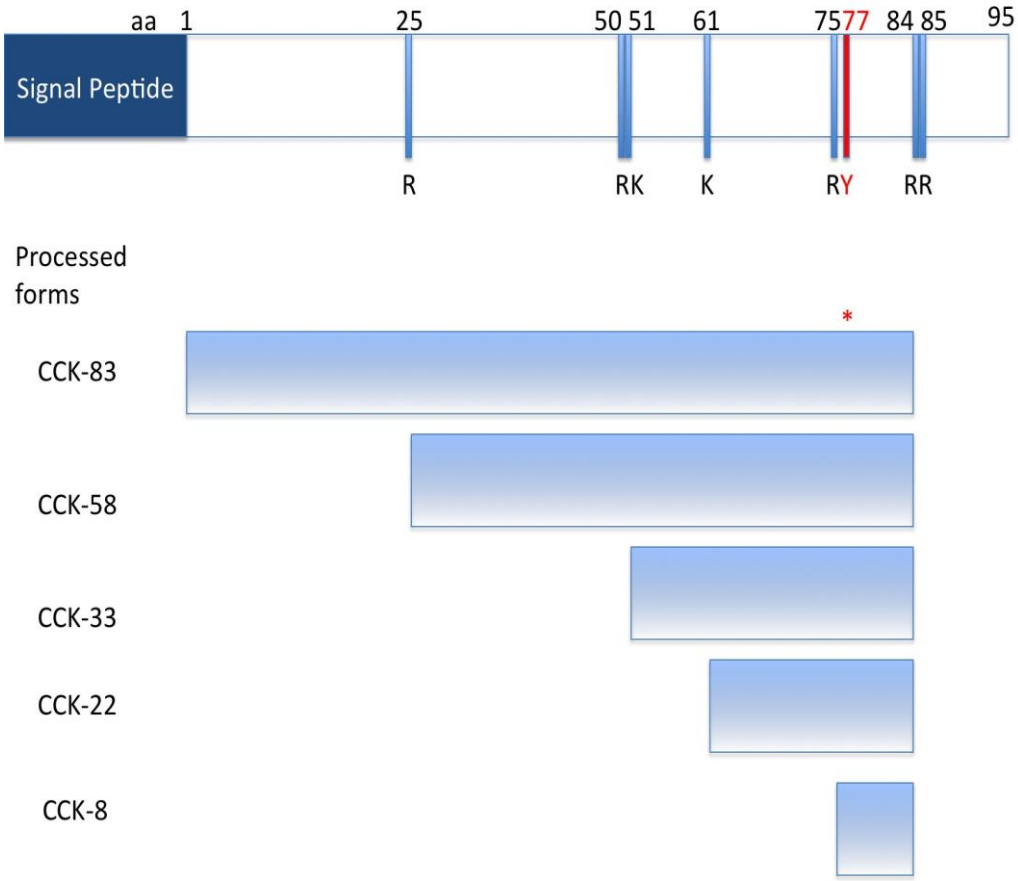

Figure 1.3: Generation of circulating bioactive CCK peptides
PreproCCK is cleaved by prohormones convertases to generate shorter peptides that are secreted from I-cells. These peptides have a common C-terminal sequence and their length varies (from 8 to 83 amino-acids) depending on how extended is the N-terminal region. Sulfation at Tyr-77 (red asterisk) allows CCK peptides to bind to the CCK-A receptor. Unsulfated CCK peptides can only bind to the CCK-B receptor. Adapted and modified from (Rehfeld and Agersnap, 2012).

1.2.3 CCK measurement in plasma

The accurate measurement of CCK concentration in plasma, where gastrin circulates at concentrations 10-50 times higher than CCK peptides, is difficult. The C-terminal homology shared by CCK and gastrin, in conjunction with the relative low levels of circulating CCK, are the main reasons why accurate measurement of bioactive CCK in plasma is problematic. There are very few antibodies that can be used in a reliable radioimmunoassay (RIA) for the detection of sulfated plasma CCK in plasma samples. The majority of antibodies used for CCK measurement RIA assays significantly cross-react with gastrin. A further problem is the high heterogeneity of circulating bioactive CCK molecules and the difficulty in measuring the different CCK peptide species. An ideal antibody for the measurement of plasma CCK should be highly sensitive, bind selectively to sulfated CCK peptides and recognize different circulating forms with equimolar affinity.

Unfortunately, an antibody-based assay that is able to fulfil these criteria has not been developed. Development of mass spectrometry assays may represent a highly selective and sensitive method for the measurement of different forms of circulating CCK, as has been demonstrated for other peptides including Brain Natriuretic Peptide (BNP) (Berna *et al*., 2008). A method that is based on the liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of immunoprecipitated plasma CCK was developed for the quantification of circulating sulphated CCK-8 in Syrian Golden Hamster (Young *et al*., 2009). This assay has the required high sensitivity to detect both basal and post-prandial physiological levels of CCK and also provided a highly specific and accurate quantification of sulfated CCK-8 peptide. A mass spectrometry based method with the same characteristics would be ideal for measurement of human plasma CCK.

1.3. CCK: An anorexigenic hormone and a co-ordinator of food digestion

Ivy and Oldberg discovered CCK in 1928, describing it as a substance in extracts of upper small intestinal mucosal layer which has the ability to cause gallbladder contraction and release of bile, after *iv* injection in dogs (Ivy and Oldberg, 1928). Fifteen years later, the crucial role of CCK as stimulator of the secretion of pancreatic enzymes was revealed by Harper and Raper who described CCK as a hormone prepared from upper small intestinal -but not gastric- extracts which mediates secretion of pancreatic enzymes after *iv* infusion in cats. The described peptide was subsequently named “pancreozymin” (Harper and Raper, 1943). In 1968, purification of CCK revealed that cholecystokinin and pancreozymin were actually the same hormone (Mutt and Jorpes, 1968), and following this discovery the name “pancreozymin” eased to be used in favour of CCK.

CCK acts as anorectic and pro-digestive peptide. It was the first gut hormone classified as major anorectic peptide, when it was observed to reduce meal size in rats (Gibbs *et al*., 1973). During the following decades, multiple studies established that CCK regulates appetite in that it is a principal inducer of satiety and serving to reduce food intake in both humans and animal models, upon ingestion of nutrients. These observations have led to CCK being considered today as the archetypal satiety hormone. In addition, CCK is a principle co-ordinator of food digestion: It stimulates gallbladder contraction, release of bile, secretion of pancreatic enzymes and intestinal peristalsis; CCK also inhibits gastric emptying, gastric motility and gastric acid secretion. These parallel functions orchestrate digestion by regulating the delivery of nutrients to proximal small intestine and the release of digestive secretions, bile and pancreatic enzymes, enabling optimal digestion of ingested nutrients (Figure 1.4).

CCK can also act as an anabolic factor on endocrine pancreas to induce pancreatic growth (β-cells proliferation) and stimulate release of insulin in animal models. Additionally, it regulates insulin signalling by reducing insulin-induced hyperphagia. CCK down-regulates the increased expression of the orexigenic hypothalamic peptides orexin and melanin-concentrating hormone (MCH) in response to insulin, thus suppressing hunger (Gallmann *et al*., 2005). Similarly, CCK attenuates the orexigenic stimulation of food intake (hunger) induced by the hormone ghrelin by blocking ghrelin-induced activation of hypothalamic arcuate nucleus (ARC) neurons (Kobelt *et al*., 2005).

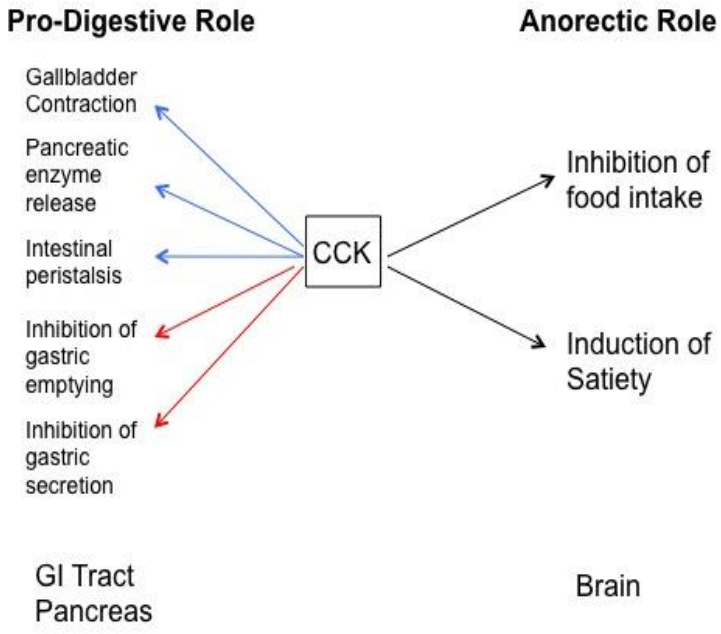

Figure 1.4: Biological actions of CCK
CCK has an anorectic and a pro-digestive role. It induces satiety via gut-to-brain signalling and it co-ordinates digestion via CCK-A receptor mediated signalling to the gastrointestinal tract (blue arrows for excitory action, red arrows for inhibitory action) and the pancreas.

1.3.1 Endocrine function

CCK is released postprandially and its peak levels in plasma are about 5-fold higher than its basal levels. The postprandial CCK concentration in plasma is ~ 5 pMol/L (whereas the fasting CCK concentration is ~1 pMol/L) and peaks about 15 minutes after the consumption of a meal. CCK plasma levels decrease after 1 hour, although they remain significantly elevated in comparison with basal levels for 2 hours after a meal (Liddle *et al*., 1985). CCK release in plasma is a prerequisite for its endocrine function as a humoral mediator that targets directly gallbladder and pancreatic acinar cells.

1.3.2 Neuronal function

1.3.2.1 Satiety

The endocrine action of CCK is not the main mechanism responsible for its satiety effect because circulating CCK peptides cannot penetrate the blood-brain barrier (Passaro *et al*., 1982) and the presence of an intact vagus nerve is required for the satiety effect of CCK (Smith *et al*., 1981). CCK has an important neuronal function mediated by vagal afferent neurons and the vagus nerve. It acts as a neuropeptide/neurotransmitter at local vagal afferent neuron termini, which innervate the lamina propria of small intestinal mucosal layer (Berthoud *et al*., 1995, Dockray, 2009a). CCK binds to CCK-A receptors that are expressed in capsaicin-sensitive vagal afferent fibres (Moran *et al*., 1990, Moran *et al*., 1987) and it is this reflex neuronal pathway that stimulates pancreatic secretion (Li and Owyang, 1994) and inhibits gastric emptying (Forster *et al*., 1990, Holzer *et al*., 1994, Raybould and Tache, 1988). CCK also induces satiety by activating the same vagal afferents-vagus nerve pathway projecting to the CNS (Dockray, 2009b, Moran *et al*., 1997, South and Ritter, 1988) and is a critical component of the gut-to-brain signalling network that regulates appetite.

CCK is the first messenger of the complex pathway that transmits information from nutrient sensing in the duodenum to the hypothalamic centres that integrate information from the periphery to regulate food intake and energy homeostasis. CCK generates a signal that is transmitted, via vagal afferent neurons and nodose ganglia, to the dorsal vagal complex (DVC) of the brainstem. In the brainstem, CCK signalling by inducing c-fos expression activates neurons localized in the sensory nucleus of the tractus solitarius (NTS) and the area postrema (AP) (Chen *et al*., 1993, Day et al., 1994, Monnikes *et al*., 1997). These activated brainstem areas display neuronal projections to the parabranchial nucleus in the pons and hypothalamus leading to a transmission of CCK induced signal to hypothalamic feeding circuits that ultimately regulate appetite (Lassman *et al*., 2010). Activation of human brain areas after intragastric infusion of lipids is very rapid, it occurs during the first 2-4 minutes after infusion, suggesting that the central actions of CCK are caused via a vagus nerve-mediated response, rather than due to CCK peptide in the peripheral circulation (Lassman *et al*., 2010). The regulation of food intake by CCK is dependent on an N-methyl-D-aspartate (NMDA) receptor-mediated transmission of the lipid/CCK-induced signal in the NTS (Gillespie *et al*., 2005, Lam *et al*., 2010)

1.3.2.2 Regulation of blood glucose levels

Independently of the gut-to-brain signalling that inhibits food intake, ingestion of lipids and subsequent release of CCK in duodenum regulates blood glucose levels via a gut-to-brain-to-liver signalling pathway (Cheung *et al*., 2009, Wang *et al*., 2008). This negative feedback mechanism requires the formation of LCFA-coA forms of LCFA in the duodenum and the activation of a protein kinase C (PKCδ) signalling network (Breen *et al*., 2011, Kokorovic *et al*., 2011). This leads to the release of CCK that activates a neuronal network, similarly with the gut-to-brain response, by binding to

CCK-A receptors of vagal afferent nerves. In this way, the signal is relayed via the NTS to the hypothalamic centres that regulate energy homeostasis. The final segment of the gut-to-brain-to-liver network (brain to liver communication) requires the presence of the intact vagal hepatic branch fibres (Figure 1.5) (Cheung *et al*., 2009, Wang *et al*., 2008). It is remarkable that high-fat feeding attenuates the responses to lipid sensing in the duodenum, including the activation of NTS, the inhibition of food intake and the suppression of hepatic glucose production (Covasa *et al*., 2000, Covasa and Ritter, 1999, Wang *et al*., 2008)

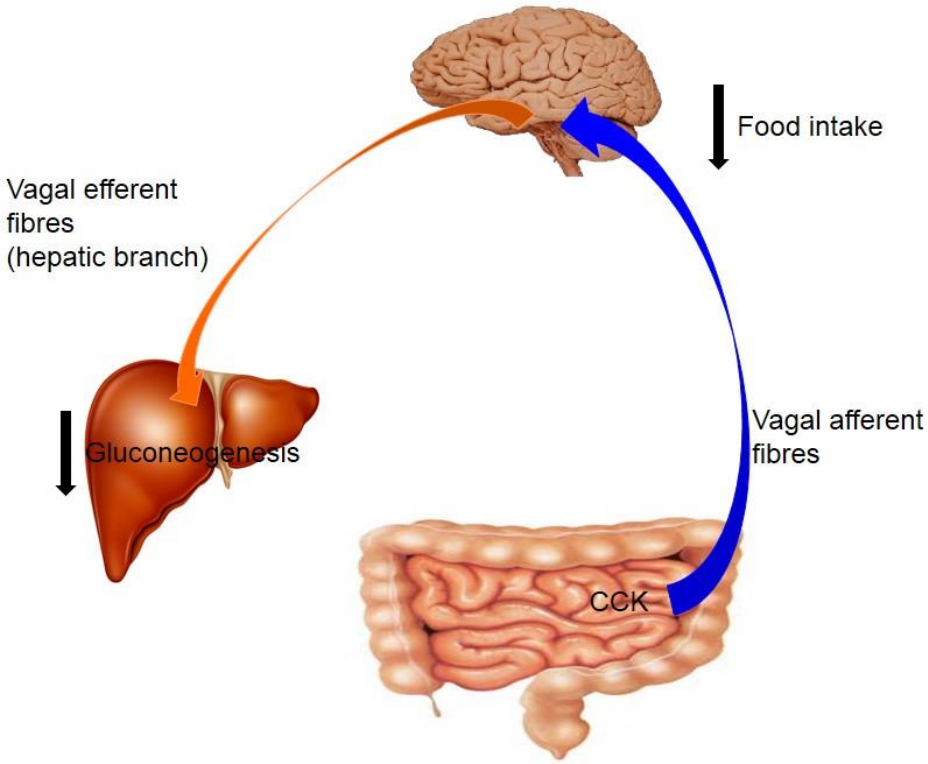

Figure 1.5 Overview of gut-to-brain-to-liver axis that regulates glucose homeostasis
Presence of LCFA in duodenum triggers CCK release. CCK activates CCK-A receptors in the vagal afferent neurons and transmits information to the brain stimulating satiety. In parallel, it inhibits glucose production in liver via the hepatic branch of the vagus nerve.

1.3.2.3 Modulation of vagus nerve signalling

Vagal afferent neurons express CCK-A receptors that are activated by CCK to inhibit food intake and also CCK-B receptors (Broberger *et al*., 2001, Moriarty *et al*., 1997). Additionally, they express a range of GPCRs that when active, either inhibit food intake such as PYY3-36 receptor neuropeptide Y receptor type 2 (Y2R) (Burdyga *et al*., 2008, Koda *et al*., 2005) or stimulate appetite such as cannabinoid receptor CB1 (Burdyga *et al*., 2004) and the melanin concentrating hormone receptor MCH1-R (Burdyga *et al*., 2006a, Burdyga *et al*., 2006b). Vagal afferents show plasticity that is dependent on the availability of nutrients and that reflects the changes in the expression profile of GPCRs during feeding (normal energy intake) or fasting (energy restriction).

This versatile expression profile of GPCRs in vagal afferents, which switches their function from inhibition to stimulation of appetite and vice versa, is dependent on the levels of circulating CCK. During feeding, increased CCK levels tonically inhibit expression of CB1 receptors and MCH-1 receptors, and upregulate expression of Y2R to promote anorectic signals to the brain (Burdyga *et al*., 2008, Burdyga *et al*., 2004, Burdyga *et al*., 2006a). These actions can be potentiated by leptin (de Lartigue *et al*., 2010) and they can be blocked by locally released endocannabinoids such as anandamide (AEA) and 2-arachidonyl glycerol (2-AG) and the orexigenic peptide ghrelin (Burdyga *et al*., 2006b)

During fasting, the levels of circulating CCK are low whereas there is an increased release of intestinal endocannabinoids and ghrelin, leading to the upregulation of CB1 receptors (mainly) and (if fasting is prolonged) MCH-1 receptors, and the down-

regulation of Y2R (Burdyga *et al*., 2008, Burdyga *et al*., 2004, Burdyga *et al*., 2006a). These changes in expression of key GPCRs serve to switch the function of vagal afferents to stimulate food intake and can be reversed by increased levels of CCK, occurring after exogenous administration or after feeding.

Therefore, CCK plays a key role as the gatekeeper peptide that regulates the plasticity of vagal afferent neurons in response to energy intake (Figure 1.6). The presence or absence of CCK modifies the expression levels of key GPCRs that mediate gut-to-brain signalling and switches the sensitivity of vagal afferents to satiety or orexigenic signals (Dockray, 2009a, Dockray and Burdyga, 2011).

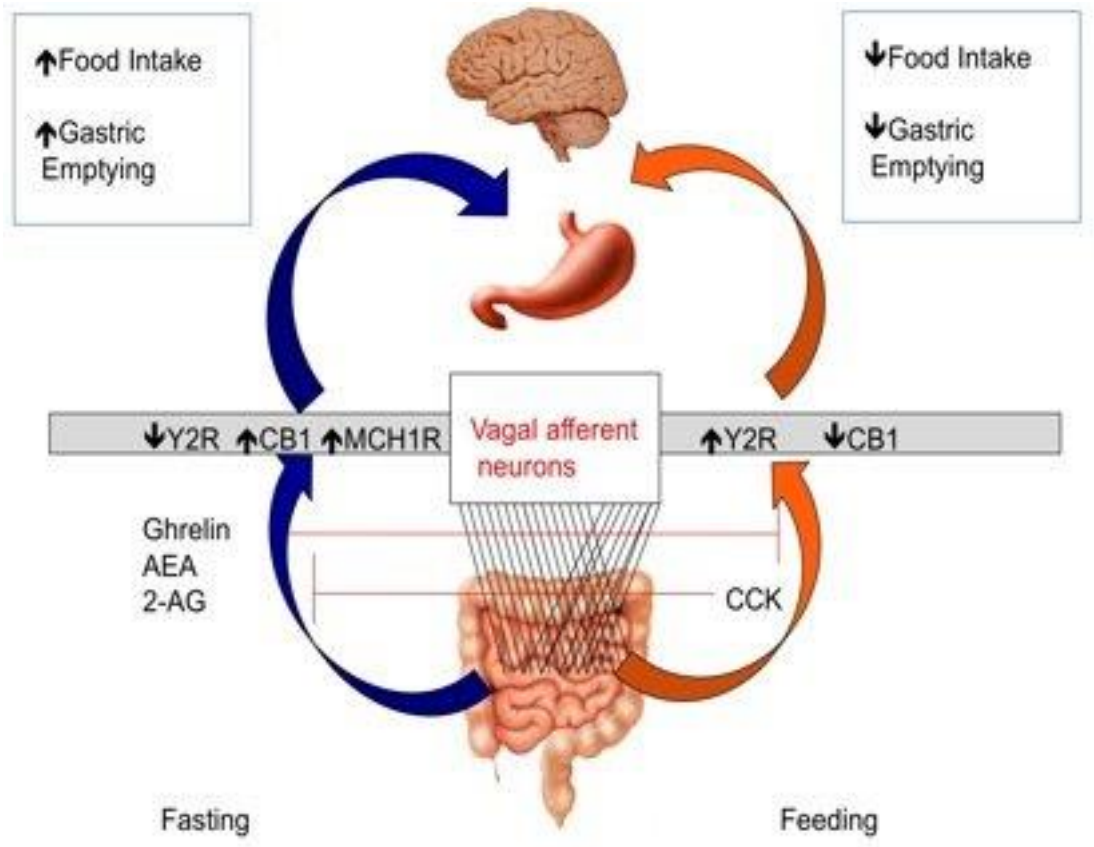

Figure 1.6: CCK is a master controller of GPCRs expression in vagal afferent neurons
The expression pattern of vagal afferent neurons GPCRs associated with the regulation of appetite is dependent on energy intake and the presence of nutrients in duodenum. Nutrient sensing causes release of CCK that switches on the expression of GPCRs that promote inhibition of gastric emptying and food intake. During energy restriction, release of ghrelin and intestinal endocannabinoids (AEA, 2-AG) is stimulated, resulting in the expression of vagal afferent GPCRs associated with stimulation of food intake and gastric emptying. Increased levels of CCK can block this action of orexigenic peptides. Similarly, increased levels of ghrelin and endocannabinoid peptides can reverse the CCK-induced stimulation of orexigenic GPCRs in vagal afferents. Adapted and modified from (Dockray and Burdyga, 2011)

1.3.3 Paracrine function

CCK may have an important paracrine action in regulating the release of other hormones from intestinal EEC cells. Specifically, studies in humans have demonstrated that CCK mediates the stimulation of GLP-1 and PYY release from distal intestinal L-cells in response to fat (Beglinger *et al*., 2010, Degen *et al*., 2007). GLP-1 and PYY secretion from L-cells in response to intraduodenal infusion of LCFA is attenuated-partly, at least- by *iv* administration of CCK-A receptor antagonist dexloxiglumide, suggesting a CCK-mediated mechanism of incretin secretion. In this model, LCFA stimulate I-cells to release CCK that subsequently triggers GLP-1 and PYY release from L-cells. This action may be direct by binding to CCK-A receptors expressed on L-cells or indirect via the activation of vagal afferent fibres (Beglinger *et al*., 2010, Degen *et al*., 2007). A similar mechanism may explain that LCFA-induced inhibition of ghrelin release is mediated by CCK signalling via CCK-A receptors (Degen *et al*., 2007).

Gut hormones are supposed to act via a paracrine mechanism and regulate important physiological processes in their neighbouring enterocytes (Schwartz and Holst, 2010). There are paracrine regulatory loops of communication between L-cells and enterocytes by which PYY regulates electrolyte secretion (Cox *et al*., 2010) and GLP-2 stimulates intestinal lipid absorption and formation/secretion of chylomicrons via CD36 (Hsieh *et al*., 2009). It has been suggested that CCK is important for a regulatory mechanism that minimizes the absorption of (bitter tasting) toxins from the intestine. These potentially toxic bitter substances stimulate release of CCK from I-cells via bitter taste receptor T2R38 (Jeon *et al*., 2008). Secreted CCK in turn acts via a CCK-B receptor mediated paracrine mechanism to increase ABCB1 (ATP-binding cassette protein B1) expression in the apical membrane of enterocytes and induce

ABCB1-mediated efflux from epithelial cells (Jeon *et al*., 2011). This is a protective mechanism to limit the absorption of toxic substances from the gut.

1.4 Study of native EEC and I-cells

1.4.1 Past approaches for study of native I-cells

The study of native EEC cells and specifically I-cells is difficult because of their rarity, scattered distribution throughout the intestine and their relatively indistinct morphology compared with neighbouring enterocytes. The inability to purify I-cells restricted previous studies to the measurement of secreted CCK in response to stimuli in mixed cultures, as an indirect assessment of I-cells function. Experimental protocols were then developed for the isolation of intestinal mucosal cells populations, enriched in I-cells. These isolation techniques resulted in primary mixed mucosal cell populations (containing all types of intestinal epithelial cells) and were enriched in CCK-secreting cells, although they had limited survive.

As an example, one approach was based on counterflow elutriation of canine jejunal epithelium that provides a ~20-fold enriched in I-cells primary mixed culture that could be maintained for 40 hours (Barber *et al*., 1986, Koop and Buchan, 1992). These mixed cultures were used for functional experiments that served to demonstrate that CCK can be released in response to aromatic L-amino acids (Barber *et al*., 1986, Koop and Buchan, 1992) in a calcium dependent mechanism (Barber *et al*., 1986).The difficulties inherent in generating primary cultures of highly enriched in I-cells forced researchers to develop alternative experimental protocols, based on the perfusion of non-enriched epithelial cultures with different potential stimuli. In these protocols, measurement of released CCK after perfusion was used to evaluate the

response of I-cells (Bouras *et al*., 1992, Herzig *et al*., 1996, Sharara *et al*., 1993).

Attempts to isolate pure populations of I-cells were based on the development of compounds that could specifically bind to I-cells, tag them and enable their sorting. One report present an ingenious method to isolate a relatively pure population of I-cells by selecting activated cells, after loading of dissociated epithelial cells with the calcium indicator penta acetoxymethyl ester of Indo-1 (Indo1-AM). The activated cells represented a highly-enriched population of CCK-containing cells that were then subsequently perfused (Liddle *et al*., 1992). Despite its novelty, this approach was technically challenging and did not become an established method of I-cells sorting. Therefore, studies of I-cells were limited to perfusion systems assessing the nutrient-induced activation of CCK release from heterogeneous mixed populations.

1.4.2 STC-1 cells and GLUTag cells: I-cells models?

The difficulties to establish and maintain primary small intestinal cell cultures shifted the emphasis of enteroendocrine research to immortalized cell lines. The murine enteroendocrine cell line STC-1 (stanniocalcin-1) has been the most commonly used cell line for the I-cells characterization and the investigation of CCK release mechanisms. The STC-1 cell-line is derived from an invasive intestinal neuroendocrine tumour that occurred in a transgenic mouse expressing the simian virus 40 large T antigen (SV40 Tag) under the control of rat insulin promoter (RIP1Tag2 transgenic mice) (Grant *et al*., 1991, Rindi *et al*., 1990). STC-1 cells were demonstrated to secrete CCK in a calcium-dependent way (Mangel *et al*., 1995) and were therefore considered as a reliable model system to investigate physiological chemosensation mechanisms and intracellular signalling pathways that regulate CCK release. STC-1 cells were extensively used and were shown to release CCK in the form of CCK-8, in response to LCFA (McLaughlin *et al*., 1998, Tanaka *et al*., 2008),

the neuropeptide bombesin (Chang *et al*., 1994a), aromatic amino acids (Mangel *et al*., 1995), peptones and peptidomimetics (Nemoz-Gaillard *et al*., 1998).

STC-1 cells do not only secrete CCK but they express and release a spectrum of gut hormones including neurotensin, secretin (Rindi *et al*., 1990), GLP-1 (Hirasawa *et al*., 2005, Rindi *et al*., 1990), GIP (Jepeal *et al*., 2003, Kieffer *et al*., 1995) and PYY (Geraedts *et al*., 2009, Hand *et al*., 2012). This multihormone expression profile of STC-1 cells does not seem to reflect the hormonal content of native I-cells and is considered as a major drawback of using this cell line model. Despite this disadvantage, the STC-1 cell line is still considered as the best I-cell line model to study mechanisms of CCK secretion.

Another model that has been used to investigate the CCK release mechanisms is the GLUTag cell line. GLUTag cells are a well differentiated murine enteroendocrine cell line that principally expresses the glucagon gene (Lee *et al*., 1992) and secretes the glucagon-related peptides in response to LCFA (Brubaker *et al*., 1998), carbohydrates (glucose) (Gribble *et al*., 2003, Reimann and Gribble, 2002) and amino-acids such as glutamine (Reimann *et al*., 2004). GLUTag cells have the ability to release CCK in response to LCFA (Sidhu *et al*., 2000) but are not frequently used as an alternative I-cells model because they predominantly express preproglucagon-derived peptides.

A recent study questions the traditional characterization of STC-1 cells as the principal CCK-secreting cell line. Gribble/Reimann and their colleagues analyzed the transcriptomic hormonal profile of STC-1 and GLUTag cells, using gene expression/DNA microarray analysis and found that CCK expression levers are higher in GLUTag than in STC-1 cells. Importantly, CCK is not the predominant expressed hormone in STC-1 cells that rather mainly express GIP and secretin (Habib *et al*., 2012). This transcriptomic analysis also confirmed the multi-hormone expression

profile of both STC-1 and GLUTag enteroendocrine cells lines and highlighted their similar characteristics in terms of CCK expression by revealing that CCK is not the predominantly expressed hormone in STC-1 cells.

1.4.3 The use of transgenic animal models for the study of native EEC and I-cells

Enteroendocrine cell lines are useful for the understanding of pathways that trigger hormone release but it is doubtful if they reflect the biology of native EEC cells. STC-1 cells are, at best, an approximate model of native I-cells and CCK release mechanisms revealed from their study should be confirmed on native cells.

Genetically engineered transgenic mice that express fluorescent proteins driven by hormone gene promoters are useful tools that became recently available and made the –previously impossible- task of isolating native EEC cells achievable. These mice have a tissue-specific expression pattern of fluorescent-labelled EEC cells, depending on the transcription profile of the relative hormone.

The first animal model used for isolation of native EEC cells was a transgenic mouse with yellow fluorescent protein Venus-tagged cells that express preproglucagon, which enabled the purification and characterization of native L-cells (Reimann *et al*., 2008). Subsequently, transgenic mice models with Venus tagged K- and green fluorescent protein (GFP) tagged I-cells were developed. The availability of transgenic mice with fluorescently labelled L-, K- and I-cells enables the molecular characterization of these EEC cells subtypes, advancing EEC cells research (Habib *et al*., 2012, Liou *et al*., 2011b, Parker *et al*., 2009, Reimann *et al*., 2008, Tolhurst *et al*., 2009, Wang *et al*., 2011, Liou et al., 2011a, Tolhurst *et al*., 2012a, Tolhurst *et al*.,

2011).

These transgenic animal models enable us to isolate and characterize native EEC cells in order to investigate their gene expression profile, define their hormonal content and shed light on their chemosensing properties and nutrient sensing machinery.

1.5 Intestinal sensing of nutrients

1.5.1 Chemosensation in the gut

The intestine is programmed to digest a wide range of food components that may be digestible or indigestible, beneficial or detrimental (harmful chemicals or toxins). Chemosensory cells in the gastrointestinal tract sense a spectrum of nutrients and products of bacterial metabolism. The intestinal chemosensing system can differentiate between nutrients and also has the capacity to interpret the identity and the concentration of nutrients (Parker et al., 2014). Having registered the type and quantity of sensed compounds, the enteroendocrine system integrates the signals and generates an appropriate response that regulate digestion, food intake and glucose metabolism. The intestinal mucosal cells that participate in chemosensation are EEC cells and tuft cells.

Tuft cells (also known as caveolated or brush cells) have a similar morphology with EEC cells, being polarized with a narrow apical membrane characterized by a tuft of microvilli that extends to the gut lumen. Despite their similar morphology with EEC cells, tuft cells represent a distinct secretory epithelial cell lineage and they are not an EEC cell subtype (Gerbe *et al*., 2011). What nets tuft cells apart from EEC cells is that tuft cells contain caveolae in the apical cytoplasm (Nabeyama and Leblond, 1974) but not secretory granules in the basolateral pole (Hofer and Drenckhahn, 1996). Tuft

cells express proteins involved in taste receptor signalling and they are assumed to secrete opioids to the gut lumen (Gerbe *et al*., 2011, Kokrashvili *et al*., 2009). It is unknown if tuft cells can directly sense nutrients and regulate intestinal opioid release in response to them. Therefore, EEC cell lineage has the pivotal role in intestinal chemosensing.

1.5.2 An overview of nutrient sensing mechanisms

The sensory transduction mechanism that transmits the chemical stimuli from intestinal lumen nutrients to activate the neurons of the enteric nervous system is complex and not yet fully resolved. Several competing, yet not mutually exclusive, hypotheses exist about how ingested nutrients generate signals that regulate appetite. Although EEC cells have a crucial role in nutrient sensing, it has been suggested that nutrients may cross the intestinal epithelium via diffusion or active transport by specific transport proteins to directly activate vagal afferent neurons (Bertrand, 2009, Liu *et al*., 1999).

An alternative mechanism is based on the activation of intracellular signalling pathways that regulate hormone release by increased concentrations of nutrients absorbed from the intestinal epithelium (Tolhurst *et al*., 2012b). Solute carrier transporters (SLCs) facilitate diffusion or actively transport nutrients across the epithelium to stimulate signalling mechanisms coupled to peptide release. Signals may include membrane depolarization and elevation of intracellular calcium levels. EEC cells are electrically excitable (Reimann *et al*., 2008, Wang *et al*., 2011, Rogers *et al*., 2011) and their membrane depolarization can trigger hormone release. As an example, the SLC protein sodium-coupled glucose transporter 1 (SGLT1) has been suggested to act as a luminal glucose sensor that mediates GLP-1 release by stimulating an action potential that leads to voltage-dependent calcium influx in L-cells

(Gribble *et al*., 2003, Parker *et al*., 2012, Reimann *et al*., 2008). Additionally, the glucose transporter GLUT2 is implicated in the same mechanism, acting as a glucose transporter in K- and L-cells. GLUT2-mediated glucose transport causes indirectly depolarization of cell membrane, increase of intracellular calcium and secretion of GIP/GLP-1 (Mace *et al*., 2012). In parallel, amino acids (glutamine, asparagine and phenylalanine) stimulate the electrical activity of L-cells and trigger GLP-1 release in a sodium-uptake and calcium-influx dependent mechanism (Tolhurst *et al*., 2011).

Despite the important role of SLCs and amino acids as electrogenic stimuli that regulate bioactive peptide secretion, it is considered that the critical role of EEC cells as chemosensors is mediated by the expression of a wide range of GPCRs that can directly sense luminal nutrients and trigger hormone release (Symonds *et al.,* 2014).

1.5.3 G-protein coupled receptors as gastrointestinal tract chemosensors

GPCRs, also known as seven-transmembrane receptors (7TMs), form the biggest family of cell surface receptors and are responsible for the transduction of external stimuli by triggering a cascade of intracellular signalling pathways (Ross and Gilman, 1980). This signalling cascade starts with the activation of membrane-bound guanine nucleotide –binding G-proteins that are activated, following the conformational change of GPCRs after the binding of extracellular ligands. G-proteins are heterotrimers, formed by subunits α, β and γ (Prezeau *et al*., 2010). When they are activated, they generate signalling cascades that can be categorized based on their Gα subunit subtype. $G\alpha_s$ and $G\alpha_i$ are coupled to the cyclic adenosine monophosphate (cAMP) pathway and have opposite actions. $G\alpha_s$ stimulate adenylate cyclase and therefore elevates intracellular cAMP concentration, whereas $G\alpha_i$ decreases cAMP levels by inhibiting adenylate cyclase. $G\alpha_q$ recruits the phosphatidylinositol pathway and

activates phospholipase C (PLC) that in turn hydrolyzes Phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates calcium release from intracellular stores and DAG activates Protein Kinase C (PKC) (Neves *et al*., 2002).

Recently, a group of -previously orphan- GPCRs were deorphanized and it was shown that their ligands are nutrients. The expression of these GPCRs in the gastrointestinal tract and in enteroendocrine cell line models led to the hypothesis that they have a crucial role in chemosensation. One of major questions in EEC cells research is if these GPCRs are expressed in enteroendocrine cells and mediate release of gut peptides in response to nutrients (Engelstoft *et al*., 2008).

1.5.4 GPCRs involved in intestinal lipid sensing

1.5.4.1 Fat sensing in the gut

The high energy density and palatability of dietary fat contributes to excessive energy intake (Westerterp, 2006). Fat sensing in the small intestine stimulates release of the anorectic hormones CCK, GLP-1 and PYY that regulate energy intake by inducing satiety and suppresses the secretion of orexigenic hormone ghrelin (Feltrin *et al*., 2004, Feinle *et al*., 2003, Feinle-Bisset *et al*., 2005). Several studies have demonstrated that the intestinal epithelium senses products of fat digestion (free fatty acids- FA) and not intact triglycerides (Feinle-Bisset *et al*., 2005, Little *et al*., 2007, O'Donovan *et al*., 2003). Inhibition of the pancreatic lipase with tretrahydrolipstatin (THL) inhibits the release of anorectic hormones and attenuates the satiety effect induced by intraduodenal lipids (Feinle *et al*., 2001, O'Donovan *et al*., 2003).

LCFA (McLaughlin *et al*., 1999, McLaughlin *et al*., 1998) and chylomicrons (Raybould *et al*., 1998) were found to stimulate CCK release. The formation of chylomicrons in

enterocytes has been reported to be essential for lipid-induced CCK secretion (Hata *et al*., 2011, Raybould *et al*., 1998). Chylomicrons are formed from triglycerides (synthesized by absorbed FA and monoacylglycerides) packaged together with apolipoproteins and secreted basolaterally into the lymph. The mechanism by which chylomicrons are involved in CCK release is still unknown. Additional, it is unclear if I-cells have the ability to form chylomicrons.

Contrarily to the indirect action of chylomicrons, it is considered that LCFA (>12 carbons) have a direct effect on I-cells to stimulate CCK release. This LCFA-induced CCK secretion may be mediated via GPCRs that act as LCFA sensors. These LCFA sensors may be expressed in other subtypes of EEC cells including K- and L-cells to mediate LCFA-induced release of GIP, GLP-1 and PYY respectively (Ellrichmann *et al*., 2008, Enc *et al*., 2009)

1.5.4.2 LCFA receptors (GPR40/GPR120)

In the last decade, deorphanization of GPCRs identified two receptors that respond to LCFA. These receptors are Free Fatty Acid Receptor 1 (FFAR1, formerly known as GPR40) and Omega-3 Fatty Acid Receptor 1 (O3FAR1, formerly known as GPR120).

GPR40/FFAR1 was identified as a receptor for saturated and unsaturated medium (>8 carbon atoms) and long chain fatty acids (12-22 carbon atoms) (Briscoe *et al*., 2003, Itoh *et al*., 2003, Kotarsky *et al*., 2003). Notably, the potency of saturated LCFA (contrarily to unsaturated LCFA) that act as GPR40/FFAR1 agonists depends on their chain length, with pentadecanoic acid (C15) and palmitic acid (C16) being the most potent stimuli (Briscoe *et al*., 2003). O3FAR1/GPR120 was shown to act as a receptor for LCFA, being activated from both saturated (C14-C18) and non-saturated (C16-C22) FA (Hirasawa *et al*., 2005, Suzuki *et al*., 2008). Additionally, O3FAR1/GPR120 was suggested to be activated from ω-3 FA, eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA) (Oh *et al*., 2010). Both LCFA GPCRs are primarily coupled to Ga_q –phosphatidylinositol pathway, resulting in an increase in cytosolic free calcium and activation of PLC (Hara *et al*., 2009, Hirasawa *et al*., 2005, Itoh *et al*., 2003).

GPR40/FFAR1 is expressed in the pancreatic islets where it mediates FA-induced potentiation of glucose-stimulated insulin secretion (GSIS), in taste buds where it mediates fat taste transduction (Cartoni *et al*., 2010) and in the intestine (Briscoe *et al*., 2003, Itoh *et al*., 2003). O3FAR1/GPR120 is expressed in the intestine, in macrophages where it mediates anti-inflammatory action of ω-3 FA (Oh *et al*., 2010) and in adipocytes where it triggers insulin-sensitizing anti-inflammatory effects in response to ω-3 FA (Gotoh *et al*., 2007, Oh *et al*., 2010). A loss of function mutation of O3FAR1/GPR120 is associated with development of obesity and insulin resistance in humans (Ichimura *et al*., 2012). Similarly with GPR40/FFAR1, O3FAR1/GPR120 is localized in the taste buds and mediates fat taste (Cartoni *et al*., 2010, Matsumura *et al*., 2009).

Both LCFA receptors are expressed in the STC-1 enteroendocrine cell line. Functional experiments suggest that O3FAR1/GPR120 is critical for CCK secretion in STC-1 cells (Hirasawa *et al*., 2005, Tanaka *et al*., 2008). This O3FAR1/GPR120-mediated CCK release is dependent on Protein Kinase A (PKA) and the activation of L-type calcium channels, suggesting a distinct non Ga_q-mediated mechanism. In mice, GPR40/FFAR1 was reported to colocalize with a range of gut hormones, including CCK, GIP, GLP-1, ghrelin, PYY, secretin, serotonin and substance P (Edfalk *et al*., 2008). This expression pattern suggests that GPR40/FFAR1 is specifically expressed in EEC cells. Studies in transgenic animal models with fluorescent-tagged EEC cells suggest that mRNA transcripts encoding GPR40/FFAR1 and

O3FAR1/GPR120 are highly enriched in K-, L- and I- cells (Iwasaki *et al.*, 2014, Liou *et al.*, 2011a, Reimann *et al.*, 2008).

It has been suggested that GPR40/FFAR1 mediates LCFA-induced CCK release from native I-cells (Liou *et al*., 2011a). GPR40/FFAR1 Knockout (KO) mice have reduced linoleic acid-stimulated CCK secretion suggesting that GPR40/FFAR1 is critical for LCFA-triggered CCK release (Liou *et al*., 2011a). Furthermore, the absence of GPR40/FFAR1 expression attenuates GLP-1 and GIP release in response to dietary fat, indicating a common mechanism for LCFA-induced secretion of hormones in different subtypes of EEC cells (Edfalk *et al*., 2008). Studies in enteroendocrine cell lines and native EEC cells/ whole organisms suggest different mechanism of FA sensing. This discrepancy, that may represent the difference between these experimental models, highlights the need for more functional experiments for the identification of the key GPCR in FAs sensing.

1.5.4.3 SCFA receptors (GPR41/GPR43)

SCFA receptors are activated by FA that have a carbon chain with less than 6 carbon atoms, including formate (C1), acetate (C2), propionate (C3), butyrate (C4) and pentanoate (C5) (Brown *et al*., 2003, Le Poul *et al*., 2003, Nilsson *et al*., 2003). FFAR3/GPR41 is preferentially activated by propionate, butyrate and pentanoate and signals via $G\alpha_i$-mediated pathway. FFAR2/GPR43 preferentially binds acetate and propionate and acts via a $G\alpha_q$-phosphatidylinositol or a $G\alpha_i$-mediated intracellular signalling pathway (Brown *et al*., 2003, Le Poul *et al*., 2003, Nilsson *et al*., 2003). FFAR3/GPR41 is expressed in adipocytes and in the gastrointestinal tract (small intestine and colon) (Brown *et al*., 2003, Samuel *et al*., 2008, Xiong *et al*., 2004). In adipocytes, FFAR3/GPR41 mediates SCFA-induced leptin release (Xiong *et al*., 2004). FFAR2/GPR43 is expressed in the gastrointestinal tract and enriched in adipocytes, in the spleen, the bone marrow and the polymorphonuclear cells (Brown

et al., 2003, Ge *et al*., 2008, Hong *et al*., 2005, Le Poul *et al*., 2003). It is also an important regulator of lipolytic activity in adipocytes (Ge *et al*., 2008).

FFAR3/GPR41 and FFAR2/GPR43 localized in the gastrointestinal tract mainly respond to SCFA that are locally generated by the gut microflora. Gut microbiota hosts bacteria from up to 1000 species and generates a range of metabolites that influence the immune system and establish a metabolic communication with the host (Nicholson *et al*., 2012). Bacterial fermentation of dietary fibre (nondigestible polysaccharides) by the gut microbiota generates SCFA that reach their highest concentration in colon and distal small intestine (Samuel *et al*., 2008). Immunohistochemical studies suggest that both SCFA GPCRs are expressed in colonic L-cells (Karaki *et al*., 2006, Karaki *et al*., 2008, Tazoe *et al*., 2009). Additionally, FFAR2/GPR43 was reported to be expressed in 5-Hydroxytryptophan (5-HT) containing mucosal mast cells (Karaki *et al*., 2006). Quantitative RT-PCR in isolated native L-cells showed the both FFAR3/GPR41 and FFAR2/GPR43 mRNA transcripts are highly enriched in both colonic and small intestinal L-cell populations (Tolhurst et al., 2012a, Nøhr et al., 2013). It has been suggested that SCFA stimulate GLP-1 secretion, regulating glucose tolerance (Freeland and Wolever, 2010, Lin *et al*., 2012, Tolhurst *et al*., 2012a, Zhou *et al*., 2008) and this response is mediated by FFAR2/GPR43 (Tolhurst *et al*., 2012a).

Small intestinal CCK-containing cells are highly enriched in mRNA transcript encoding FFAR3/GPR41 (Samuel *et al*., 2008). It has been suggested that FFAR3/GPR41 triggers SCFA-induced release of PYY that regulates intestinal transit time, absorption and energy extraction from SCFA, and hepatic lipogenesis. Therefore, FFAR3/GPR41 has a critical role in the local and systemic effects of the gut microbiota, acting as a link between gut microbes, digestion and regulation of energy balance (Samuel *et al*., 2008). It is unknown if FFAR3/GPR41 is expressed in duodenal I-cells, an EEC-cell

subpopulation localized in an intestinal segment where the lumen concentration of SCFA is very low in comparison with distal small intestine and colon.

1.5.4.4 Lipid derivatives receptors- GPR119

Lipid sensing in the gut is not limited only to products of triglycerides digestion (FA) but includes fat metabolites. Lipid derivatives that are locally formed and released are sensed by EEC cells in order to regulate secretion of gut hormones. These lipid-derived compounds include endocannabinoid peptides and acylethanolamines, such as oleoylethanolamide (OEA).

A key receptor activated by FA-derivatives is GPR119. GPR119 is expressed in pancreatic β-cells and in the intestine (Chu *et al*., 2008, Chu *et al*., 2007). Intestinal L- and K- cells are highly enriched in GPR119 mRNA transcript, suggesting that GPR119 is specifically expressed in EEC cell subtypes (Parker *et al*., 2009, Reimann *et al*., 2008). GPR119 activation is coupled to Ga_s signalling pathway (Chu *et al*., 2007, Soga *et al*., 2005) and mediates important anti-diabetic pharmacological actions. Activation of pancreatic GPR119 stimulates glucose-stimulated release of insulin (Chu *et al*., 2008, Chu *et al*., 2007, Flock *et al*., 2011, Soga *et al*., 2005), whereas activation of intestinal GPR119 enhanced GLP-1 release from L-cells, GIP release from K-cells and delays gastric emptying (Lauffer *et al*., 2009, Overton *et al*., 2006, Overton *et al*., 2008). These actions promote glucose tolerance and highlighted GPR119 as an attractive target for the development of anti-diabetic drugs.

GPR119 binds OEA (Overton *et al*., 2006), lysophosphatidyl-choline (LPC) (Soga *et al*., 2005) and 2-oleoylglycerol (2-OG) (Hansen *et al*., 2012). OEA is an anorectic fatty-acid amide that is mobilized from intestinal enterocytes in response to ingested fat (Schwartz *et al*., 2008). OEA induces satiety in a vagal- afferent mediated pathway (Rodriguez de Fonseca *et al*., 2001) that is CD36-dependent and CCK-independent

(Schwartz *et al*., 2008). It is debatable if the satiety signal generated by OEA is mediated via paracrine activation of GPR119 receptor in EEC cells that leads to the secretion of anorectic peptides such as PYY and GLP-1.

Activation of GPR119 by 2-OG is another mechanism that may link presence of fat in the gut lumen with release of intestinal hormones. 2-OG belongs to the family of 2-monoacylglycerols and is a product of triglyceride digestion by pancreatic lipase, which is generated in high amounts in the gut lumen. 2-OG may activate directly GPR119 expressed in EEC cells to stimulate fat-induced release of gut hormones. Increased secretion of GLP-1 and GIP (but not CCK), after oral administration of 2-OG supports this hypothesis for L- and K-cells (Hansen *et al*., 2012). It has not been examined if I-cells express GPR119 and if GPR119 stimulation by OEA has any effect in circulating CCK levels.

1.5.4.5 Endocannabinoid receptors – CB1

Other FA-derived compounds that have a major role in the adaptation of the gut to energy intake are the classic endocannabinoid peptides N-arachidonoylethanolamine/anandamide (AEA) and 2-arachidonoylglycerol (2-AG). These key peptides of the gut endocannabinoid system are locally produced in the intestinal epithelium. Their synthesis and release is stimulated by food deprivation and inhibited by food intake (Izzo *et al*., 2009, Izzo and Sharkey, 2010, Gomez *et al*., 2002, Kirkham *et al*., 2002). This “on-demand” synthesis pattern of intestinal endocannabinoids suggests that they act as mediators of the state (empty or full) of the digestive system and when they are released, they transmit orexigenic signals (Maccarrone et al., 2015).

The endocannabinoid system has a pivotal role in the regulation of energy balance and the homeostatic and hedonic control of food intake via central (brain) or

peripheral pathways (Bellocchio *et al*., 2008). The intestinal endocannabinoid system is adapted to the diet and has currently a recognized role in the modulation of food intake, via a vagal-afferent mediated gut-to-brain signalling network. Activation of intestinal cannabinoid receptor CB1 is critical for the gut endocannabinoid system signalling (Di Marzo, 2011).

CB1 receptor has been identified as one GPCR for Δ9-tetrahydrocannabinol (Δ^9-THC), the psychotropic substance of *cannabis sativa* (Matsuda *et al*., 1990). Δ^9-THC is responsible for the stimulation of appetite, particularly for palatable high-fat food, an effect known as the “munchies” (Greenberg *et al*., 1976). Δ^9-THC has been shown to activate CB1 receptor, promoting food intake and weight gain (Matias and Di Marzo, 2007). On the other hand, blockade of CB1 receptor has been shown to suppress appetite (Colombo *et al*., 1998, Di Marzo *et al*., 2001) indicating CB1 receptor as a potential therapeutic target for the treatment of obesity. CB1 receptor is widely expressed in the CNS but also in peripheral neurons, in the liver, in the kidney and in the intestine.

There is evidence that CB1 receptor is expressed in the gastrointestinal tract but its expression pattern is rather obscure. It is controversial if CB1 receptor is expressed in intestinal epithelial cells. One study reported the expression of CB1 receptor in human colonic epithelium (Wright *et al*., 2005) whereas CB1 receptor expression was not detected in human duodenal epithelium (D'Argenio *et al*., 2007). CB1 receptor is mainly expressed in the enteric nervous system with a high density in the myenteric and the submucosal plexus (Wright *et al*., 2005). Its expression in vagal afferent fibres (Burdyga *et al*., 2004, Burdyga *et al*., 2006b, de Lartigue *et al*., 2010) has an important role in the transmission of orexigenic effects from the gut to the brain.

Energy restriction leads to a parallel increase of intestinal AEA release and upregulation of vagal afferent CB1 receptor levels that mediate the AEA-induced orexigenic effect to the brain (Burdyga *et al*., 2004, Gomez *et al*., 2002). These co-ordinated effects generate a strong appetite-promoting response that can be attenuated by peripheral CB1 antagonists (Gomez *et al*., 2002, LoVerme *et al*., 2009). High-fat diet has been shown to upregulate AEA levels and potentiate the orexigenic effect of intestinal endocannabinoids (Izzo *et al*., 2009). Additionally, diet-induced obesity animal models have enhanced expression of vagal afferent CB1 receptor and show an increased hunger effect in response to high-fat diet (Paulino *et al*., 2009).

A recent study shed light to the pathways by which intestinal endocannabinoid system stimulates intake of palatable high-fat food. It is suggested that a fatty meal can specifically induce production of AEA and 2-AG in the proximal small intestine. This effect is mediated by vagal afferent/efferent neurons and is dependent on the orosensory properties of fat. Increased levels of locally released endocannabinoids, in turn, stimulate further fat intake in a CB1-mediated response (DiPatrizio *et al*., 2011). The exact mechanism about how activation of intestinal CB1 receptors reinforces food (fat) intake is not defined. This mechanism may involve distinct but integrated pathways; one direct by activation of vagal afferent neurons and brainstem regions responsible for regulation of food intake and one indirect based on the inhibition of secretion of anorectic gut peptides (Di Marzo, 2011, DiPatrizio *et al*., 2011). Endocannabinoid peptides may inhibit CCK release from I-cells in a CB1-mediated way, to promote further fat intake (Di Marzo, 2011).

1.6 Hormonal content of duodenal EEC cells (I-cells?)

A presentation of other subtypes of duodenal EEC cells and the predominant hormones that they contain/secrete. Although the current EEC cells classification is based on the “one cell-one hormone” dogma, some of these EEC cells subsets have been previously suggested to overlap with I-cells.

1.6.1 S cells/ Secretin

Secretin-containing EEC cells are localized throughout the small intestine (Lam *et al*., 2006) with decreasing density towards ileum (Bryant and Bloom, 1979). Release of secreting is stimulated by gastric acid (Glad *et al*., 1996) and dietary nutrients, including fat and protein. Secretin has a protective role in the gastrointestinal tract against gastric acid and in parallel regulates food digestion. It inhibits directly gastric acid release (You and Chey, 1987) and gastric emptying (Jin *et al*., 1994), regulating the flow of gastric acid to duodenal epithelial cells. Additionally, secretin is a key secretagogue for the release of bicarbonate in the pancreatic juice (Jin *et al*., 1994, Konturek *et al*., 2003) and also stimulates the release of bicarbonate-enriched bile (Banales *et al*., 2006). Overall, secretin protects gastrointestinal epithelium from the excessive release of gastric acid and contributes to the co-ordination of digestion by the stimulating the release of bicarbonate-enriched pancreatic juice.

1.6.2 N cells/ Neurotensin

Neurotensin is released from N-cells, localized throughout the small intestine. The release of neurotensin is stimulated by gastric acid (Wallin *et al*., 1995) and lipids (Ferris *et al*., 1985). Lipid-induced neurotensin release is dependent on the formation

of LCFA by digestion of triglycerides and therefore can be inhibited by orlistat (Drewe *et al*., 2008). It has been suggested that the first phase of neurotensin release in response to LCFA is mediated by CCK signalling via CCK-A receptors (Drewe *et al*., 2008). Neurotensin has a multiple contribution in the regulation of digestion and the physiology of the gastrointestinal tract. It has an important role in the enterohepatic circulation and controls lipid digestion by enhancing bile acid secretion (Gui *et al*., 2000) and increasing bile acid absorption in ileum (Gui *et al*., 2001, Gui and Carraway, 2001). It also promotes the release of pancreatic enzymes (Wood *et al*., 1988) and increases small intestinal blood flow (Hammer *et al*., 1988), whereas it inhibits secretion of gastric acid (Blackburn *et al*., 1980). Furthermore, neurotensin can promote insulin release from pancreas (under low/basal glucose concentrations) (Beraud-Dufour *et al*., 2010) and has a cytoprotective and anti-apoptotic role, protecting pancreatic islets from cytotoxic agents (Coppola *et al*., 2008). In addition to its pro-digestive functions, neurotensin has been characterized as a peptide that transmits short-term anorectic signals and inhibits appetite (Boules *et al*., 2000, Cooke *et al*., 2009).

1.6.3 K cells/GIP

GIP (glucose-dependent insulinotropic peptide formerly known as gastric inhibitory polypeptide) is a 42-amino acid peptide released by K-cells, localized in upper small intestine (mainly duodenum) (Diakogiannaki *et al*., 2012, Takeda *et al*., 1987). It is the first gut hormone that was discovered to mediate the incretin effect, after the demonstration that it can enhance insulin release in healthy volunteers (Dupre *et al*., 1973) and it can induce insulin secretion when administered on pancreatic islets (Taminato *et al*., 1977). GIP release is stimulated by glucose (Elliott *et al*., 1993, Vollmer *et al*., 2008) and also lipids (Karhunen *et al.*, 2008, Vollmer *et al.*, 2008, Shibue *et al.*, 2015, Sommer and Mostoslavsky, 2014). Circulating GIP in plasma

reaches its peak concentration within 15-30 minutes after the consumption of a meal (Falko *et al*., 1975). LCFA generation in the gut lumen is critical for GIP release (Pilichiewicz *et al*., 2003), with monounsaturated FA being the most potent stimuli (Thomsen *et al*., 1999). Additionally, amino acids can trigger GIP release (Greenfield *et al*., 2009, Thomas *et al*., 1978). In parallel with the insulinotropic effect, GIP stimulates proliferation of pancreatic β cells (Xu *et al*., 1999) and has an anti-apoptotic effect on pancreatic islets (Kim *et al*., 2008, Kim *et al*., 2005). GIP also acts directly on the adipose tissue to regulate insulin sensitivity of adipocytes (Knapper *et al*., 1995) and to promote fat storage (Song *et al*., 2007) .

1.6.4 L cells

L-cells are localized throughout the gastrointestinal tract. They are present in low density in the upper small intestine and concentrated in the distal intestine (terminal ileum and colon). L-cells contain and release preproglucagon derived peptides (GLP-1, GLP-2 and OXM that are produced by PC1/3-mediated cleavage of preproglucagon) and PYY. Gut peptides released by L-cells are anorectic and their levels increase postprandially (Anini *et al*., 1999, Dhanvantari *et al*., 1996, Orskov *et al*., 1986)

1.6.4.1 Proglucagon-derived peptides

1.6.4.1.1 GLP-1

GLP-1 is a peptide generated from post-translational processing of pre-proglucagon and secreted form intestinal L-cells. GLP-1 is an incretin that has been shown to stimulate insulin release from isolated pancreatic islets (Schmidt *et al*., 1985) and healthy humans (Kreymann *et al*., 1987). It has been shown that, due to the rapid degradation by DPP-4, less than 15% of released GLP-1 is circulating in plasma (Deacon *et al*., 1996). Therefore, it is suggested that GLP-1 triggers insulin secretion

via a vagal-afferent mediated pathway (Nakabayashi *et al*., 1996). The insulinotropic action of GLP-1 is attenuated by chronic hyperglycaemia (Hojberg *et al*., 2008). Similarly with GIP, GLP-1 induces β- cells growth and acts as a β-cell cytoprotective (anti-apoptotic peptide) (Farilla *et al*., 2002, Wang and Brubaker, 2002).

GLP-1 plasma levels rise in minutes after the consumption of a meal and decrease during fasting (Nauck *et al*., 1996). Similarly with CCK and contrarily to GIP, GLP-1 reduces food intake (Flint *et al*., 1998, Naslund *et al*., 1998, Rinaman and Rothe, 2002). The anorexigenic effect of GLP-1 is abolished by the ablation of the vagal-brainstem-hypothalamic pathway, suggesting that vagus-mediated gut-to-brain signalling is responsible for the appetite-suppressing action of GLP-1 (Abbott *et al*., 2005a). In parallel, GLP-1 acts locally in the gastrointestinal tract to inhibit gastric acid release (O'Halloran *et al*., 1990, Wettergren *et al*., 1994) and to decelerate gastric emptying (Flint *et al*., 2001, Naslund *et al*., 1999). Additionally, GLP-1 inhibits glucagon release by binding to GLP-1 receptors expressed in pancreatic α-cells (Nauck *et al*., 1993)

1.6.4.1.2 GLP-2

GLP-2 is secreted following nutrient ingestion and acts mainly on the gastrointestinal tract. It induces growth of intestinal epithelium by stimulating proliferation of stem cells residing in intestinal crypts and by inhibiting apoptosis of epithelial cells. Therefore, it induces an increase in the height of crypt-villus axis, the surface area and the weight of the intestinal epithelium (Drucker, 2005, Drucker *et al*., 1996, Rowland and Brubaker, 2011). GLP-2 also inhibits gastric emptying, although not so efficiently as GLP-1 (Nagell *et al*., 2004). An important action of GLP-2 is that it facilitates the absorption of nutrients by stimulating the expression of nutrient transporters in enterocytes. It had been shown that GLP-2 stimulated intestinal lipids absorption and the subsequent assembly and release of chylomicrons from enterocytes, in a CD36-

mediated way (Hsieh *et al*., 2009). This paracrine loop links nutrient delivery to the intestine with the mechanisms that regulate the absorption and usage of ingested lipids in enterocytes.

1.6.4.1.3 OXM

OXM is a 37-amino acid peptide that was identified as an inhibitor of gastric acid release (Bataille *et al*., 1981) but acts principally as an anorexigenic hormone. OXM has been shown to suppress food intake (Cohen *et al*., 2003, Dakin *et al*., 2002). It is suggested that OXM stimulates weight loss in rodents and humans by a combined action that involves inhibition of food intake and increase of energy expenditure (Liu *et al*., 2010, Wynne *et al*., 2006). Additionally, OXM can stimulate insulin release but with lower potency in comparison with GLP-1 (Du *et al*., 2012b).

1.6.4.2 PYY

PYY is a 36-amino acid peptide released from (distal) intestinal L-cells. Recently, it has been reported that PYY is expressed in ~45% of terminal ileal L-cells and in ~70% of colonic L-cells (Habib *et al*., 2012). A targeted ablation of colonic PYY-expressing cells results in the reduction of GLP-1 levels by 95.9% confirming the co-expression of GLP-1 and PYY in L-cells (Sam *et al*., 2012). PYY is released postprandially and its concentration increases in less than 15 minutes and peaks within 1-2 hours, after a meal (Adrian *et al*., 1985). PYY is circulating in plasma as full length PYY_{1-36} or as the truncated form PYY_{3-36} that represents the predominant circulating PYY peptide. PYY_{3-36} is the product of PYY_{1-36} cleavage by DPP4 (Mentlein *et al*., 1993) and an agonist of the Y2 receptor (Medeiros and Turner, 1994). In contrast with PYY_{3-36}, PYY_{1-36} binds to Y1 and Y5 receptors (Dumont *et al*., 1995)

PYY was firstly characterized as a mediator of the “ileal brake” that inhibits intestinal motility in response to sensing of ingested nutrients (Lundberg *et al*., 1982), and as an

inhibitor of pancreatic exocrine release (Tatemoto, 1982). Different circulating forms of PYY have opposite effects on the regulation of appetite. Thus, PYY_{1-36} promotes food intake via Y1 receptor (and possibly Y5 receptor) (Kanatani *et al*., 2000) and PYY_{3-36} inhibits appetite via the inhibition of secretion of neuropeptide Y (NPY) from NPY-expressing neurons localized in the ARC nucleus (Batterham *et al*., 2002, Abbott *et al*., 2005b). PYY_{3-36} has been shown to have a significant anorectic effect in rodents and humans (Batterham *et al*., 2003, Batterham *et al*., 2002) but this effect was not replicated in other studies (Boggiano *et al*., 2005, Tschop *et al*., 2004) so it remains controversial how potent is the anorectic effect induced by PYY_{3-36}. Despite this controversy, there is evidence that PYY_{3-36} stimulates neuronal activity in regions of brain associated with regulation of appetite (Batterham *et al*., 2007) and increased circulating levels of PYY_{3-36} contribute to weight-loss after bariatric surgery (Korner *et al*., 2006). Additionally, it has been reported that postprandial PYY release is attenuated in obese people, resulting in increased food intake (Pfluger *et al*., 2007). It is unclear if the effect of PYY_{3-36} is mediated via Y2 receptors expressed in vagal afferent neurons (Abbott *et al*., 2005b, Koda *et al*., 2005). The nutrient-dependent and CCK-mediated regulation of the expression of Y2 receptor in vagal afferent fibres may influence the capacity of PYY_{3-36} signalling to the brain (Burdyga *et al*., 2008).

1.6.5 X/A (P/D1) cells -Ghrelin

Ghrelin was discovered in 1999 as a ligand for the growth-hormone secretagogue receptor 1α (GHS-R1α) (Kojima *et al*., 1999) and is considered so far as the only circulating orexigenic peptide. Ghrelin is released by X/A cells (P/D1 in humans), localized mainly within gastric oxyntic glands, that produce ~65-80% of circulating plasma levels (Hosoda *et al*., 2006, Ariyasu *et al*., 2001). X/A cells are also found scattered throughout small intestine and colon, with decreased density towards distal intestine (Sakata *et al*., 2002). Ghrelin is a 28-amino acid peptide that can be acylated

(acyl ghrelin) or non-acylated (des-acyl ghrelin). Des-acyl ghrelin represents the vast majority (80-90%) of circulating ghrelin. The acylation of ghrelin is required for its activity (binding to ghrelin receptor / GHS-R1α) and is mediated by the enzyme ghrelin O-acyltransferase (GOAT) that links a MCFA (C8-C10) to Ser3 amino acid of ghrelin (Gutierrez *et al*., 2008, Yang *et al*., 2008).

Levels of circulating ghrelin increase during fasting and peak before the anticipated meal (Cummings *et al*., 2001, Tschop *et al*., 2001) whereas they are suppressed following nutrient ingestion (Tschop *et al*., 2001). Ghrelin induces food intake (Tschop *et al*., 2000, Wren *et al*., 2001) by increasing frequency of meals while it does not affect meal size. However, mouse models characterized by lack of ghrelin or lack of ghrelin receptor do not show altered long-term food intake indicating that ghrelin is probably involved in the short-term regulation of food intake (Kirchner *et al*., 2009, Pfluger *et al*., 2008, Zigman *et al*., 2005).

1.7 Aims

The aim of this study was to isolate and characterize duodenal I-cells on a molecular level.

A transgenic CCK-eGFP (enhanced GFP is expressed under the control of the Cck gene promoter) animal model was used to isolate native duodenal I-cells.

Aim 1: Develop and optimize a protocol for the isolation of duodenal I-cells

A targeted transcriptomic semi-quantitative RT-PCR analysis was subsequently performed on cDNA template prepared from sorted I-cells and non I-cells to investigate the hypotheses a) that I-cells contain mRNA transcripts encoding key fatty acid and endocannabinoid peptide receptors and b) that I-cells do not contain only CCK but rather express multiple gut hormones.

Aim 2: Investigate if duodenal contain mRNA transcripts encoding key GPCRs that act as LCFA, SCFA, fatty-acid derivatives and endocannabinoid receptors

After the identification of mRNAs encoding key chemosensors in I-cells, it was examined if gene expression of these GPCRs is regulated by the energy status of the organism.

Aim 3: Investigate if mRNA levels of GPCRs expressed in I-cells are regulated by fasting/feeding

Aim 4: Define the hormonal content of duodenal I-cells

Finally, an antibody against human pro-CCK was made in order to be used for the future plan to isolate human duodenal I-cells.

Aim 5: Development of an antibody against human pro-CCK, a human I-cell specific intracellular protein

CHAPTER 2

Duodenal I-cells contain mRNA transcripts encoding key fatty acid and endocannabinoid receptors

This chapter is an extended and modified version of the article SYKARAS, A. G., DEMENIS, C., CASE, R. M., MCLAUGHLIN, J. T. & SMITH, C. P. 2012. Duodenal Enteroendocrine I-Cells Contain mRNA Transcripts Encoding Key Endocannabinoid and Fatty Acid Receptors. *PLoS ONE,* 7**,** e42373.

Author contributions: I generated data depicted in all figures except for figure 2.1. I wrote the first draft and the revised version of the manuscript.

2.1 Abstract

Gut hormones released by enteroendocrine cells have a pivotal role in the regulation of food digestion and the control of appetite and energy balance. Cholecystokinin is the archetypal satiety hormone that is secreted from a subtype of enteroendocrine cells, classically termed I-cells, in response to ingested nutrients (principally fat but also amino acids). I-cells are mainly localized in the duodenum and have a potentially key role in the nutrient sensing and the co-ordination of food digestion. Despite their important biological function, native I-cells have not been isolated and studied yet. In this study, we developed a robust protocol for the isolation and characterization of native duodenal I-cells. Additionally, we performed a targeted gene expression analysis by using semi-quantitative RT-PCR and we determined that mouse duodenal I-cells are enriched in mRNA transcripts encoding key fatty acid sensors and endocannabinoid receptors. These include the long chain fatty acid receptors GPR40/FFAR1, GPR120/O3FAR1; the short chain fatty acid receptors GPR41/FFAR3 and GPR43/FFAR2; the oleoylethanolamide receptor GPR119 and the classic endocannabinoid receptor CB1. Our analysis provides an experimental method for the isolation of native I-cells and indicates that I-cells may express key G-protein coupled receptors that mediate nutrient sensing. Our data suggest that I-cells may respond to a wide range of fatty acids that are present in the gut lumen after food digestion and also have the ability to sense fatty-acid derivatives or endocannabinoid peptides, that are synthesized by intestinal epithelial cells (enterocytes).

2.2 Introduction

Endocrine cells distributed throughout the intestinal tract integrate dietary and pathological cues and, via hormonal and neural signals, orchestrate multiple tissues to co-ordinate food digestion and regulate appetite. Collectively these cells are termed enteroendocrine (EEC) cells and they constitute ~1% of the intestinal epithelial cell population (Diakogiannaki *et al*., Engelstoft *et al*., 2008, Rindi *et al*., 2004, Sternini *et al*., 2008, Moran *et al*., 2008).

I-cells are a subset of duodenal EEC cells that express the anti-orexigenic and principal satiety peptide hormone cholecystokinin (CCK) (Buchan *et al*., 1978, Smith *et al*., 1985, Polak *et al*., 1975). CCK is released by I-cells in response to luminal nutrients, in particular fatty acids and amino acids (Liddle *et al*., 1985). CCK co-ordinates digestion by inhibiting gastric emptying, and by stimulating gallbladder contraction and pancreatic enzyme secretion (Chandra and Liddle, 2007). I-cells are therefore pivotal in the intestinal response to nutrients in so far as they are suggested to sense luminal gut nutrients by membrane bound G-protein coupled receptors (GPCRs) (Engelstoft *et al*., 2008, Liou *et al*., 2011a), integrate nutrient signals and transmit these signals both centrally and peripherally by hormone release, and to the brain by vagal afferent-mediated signalling.

Transcripts encoding the long chain fatty acid receptors (LCFA) free fatty acid receptor 1 (FFAR1, formerly known as GPR40) (Briscoe *et al*., 2003, Itoh *et al*., 2003) and omega-3 fatty acid receptor 1 (O3FAR1, formerly known as GPR120) (Hirasawa *et al*., 2005, Oh *et al*., 2010) are present in I-cells (Liou *et al*., 2011a). Signalling by GPR40/FFAR1 has been suggested to regulate CCK release from I-cells (Liou *et al*.,

2011a). Interestingly, in humans McLaughlin *et al* have reported release of CCK in response to intragastric fatty acids with chain lengths matching the ligand profiles of GPR40/FFAR1 and GPR120/O3FAR1 (McLaughlin *et al*., 1999). In addition to GPR40/FFAR1 and GPR120/O3FAR1, other GPCRs have been implicated in EEC cell nutrient sensing and appetite regulation. These include the short chain fatty acid (SCFA) receptors free fatty acid receptor 3 (FFAR3, formerly known as GPR41) and free fatty acid receptor 2 (FFAR2, formerly known as GPR43) (Brown *et al*., 2003, Le Poul *et al*., 2003, Tolhurst *et al*., 2012a). GPR41/FFAR3 is highly enriched in duodenal and colonic L-cells and also in CCK-containing cells of the small intestine (Samuel *et al*., 2008). It has been proposed that GRP41/FFAR3 acts as a sensor of SCFA generated by bacterial fermentation of polysaccharides (Samuel *et al*., 2008). GPR43/FFAR2 is expressed in duodenal and colonic L-cells and mediates GLP-1 release in response to SCFA.

GPCRs belonging to the endocannabinoid receptors family are also known to be expressed in the small intestine, but their localization and distribution within duodenal epithelium remains undetermined. These include GPR119 that binds oleoylethanolamide (OEA), an anorectic lipid amide that is a derivative of fatty acid digestion (Overton *et al*., 2006) and 2-oleoylglycerol, a product of digestion of dietary triacylglycerol (Hansen *et al*., 2012). Activation of GPR119 stimulates glucagon-like peptide 1 (GLP-1) release from L-cells (Lauffer *et al*., 2009, Overton *et al*., 2006, Overton *et al*., 2008), enhances glucose-stimulated insulin secretion and inhibits gastric emptying (Chu *et al*., 2008, Chu *et al*., 2007, Flock *et al*.). In addition to GPR119, the cannabinoid receptor 1 (CB1) is a GPCR that has a key role in the regulation of appetite. There is evidence that CB1 receptor is expressed in vagal afferent neurons where it mediates the transmission of orexigenic signals to brain (de

Lartigue *et al*., Burdyga *et al*., 2006b, Burdyga *et al*., 2004), but its expression in duodenal epithelium remains obscure.

The study of enteroendocrine cells is difficult because of their diffuse and sparse distribution, and their relatively indistinct morphology. In the past, research has focused on surrogate models, such as the enteroendocrine cell lines STC-1 and GLUTag, which represent at best approximations of native enteroendocrine cells. The recent engineering of transgenic mouse models with genetically tagged genes that encode gut hormones, enabling fluorescent delineation of native EEC cells, has ushered in a new era of EEC research research (Habib *et al*., 2012, Liou *et al*., 2011b, Parker *et al*., 2009, Reimann *et al*., 2008, Tolhurst *et al*., 2009, Wang *et al*., 2011, Liou *et al*., 2011a, Tolhurst *et al*., 2012a, Tolhurst *et al*., 2011).

In this study we describe a robust method to isolate I-cells and use these isolated cell populations to probe the I-cell transcriptome for key nutrient sensors and endocannabinoid receptors. As a proof of principle, we perform a targeted gene expression analysis of I-cells. Our results confirm that duodenal I-cells contain mRNA transcripts that encode LCFA receptors GPR40/FFAR1 and GPR120/O3FAR1 and are highly enriched in mRNA transcripts of SCFA receptors (Gpr41/Ffar3 and Gpr43/Ffar2) and the endocannabinoid receptors GPR119 and CB1.

2.3 Methods

2.3.1 Ethics Statement

All animal procedures used in this study were ethically approved by the University of Manchester Ethical Review Process Committee, in accordance with the UK Home Office regulations, under licence 40/3409. All animal procedures used in this study were in accordance with Animals Scientific Procedures Act 1986 (UK) and UK Home Office regulations.

2.3.2 Experimental animals

CCK-eGFP mice were purchased from MMRRC (Mutant Mouse Regional Resource Center, USA). CCK-eGFP (Tg(CCK-EGFP)BJ203Gsat/Mmmh) is a transgenic mouse model that expresses enhanced Green Fluorescence protein (eGFP) under the control of *Cck* gene promoter and was generated as part of the GENSAT (Gene Expression Nervous System Atlas) project at Rockefeller University (Gong *et al*., 2003). Mice were bred in-house and were kept on a 12h light: dark cycle with ad libitum food and water. Adult male mice (8-16 weeks old) were used for all the experiments. The presence of the transgene was verified by genotyping, according to the instructions provided by MMRRC (http://www.mmrrc.org/catalog/sds.php?mmrrc_id=249)

2.3.3 Imaging of CCK-eGFP cells

Cryosections were prepared from mouse duodenum, fixed in 4% paraformaldehyde (PFA) and cryoprotected overnight in 30% sucrose/PBS. 6µm cryosections were mounted onto superfrost plus slides. Sections were thawed at 60^{o}C for 30 mins, soaked in 4% PFA 15 mins and washed in dH_20. Antigen sites were blocked for 30

minutes in 50mM NH_4Cl-PBS, and permeabilised in buffer (1% BSA, 0.2% gelatine, 0.05% saponin-PBS). Sections were then incubated overnight with anti-rabbit anti-CCK antiserum L421 at 1/500 dilution in antibody buffer (0.1% BSA, 0.3% Triton-X in PBS). After incubation with primary antibody, sections were returned to room temperature for 60 minutes, sections were washed three times in 0.1% BSA, 0.2% gelatine, 0.05% saponin in PBS buffer, after which goat anti-rabbit IgG Alexafluor594 (A-11012) secondary antibody was applied at 1/1000 dilution in antibody buffer for 90 minutes at room temperature. Slides were washed 3 times in PBS, incubated for 15 minutes with nuclei acid stain Hoechst 33342 (Invitrogen, UK) at a final concentration of 0.5 ng/µl, washed with dH_20 and mounted with DAKO glycergel. They were visualised on Olympus BX51 upright microscope using a 20X or 40X objective. Specific band pass (BP) filter sets for Hoechst 33342 (excitation BP 350/50 nm, emission BP 460/50 nm), GFP (excitation BP 480/40nm, emission 535/50 nm) and Texas Red (excitation BP 560/55, emission 645/75 nm) were used to avoid channel bleed-through. Images were captured using a coolsnap ES camera (Photometrix) through MetaVue Software (Molecular Devices) and processed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/)

2.3.4 Preparation of isolated duodenal epithelial cells.

Four adult male CCK-GFP mice were used in each experiment. CD-1 adult mice or mice that were verified by genotyping that did not express the transgene (eGFP-negative) were used as control. Mice were anaesthetized with CO_2 and then killed by dislocation of the neck. Duodenum (the first 5-6 cm of the small intestine, after the pyloric sphincter) was removed and put into ice-cold PBS. After the removal of luminal contents, the tissue was rinsed with PBS several times and was dissected

longitudinally and then cut laterally into 5-10 mm pieces, in order to achieve a maximum expose of the epithelium to the dissociation buffer.

A chemical/mechanical method was used for the dissociation of the epithelium. Tissue fragments were placed into 100mm Petri-dish containing ice-cold calcium-magnesium free Hanks Balanced Salt Solution (CMF-HBSS) supplemented with 5% Foetal Bovine Serum (FBS) and 0.5 mM ditheiothreitol (DTT) and shaken gently for 10 minutes at 4^0C. Then, duodenal pieces were transferred into conical tubes containing CMF-HBSS with 5% FBS, 0.6 mM DTT and 1mM 2,2', 2'', 2'''-(Ethane-1, 2-diyldinitrilo) tetraacetic acid (EDTA). Epithelium was dissociated into single-cells by shaking at 37^0C for 20 minutes (175 rpm for the first 10 minutes and 100 rpm for another 10 minutes). Dissociated single cells were pelleted by centrifugation for 5 minutes at 150-250g at room temperature. The resulting cell pellet was resuspended into FACS-sorting buffer (PBS containing 3% FBS) and the resulting cell suspension culture was filtered through a 40 μm cell strainer to ensure that only dissociated single cells will be subject to Fluorescence Activated Cell Sorting (FACS) analysis (Protocol A).

We have also used successfully an alternative protocol for the isolation of duodenal epithelial single-cells. This alternative protocol is based on the same principle (chemical/mechanical dissociation) but the dissociation buffer contains only EDTA instead of DTT/EDTA. In this protocol, the tissue fragments were placed directly into conical tubes containing CMF-HBSS supplied with 5% FBS and 1mM and dissociated for 30 minutes (15 min at 175 rpm and 15 minutes at 100 rpm). Then, single-cells prepared for FACS as in protocol A (Protocol B).

2.3.5 FACS sorting of eGFP+ and eGFP- cells

FACS analysis was performed by using a BD FACS Aria (DIVA version 5 software) cell sorter (BD Biosciences). Propidium Iodide (PI) or Sytox Red (Invitrogen, UK) were added to the single-cells suspension in order to identify dead cells. Dead cells (cells stained with PI or SytoxRed) were detected and excluded from further analysis by using either a 488 nm laser (617/25 bandpass filter) for PI, or a 633 nm laser (660/20 bandpass filter) for Sytox Red. A forward scatter versus side scatter plot analysis of the PI-negative cell population was used in order to exclude debris from further analysis. The viable single-cell population was then analyzed on the basis of eGFP fluorescence intensity. A 488 nm laser was used for excitation and fluorescent signal was detected at a 530/30 nm bandpass. Dispersed duodenal single cells from a CD-1 or a wild type mouse (cells that do not display eGFP fluorescence) were used as a control sample that enabled us to establish the background level of autofluorescence, permitting gating of the most intense fluorescent cells. Finally, living cells were sorted (directly into lysis buffer for RNA preparation) into two populations, one cell population of eGFP+ cells and a second population of (approximately equal number) of eGFP- cells.

2.3.6 RNA isolation/RNA (cDNA) amplification

RNA was prepared from sorted cells using RNA aqueous micro kit (AM1931, Ambion, UK) according to the manufacturer's protocol. Briefly, eGFP+ and eGFP- cells were sorted directly into 500 µl of cell lysis buffer. Then, 250 µl of 100% ethanol were added to the lysate and the mixture was passed through a micro filter cartridge. RNA bound to the filter was washed twice and eluted to a final volume of 20 µl. In order to confirm the integrity of RNA and calculate its relative concentration, we used RNA 6000 Pico Chip Assay on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was treated

with DNAseI (Ambion, UK) before reverse transcription to avoid contamination of cDNA with genomic DNA.

For amplification of cDNA generated by RNA extracted from sorted cells, we used the Ovation Pico WTA System (Nugen), which requires as template 500pg-50ng of RNA. Amplification started with 5 μl of RNA as template (total amount of RNA was 2-5 ng) and involved three steps. The first step was the generation of first strand cDNA using a mix of oligodT and random primers, ensuring amplification of the whole transcriptome. The second step involved the generation of a second DNA strand, which results in double-stranded cDNA that contains a DNA/RNA hetero-duplex in one end. This cDNA was purified by binding to magnetic beads and washed with ethanol that removed all contaminants. Then, it was amplified in a linear isothermal way by using a mixture of a specific DNA/RNA chimeric primer, DNA polymerase and RNase H. Amplification was performed at several cycles, where primer binds to cDNA and initiates replication, whereas RNase H cleaves remaining RNA on the generated product and exposes a new binding site for primers to start another round of amplification. Amplified cDNA was purified using Qiagen PCR Purification Kit (Qiagen, UK) and eluted at a final volume of 30 μl. The concentration of amplified cDNA was measured using Nanodrop (Nanodrop Technologies).

2.3.7 Semi-quantitative reverse-transcription PCR (RT-PCR) analysis

Reverse transcriptase PCR was performed using unamplified RNA from sorted cells. cDNA was synthesized from RNA using Superscript III reverse transcriptase (Invitrogen, UK). In parallel, identical reactions were setup in which Superscript

enzyme was omitted and replaced by ddH_2O. These samples were used as no reverse transcriptase (–RT) controls, to ensure that PCR products were not a result of genomic DNA contamination. The final volume of RT+ and RT- reactions were 20 μl of cDNA and 1 μl was used as template in each PCR reaction except from the PCR reaction for CB1 in which 1.5 μl was used as starting material.

Amplified cDNA was diluted 1/50 and 1 μl of diluted cDNA was used as template in each reaction, except RT-PCR for Gpr119 (1.2 μl), Gpr120 (1.2 μl), CB1 (1.2 μl) and Gpr43 (1.2 μl). RNA that had not been amplified was diluted accordingly and served as negative (-RT) control. The quantity of starting cDNA template from eGFP+ and eGFP- samples was normalized using 18S rRNA housekeeping gene. PCR was performed using Biotaq (Bioline) and a typical mastermix for each reaction contained 10x NH_4 reaction buffer (670mM Tris-HCl pH 8.8, 160mM $(NH_4)_2SO_4$, 100mM KCl, 0.1% stabilizer), 3mM $MgCl_2$, 0.8mM dNTPs, 4% DMSO, 0.8 μM of each primer, 1.75 Units of Biotaq and H_2O up to 25 μl. Oligomers used in the study are listed in Table 2.1. Reaction details (annealing temperature, cycles) are listed in Table 2.2. RT-PCR products were analysed by electrophoresis on a 2% TBE (Tris/Borate/EDTA buffer) agarose gel. Sequencing of PCR amplicons was performed at University of Manchester Sequencing Core Facility.

Target	Forward Primer (5→3)	Reverse Primer (5→3)	Amplicon Size (bp)
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151
Cck	CGCTGGAACTCGCCAAGCCA	GCGGCCAGAAGGAGCTTTGC	270
Akp3	CTCATCTCCAACATGGAC	TGCTTAGCACTTTCACGG	334
Muc2	ACGATGCCTACACCAAGGTC	TGATCTTCTGCATGTTCCCA	210
eGFP	ACCCTCGTGACCACCCTGACCTAC	CGTCCTTGAAGAAGATGGTGCG	133
Gpr40/ Ffar1	CAGTGTCCCACGCTAAACT	GGCAGAAAGAAGAGCAGAAT	493
Gpr40/ Ffar1 **(Fig2.8)**	ATCCGAGGCGCAGTGTCCCA	AGCCTCCGCCTGCGTAGAGG	208
Gpr41 /Ffar3	AGTGCCAGTTGTCCAATACTC	GAAGAGGGAGGTGAGGTAAAT	337
Gpr41/ Ffar3 **(Fig2.8)**	TTGCTAAACCTGACCATTTCGG	GATAGGCCACGCTCAGAAAAC	199
Gpr43/ Ffar2	CTTGATCCTCACGGCCTACAT	CCAGGGTCAGATTAAGCAGGAG	137
Gpr119	GCTGATTGCCTTTGACAGAT	GAGTGGGTTGAGTAGGGAGTT	541
Gpr119 **(Fig2.8)**	TGGCAGAGGGAGGTTCGGCA	TCCTGCAGCGTCTTAGCCATCG	177
Gpr120 /O3far1	TCTGCCACCTGCTCTTCTA	TTGTTGGGACACTCGGAT	446
Gpr120 /O3far1 **(Fig2.8)**	ACCAAGTCAATCGCACCCAC	GTGAGACGACAAAGATGAGCC	111
CB1	CGGGGGATGCGAAGGGGTTC	ACCGTGAAGGTGCCCAGGGT	472
CB1 **(Fig2.8)**	CGGGGGATGCGAAGGGGTTC	GCCCACGTAGAGGAGGTCTGTGG	149

Table 2.1 Oligomers used in the study.
Primer sequences used for RT-PCR and the expected size (bp) of the amplicons are presented. For each GPCR, two sets of primers were used. Primer sequences for GPR43 and for GPCRs RT-PCR analysis from amplified cDNA (Figure 2.8) were obtained from PrimerBank (Harvard,USA) (Spandidos *et al*., 2010).

Primer pair (figure)		Denaturation	Annealing	Extension	Number of Cycles
Gpr40/Ffar1		94 °C 60sec	54°C 60sec	72°C 60sec	41
Gpr40/Ffar1 **(Fig 2.8)**		94°C 30sec	62°C 30sec	72°C 30sec	Low 29 High32
Gpr41/Ffar3		94°C 60sec	55°C 60sec	72°C 60sec	41
Gpr41/Ffar3 **(Fig 2.8)**		94°C 30sec	60°C 30sec	72°C 30sec	Low 30 High 34
Gpr43/Ffar2		94°C 30sec	61°C 30sec	72°C 30sec	39
Gpr43/Ffar2 **(Fig 2.8)**		94°C 30sec	61°C 30sec	72°C 30sec	Low 31 High 35
Gpr119		94°C 60sec	56.5°C 60sec	72°C 60sec	41
Gpr119 **(Fig 2.8)**		94°C 30sec	61.5°C 30sec	72°C 30sec	Low 31 High 35
Gpr120/O3far1		94°C 60sec	55.5°C 60sec	72°C 60sec	42
Gpr120/O3far1 **(Fig 2.8)**		94°C 30sec	62°C 30sec	72°C 30sec	Low 31 High 35
CB1		94°C 60sec	60.5°C 60sec	72°C 60sec	43
CB1 **(Fig 2.8)**	A	94°C 30sec	68°C 30sec	72°C 30sec	Low 32 High 36
	B	94°C 30sec	64°C 30sec	72°C 30sec	
Cck **(Fig 2.4,2.7)**		94°C 45sec	60°C 45sec	72°C 45sec	30
Cck **(Fig 2.8)**		94°C 45sec	60°C 45sec	72°C 45sec	Low 21 High 24
Cck **(Fig 2.5)**		94°C 45sec	60°C 45sec	72°C 45sec	Fig 2.5A 33 Fig 2.5B 27
eGFP		94°C 30sec	56°C 30sec	72°C 30sec	Low 21 High 24
Akp3		94°C 45sec	53.5°C 45sec	72°C 45sec	35
Akp3 **(Fig 2.8)**		94°C 45sec	53.5°C 45sec	72°C 45sec	Low 22 High 25
Muc2 **(Fig 2.8)**		94°C 30sec	56°C 30sec	72°C 30sec	Low 23 High 26
18S rRNA **(Fig 2.7)**		94°C 30sec	58°C 30sec	72°C 30sec	24
18S rRNA **(Fig 2.8)**		94°C 30sec	58°C 30sec	72°C 30sec	Low 14 High 18
18S rRNA **(Fig 2.5)**		94°C 30sec	58°C 30sec	72°C 30sec	Fig 2.5A 22 Fig 2.5B 20

Table 2.2 PCR cycling parameters.
Target names of primer pairs are listed along with the figure number in brackets where corresponding results are shown. The initial denaturation variables and final extension variables for all reactions were 95°C for 5 mins and 72°C for 10min, respectively. Data for CB1

shown in figure 2.8 was generated using a split cycling program where cycles 1-12 are shown in row CB1 A and cycles >12 are shown in CB1 row B.

2.4 Results

2.4.1 Validation of experimental model/Imaging of duodenal I-cells

In order to isolate duodenal I-cells, we used a mouse model that has genetically labelled CCK-expressing cells. This BAC (Bacterial Artificial Chromosome) transgenic mouse strain expresses an eGFP reporter that is regulated by CCK promoter elements. To validate our experimental model, we first examined cryosections of duodenum and observed that eGFP expression was restricted to few scattered cells that represent less than 1% of epithelial cell population (counting slides from 3 mice) and lay intermittently between intestinal epithelial cells (enterocytes). eGFP-labelled cells had a flask-shaped morphology that is characteristic of enteroendocrine cells; characterized by a narrow apical membrane in contact with the gut lumen and a much broader basolateral membrane (Figure 2.1A). These eGFP-labelled cells can be classified as “open-type” cells because their apical membrane projects to the gut lumen. In order to verify that eGFP fluorescence specifically labels CCK-expressing cells, we performed immunostaining with a CCK-specific antibody and showed that there is colocalization of eGFP with CCK in eGFP-tagged cells (Figure 2.1B). Counting of eGFP-positive and CCK-positive cells in multiple duodenal slides revealed that 88.4% (SEM ± 1.25, n=19 slides-prepared from 3 mice) of eGFP-tagged cells expressed CCK. Additionally, we did not notice any CCK-positive cells that did not display eGFP fluorescence. The colocalization of eGFP and CCK confirmed that the CCK promoter transgene faithfully labelled duodenal CCK-containing I-cells. The CCK-staining pattern that we observed was characteristic of I-cells in that it was very intense towards the basolateral membrane of the cell, where the CCK containing secretory vesicles congregate in preparation for release (Figures 2.1B, 2.1C).

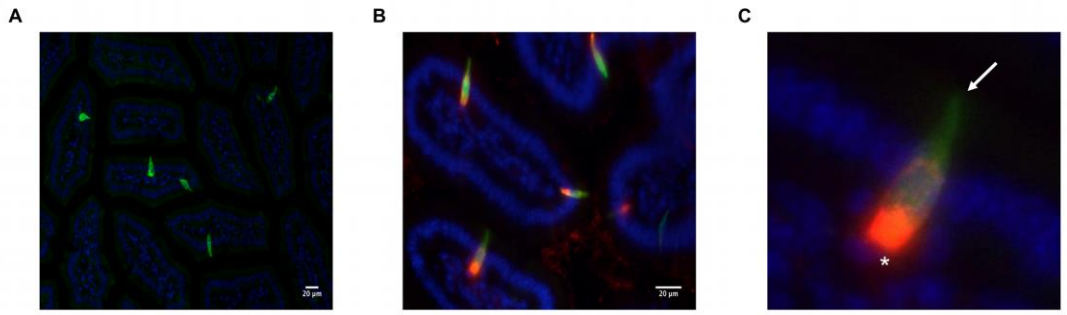

Figure 2.1 eGFP expressing cells are strongly immunopositive for CCK
Representive images of transverse sections of duodenum from CCK-eGFP mice. A: eGFP-tagged cells (green) were flask-shaped with a diffuse distribution, characteristic of 'open-type' enteroendocrine cell. B: Immunostaining with anti-CCK antibody (red) showed colocalization of CCK and eGFP in ~ 90% of eGFP-labelled cells. C: High magnification micrograph (2.5X digital zoom) revealed I-cells had a narrow apical membrane (arrow) that was in contact with gut lumen and a broad basolateral membrane (asterisk) with adjacent strong CCK staining. In all images, nuclei were counter-stained with Hoechst 33342 (blue). Courtesy of Claire Demenis and Craig P. Smith

2.4.2 FACS sorting of eGFP+ cells (I-cells)

In order to isolate native I-cells, we performed FACS-sorting analysis on a population of dissociated duodenal epithelial cells. We used a chemical method (Protocol A/Protocol B described in Methods section) to dissociate duodenal epithelium into a single-cell population of dispersed cells that can be subjected to FACS analysis (Figure 2A). Both protocols yielded very similar results in terms of the: a) morphology of dissociated cells; b) viability of dissociated cells (there was a high variability in the viability of dissociated cells in each method but not any clear difference in the cell viability between the two methods); c) forward and side scatter profile of cell population analyzed by FACS; d) percentage of eGFP+ cells; e) number of sorted eGFP+ cells f) quality of RNA prepared from sorted cells.

Imaging of dissociated cells revealed that there was no discernable difference in morphology between isolated eGFP+ enteroendocrine and epithelial cells. In each

optical field, we observed eGFP+ cells that represented less than 1% of the total cell population and displayed different levels of fluorescence intensity (Figure 2.3A). These differences were evident even in preparations of dispersed cells from a single mouse, therefore they cannot be explained by different levels of eGFP expression due to higher/lower number of transgene copies integrated in the genome of different mice. The range of eGFP fluorescence intensity rather indicates that there are sub-populations of I-cells that display different activity of the Cck gene promoter.

Dissociated cells from control mice (wild-type or CD-1) and transgenic CCK-eGFP mice were exposed to propidium iodide (PI) prior to FACS analysis, to exclude dead cells. Gating of live cells (P1) revealed a sub-population of highly fluorescent cells (P2) that were present only in cells prepared from transgenic mice (Figure 2.2B). Sorted eGFP+ cells represented 0.3-0.7% of the viable duodenal epithelial cell population in the vast majority of FACS sorting experiments that we performed. In each sorting experiment that we used duodena from four mice, we could obtain typically between 5000-15000 eGFP+ cells. Given that the percentage of eGFP+ cells was approximately equal in each preparation, the number of eGFP+ sorted cells mainly depended on the viability of dissociated cells prior to FACS.

The purity of FACS-sorted eGFP+ cells was confirmed by epi-fluorescence microscopy. By visualizing sorted cells, we estimated the purity of sorted eGFP+ cells routinely to be above 90% (Figure 2.3A, B, C).

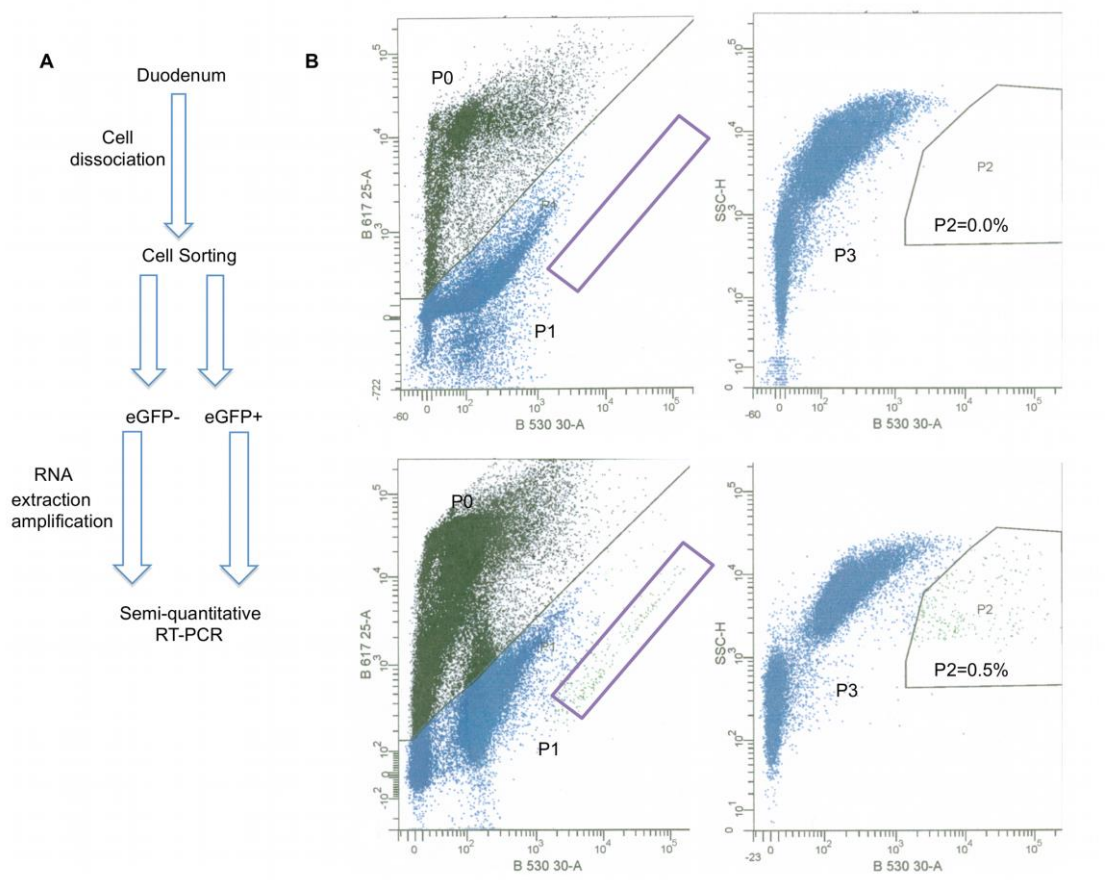

Figure 2.2 FACS-sorting of eGFP+ and eGFP-cells.
A: Schematic overview of the methods employed to isolate I-cells and perform transcript analysis. B: Dissociated duodenal cells from eGFP-CCK mice were sorted using a BD FACS Aria cell sorter (lower panels). Dissociated duodenal cells from a WT/CD-1 mouse were used as a control to set gating parameters for analysis and sorting (upper panels). Cells were first stained with PI and analyzed by eGFP (X-axis) and PI (Y-axis) fluorescence intensity (left panels). PI- positive cells (dead cells, P0) were excluded from further analysis. Live cells (Blue, P1) from CCK-eGFP mice displayed a population of highly fluorescent cells (purple rectangle) that were not present in cell from WT mice. Live cells were also analyzed based on eGFP fluorescence intensity (X-axis) and side scatter (Y-axis). Cells that expressed eGFP were gated as eGFP+ cells, representing 0.5% of total cell population (P2, right lower panel). This population was not present in cells from WT mice (P2, right upper panel). All other cells (P3) were gated as eGFP-. eGFP+ cells and approximately equal number of eGFP- cells were sorted.

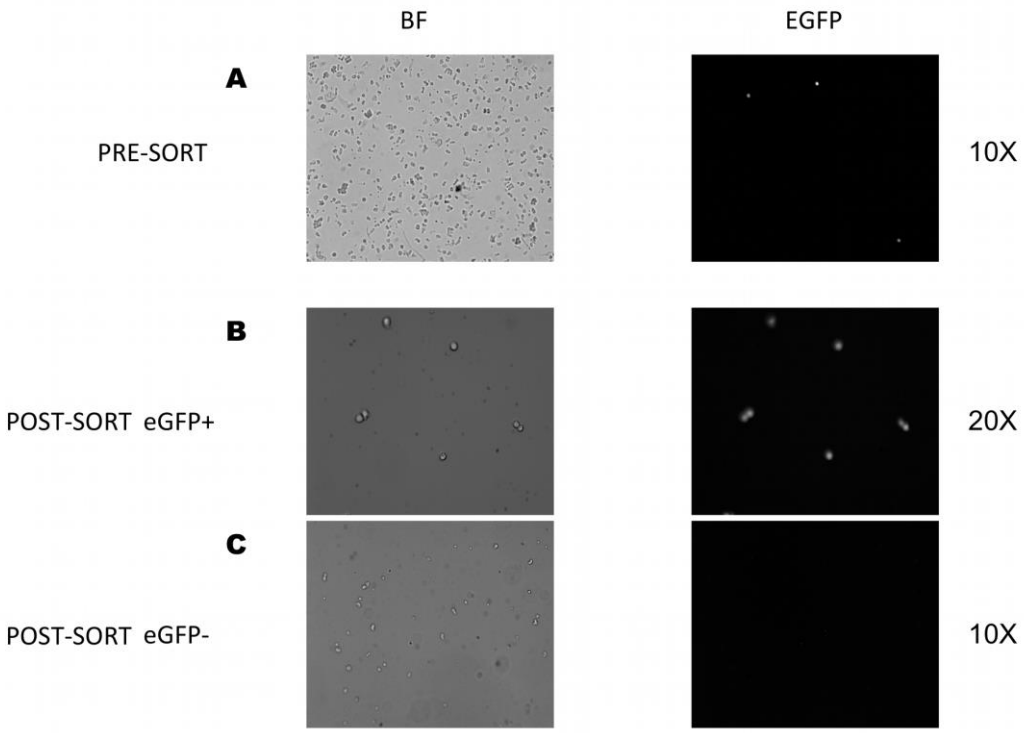

Figure 2.3 Imaging of eGFP+ and eGFP- cells pre- and post- FACS.
A: Imaging of dissociated duodenal single-cells. eGFP+ cells were less than 1% of total cell population and displayed different levels of eGFP fluorescence. B: Imaging of sorted eGFP+ cells. Sorted eGFP+ cells display eGFP fluorescence. C: Imaging of sorted eGFP- cells. Fluorescent cells were absent. Imaging confirms the successful FACS sorting of a highly enriched population of eGFP+ cells (A, B, C).

2.4.3 Sorted eGFP+ cells represent I-cells

We then investigated if sorted eGFP+ cells truly represent I-cells by performing semi-quantitative RT-PCR for Cck mRNA, a transcript that is the defining characteristic of I-cells. RNA was extracted from sorted eGFP+ and eGFP- cells. Since RNA extraction from FACS-sorted cells may result in partially or totally degraded RNA, and this degradation may compromise the results of subsequent applications such as RT-PCR, we verified the integrity of RNA prepared from eGFP+ and eGFP- using a RNA 6000 PicoChip on an Agilent 2100 Bioanalyzer. The presence of intact ribosomal bands (28S and 18S RNA) in each sample (RIN score >5) confirmed the integrity of purified RNA (Figure 2.4A). Using semi-quantitative RT-PCR, *Cck* transcripts were

only detected in eGFP+ cells and thus confirmed that eGFP+ cells represent very highly enriched I-cells (Figure 2.4B). RT-PCR for the enterocyte marker gene *Akp3* (intestinal alkaline phosphatase 3) (Narisawa *et al*., 2003) showed that this transcript was highly expressed in eGFP- cells, with a much lower expression level in eGFP+ cells, indicating a minimum contamination of eGFP+ cells with enterocytes (Figure 2.4B).

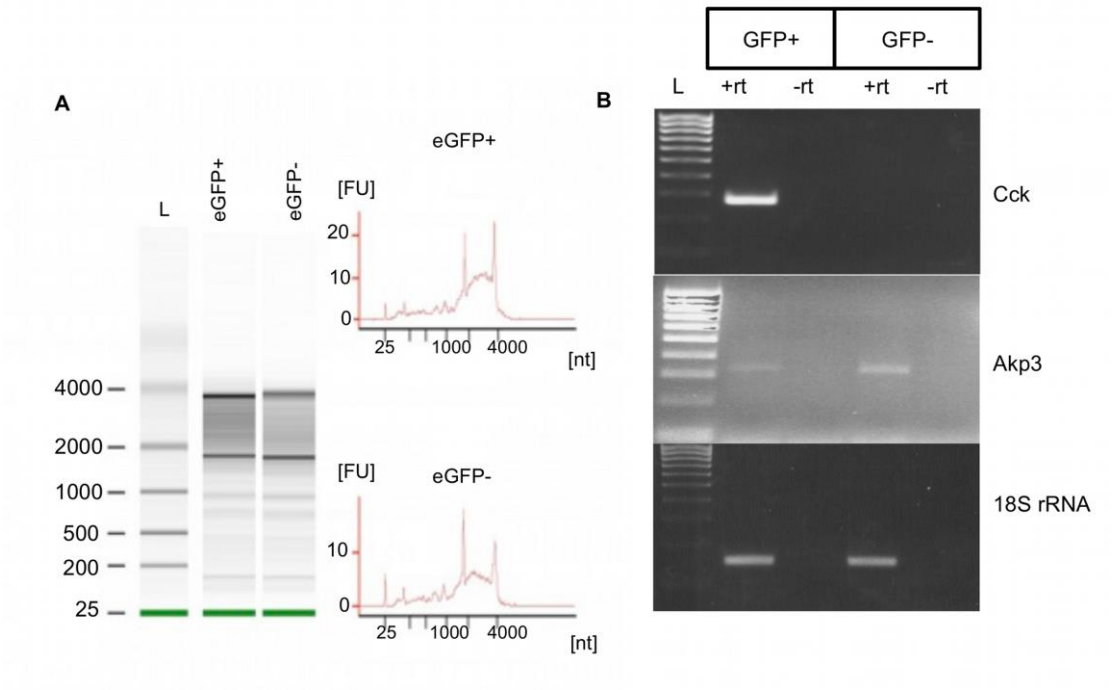

Figure 2.4 eGFP+ sorted cells represent a highly enriched population of I-cells.
A: Analysis of RNA extracted from eGFP+ and eGFP- cells. RNA was analyzed using a RNA 6000 PicoChip kit. Intact 28S and 18S ribosomal bands (gels, left panel) or distinct 28S and 18S RNA ribosomal peaks (electropherograms, right panel) confirmed integrity of extracted RNA from sorted cells. L indicates Pico 6000 RNA ladder that spans 0.2-6.0 kb. B: eGFP+ cells were highly enriched in Cck mRNA transcripts. Semi-quantitative PCR for Cck mRNA demonstrated that only eGFP+ cells contain Cck mRNA transcript, a marker of I-cells. Akp3 mRNA transcript, a marker of enterocytes, was mainly detected on eGFP- cells, showing little contamination of eGFP+ cells (I-cells) with enterocytes. 18S rRNA was used as a loading control. L declares Hyperladder IV (100-1000 bp, Bioline, UK)

Additionally, we wanted to ensure that sorted eGFP- cells were depleted of I-cells. Thus, we compared the amount of Cck mRNA transcripts in eGFP- sorted cells with unsorted dissociated duodenal cells (input material for FACS analysis). We detected Cck mRNA transcript in unsorted dissociated duodenal cells where I-cells represent less than 1% of total cells (confirming the sensitivity of the RT-PCR assay), but not in eGFP- cells (Figure 2.5A), indicating that sorted eGFP- cells were depleted of I-cells. Enrichment of I-cells in eGFP+ sorted cells (as expected) was confirmed by comparing the amount of Cck mRNA transcript in eGFP+ sorted cells with unsorted dissociated duodenal cells (Figure 2.5B).

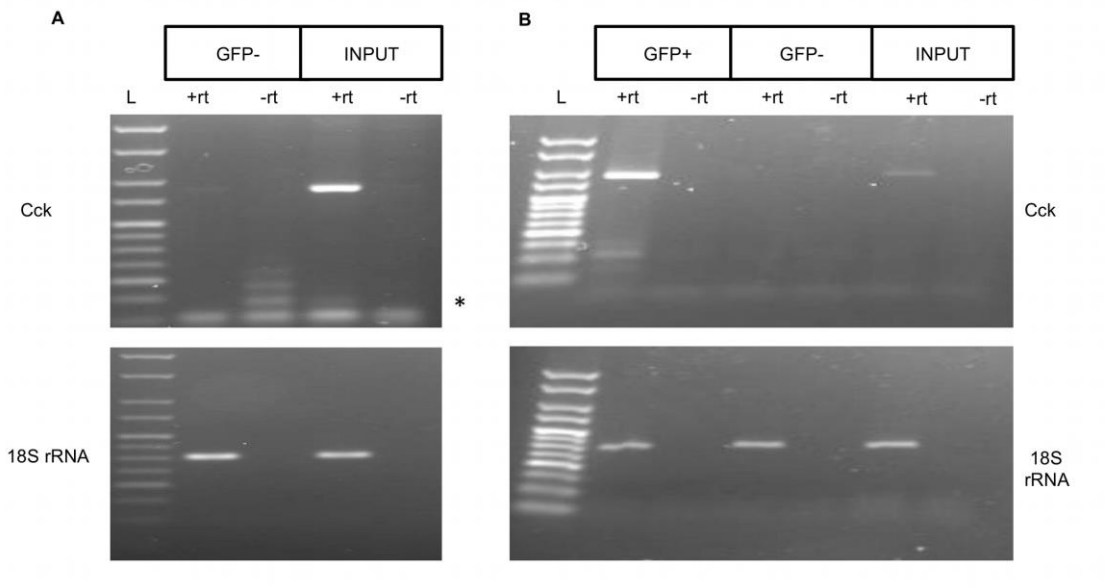

Figure 2.5 eGFP+ cells are enriched in I-cells whereas eGFP- cells are I-cells depleted. A: Semi-quantitative RT-PCR analysis of Cck mRNA transcript in dissociated duodenal cells and eGFP- cells showed that the assay is sensitive enough to detect Cck mRNA transcript in the starting population where the I-cells represent < 1% of the cell population. After 33 cycles of PCR Cck mRNA transcript was detected in the dissociated duodenal cells (INPUT), but was not present in eGFP- cells. 18S rRNA was used as loading control (21 cycles). B: Semi-quantitative analysis of Cck mRNA transcript levels revealed that eGFP+ cells are enriched in I-cells in comparison with the starting dissociated cell population (INPUT). Cck PCR products were amplified for 27 cycles to ensure that we avoid the plateau phase of the reaction for eGFP+ sample. The amount of starting cDNA templates are equal with these shown at Panel A. 18S rRNA was used as loading control (18 cycles). L = Hyperladder V (50-250bp, Bioline, UK). Asterisk indicates primer dimers.

2.4.4 I-cells contain mRNA transcripts encoding LCFA receptors (GPR40/FFAR1, GPR120/O3FAR1), SCFA receptors (GPR41/FFAR3 and GPR43/FFAR2) and endocannabinoid receptors (GPR119 and CB1)

We first investigated if mRNA transcripts of key GPCRs that have critical role in nutrient sensing are expressed in the duodenal mucosa by performing semi-quantitative RT-PCR on RNA extracted from dissociated duodenal epithelial cells. We found that duodenal epithelial cells contain mRNA transcripts encoding these nutrient sensors. Surprisingly, we also discovered that CB1 receptor mRNA transcript is also expressed in the duodenal epithelium and is not only present in vagal afferent neurons (as we confirmed by performing RT-PCR on RNA extracted from duodenal tissue that had intact muscle layer) (Figure 2.6).

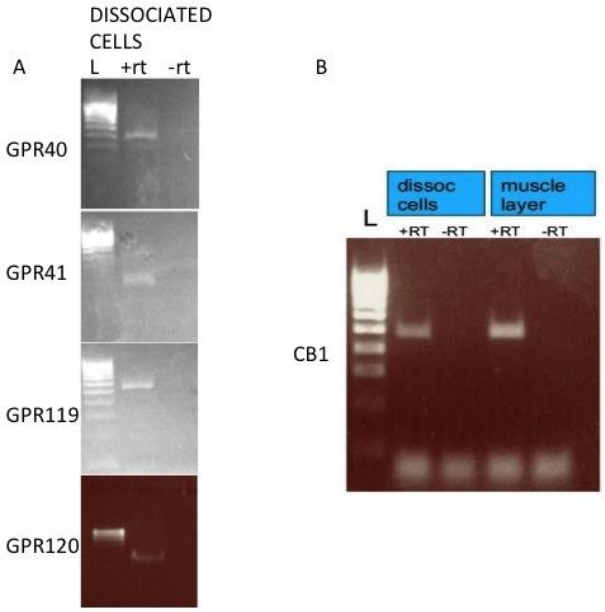

Figure 2.6 Duodenal mucosal cells contain mRNA transcripts encoding GPR40/FFAR1, GPR41/FFAR3, GPR119, GPR120/O3FAR1 and CB1 receptor
A. Semi-quantitative RT-PCR analysis reveals the presence of GPR40, GPR41, GPR119 and GPR120 mRNA transcripts in duodenal dissociated cells. B.CB1 receptor mRNA transcript is detected in dissociated duodenal mucosal cells. cDNA prepared from duodenal tissue that contains an intact muscle layer served as a positive control for CB1 receptor RT-PCR reaction. L represents Hyperladder IV and +/-rt declare the presence/absence of reverse transcriptase. –rt served as a control to exclude amplification of transcripts due to genomic DNA contamination.

Then, we used semi-quantitative RT-PCR to determine the presence in I-cells of mRNA transcripts encoding key GPCRs involved in nutrient sensing (Figure 2.7). Transcripts encoding LCFA receptors GPR40/FFAR1 and GPR120/O3FAR1 were expressed in I-cells: Gpr40/Ffar1 mRNA was highly enriched in I-cells and could not be detected in non I-cells (eGFP- cells); Gpr120/O3far1 mRNA was detected in both I-cells and non I-cells, though the amount of amplified product showed that this target was enriched in I-cells and therefore unlikely to be due to any contaminating cDNAs from non I-cells; SCFA receptor Gpr41/Ffar3 mRNA transcript was found to be abundant in I-cells and not present in non I-cells.

We then determined if OEA receptor Gpr119 and endocannabinoid receptor CB1 mRNA transcripts were present in I-cells. First, we detected transcripts for both Gpr119 and CB1 in the mixed populations of dissociated cells (figure 2.6) so then we proceeded to analyse their expression pattern in I-cells. Semi-quantitative RT-PCR showed that Gpr119 and CB1 mRNA transcripts were specifically expressed in I-cells and were not detectable in nonI-cells (Figure 2.7, left panel). In an independent sorting experiment, we performed semi-quantitative RT-PCR for Gpr43/Ffar2 and found that this transcript was present in I-cells (Figure 2.7, right panel).

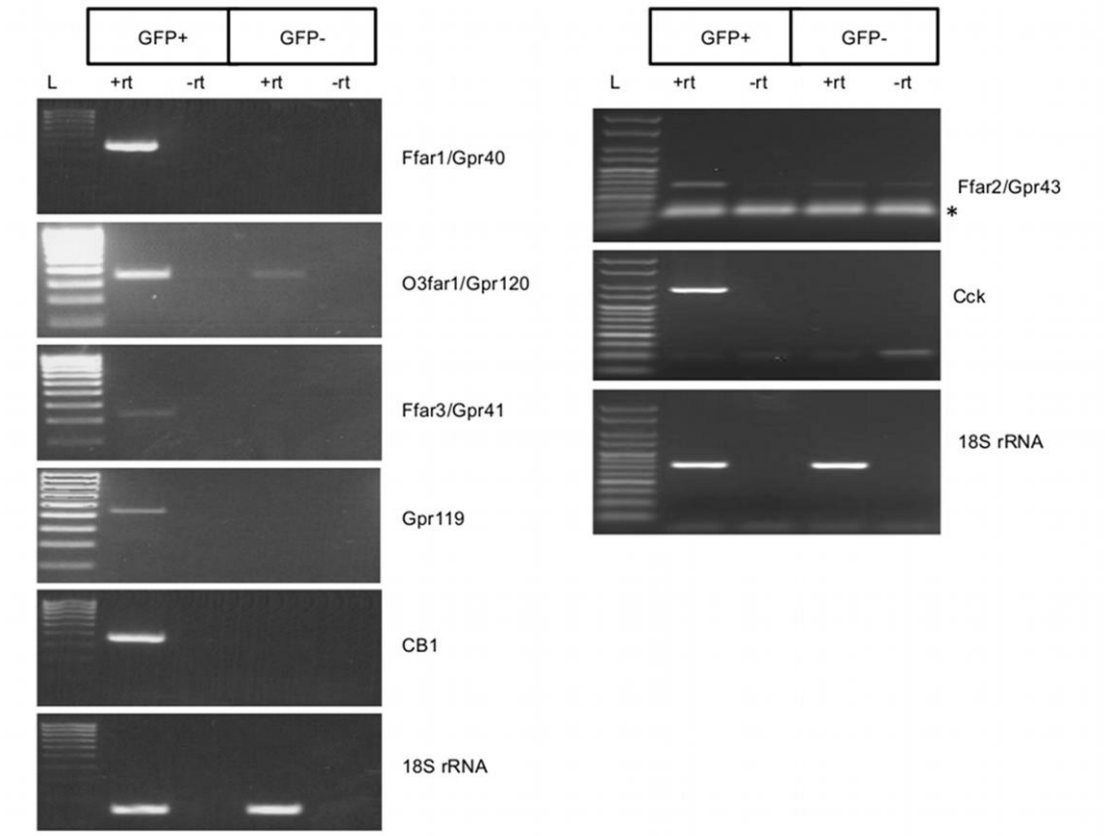

Figure 2.7 I-cells contain GPR40, GPR120, GPR41, GPR43, GPR119 and CB1 mRNA transcripts.
Semi-quantitative RT-PCR analysis revealed that eGFP+ cells (I-cells) were enriched in mRNA transcripts encoding Gpr40/Ffar1, Gpr41/Ffar3, Gpr43/FFAR2, Gpr119 and CB1 whereas Gpr120/O3far1 was enriched in eGFP+ cells, but was also present eGFP- cells; RT-PCR of 18S rRNA confirms that equal amount of cDNA template from eGFP+ and eGFP- cells was used for the analysis. L declares Hyperladder IV (100-1000bp, Bioline, UK). Asterisk indicates bands representing primer dimers.

2.4.5 Confirmation of GPCRs enrichment by semi-quantitative RT-PCR on amplified cDNA from eGFP+ and eGFP- cells

Because the amounts of RNA isolated from FACS sorted cells were limiting, we engineered an amplified cDNA library from sorted eGFP+ cells and eGFP- cells RNA to enable validation of our RT-PCR analysis and for future transcriptome analysis. RNA/cDNA amplification generated more than 5 µg of cDNA from a starting material of 2-5 ng total RNA. We then performed detailed independent semi-quantitative PCR analysis (with different sets of primers) on amplified cDNA.

Our analysis of eGFP+ and eGFP- cDNA verified that eGFP+ cells exclusively contained both Cck and eGFP mRNA transcripts compared to eGFP- cDNA pools confirming enrichment of I-cells and suggesting that eGFP- cells were not contaminated with I-cells. Thus, the exclusive presence of a transcript in eGFP+ cells strongly suggests that the encoded protein is expressed in I-cells. We also performed PCR for transcripts that are characteristic of enterocytes (*Akp3*) and goblet cells (*Muc2*, mucin 2) (Chang *et al*., 1994b), and showed that they are much more abundant in eGFP- cells than eGFP+ cells. This result demonstrated that eGFP+ do not represent a 100% pure population of I-cells but rather a highly-enriched I-cells population with little contamination from epithelial and goblet cells (Figure 2.8, left panel).

Semi-quantitative RT-PCR results confirmed our previous findings. Gpr40/Ffar1 is highly enriched in eGFP+ cells and not present in non I-cells, whereas Gpr120/O3far1 is present in both cell populations but enriched in I-cells. I-cells are also highly enriched in mRNA transcripts of SCFA receptors Gpr41/Ffar3 and Gpr43/Ffar2. Using

the amplified cDNA pools, we also confirmed that endocannabinoid receptors Gpr119 and CB1 mRNA transcripts are highly enriched in I-cells (Figure 2.8, right panel).

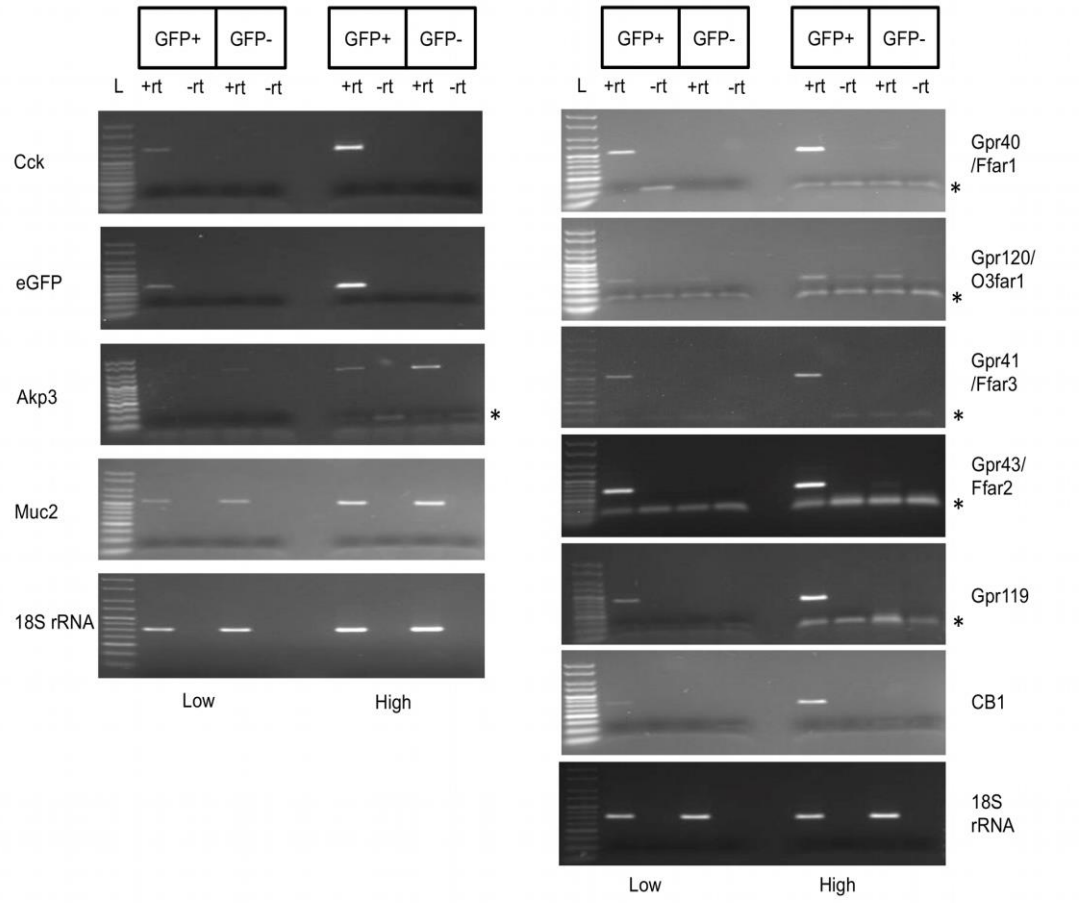

Figure 2.8 Semi-quantitative RT-PCR on amplified cDNA from sorted cells validates I-cells enriched mRNA transcripts.
Semi-quantitative RT-PCR analysis using amplified cDNA from eGFP+ and eGFP- cells. Left panel: eGFP+ cells were highly enriched in mRNA transcripts encoding eGFP and CCK whereas these transcripts were not detected in eGFP- cells. eGFP- cells were enriched in mRNA transcripts encoding AKP3 and MUC2, markers of enterocytes and goblet cells respectively. Right panel: Validation of Gpr40/Ffar1, Gpr120/O3far1, Gpr41/Ffar3, Gpr43/Ffar2, Gpr119 and CB1 mRNA transcripts enrichment in I-cells. 18S rRNA was used as a loading control. PCR products were amplified simultaneously, using different number of cycles (low-for lower number of cycles and high-for higher number of cycles). Equal volumes of each reaction were run on the same 2% agarose gel. L declares Hyperladder V (50-250bp, Bioline, UK). Asterisk indicates primer dimers.

2.4.6 Regulation of CB1 mRNA transcript by fasting/re-feeding

Finally, we examined if the expression of nutrient sensors in duodenal mucosal layer is regulated by fasting/re-feeding. We particularly focused on the hypothesis that CB1 receptor mRNA levels are regulated by energy intake, similarly with what has been reported for vagal afferent neurons. We compared CB1 receptor mRNA levels using duodenal mixed cell populations from 3 different groups of mice (fed *ad libitum,* fasted for 16 hours and fasted for 16 hours/re-fed for 5 hours, 3 mice/group). Semi-quantitative RT-PCR indicated that CB1 receptor mRNA is upregulated during fasting. Re-feeding of fasted mice down-regulates the expression of CB1 receptor mRNA transcript. Our results suggest that, similarly with vagal afferent neurons, CB1 receptor mRNA is upregulated by energy restriction. On the contrary, mRNA levels of GPR40/FFAR1, GPR41/FFAR3 and GPR120/O3FAR1 are stable during the fasting/refeeding protocol suggesting that they are not regulated by energy intake/restriction (Figure 2.9)

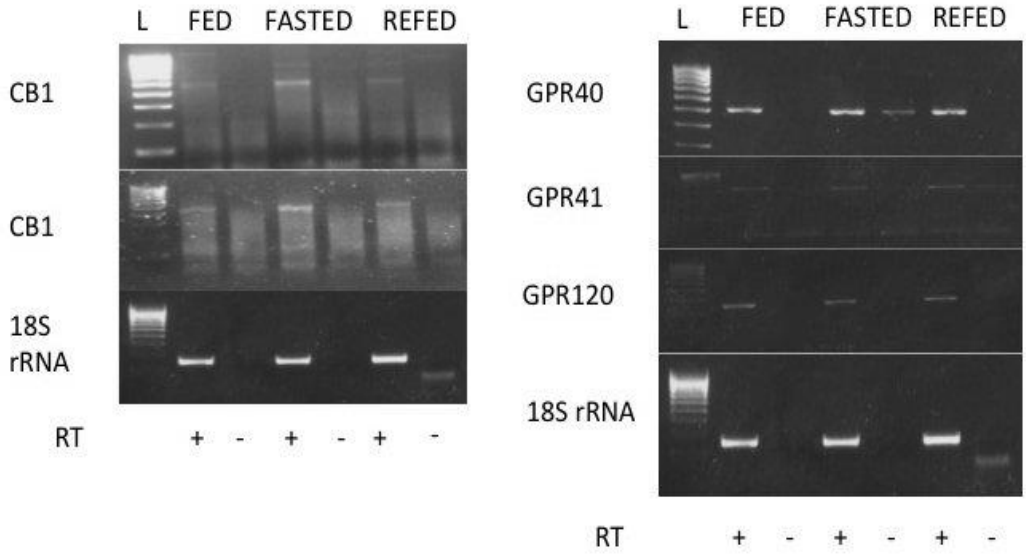

Figure 2.9 CB1 receptor mRNA transcript is upregulated during energy restriction
Semi-quantitative RT-PCR for CB1 receptor, GPR40/FFAR1, GPR41/FFAR3 and GPR120/O3FAR1 was performed on (RNA extracted from) dissociated duodenal cells from fed ad libitum, fasted and fasted/refed mice. CB1 receptor mRNA transcript is upregulated during fasting and down-regulated (to basal levels) upon acute re-feeding (left panel, 2 replicates for CB1 RT-PCR). GPR40/FFAR1, GPR41/FFAR3 and GPR120/O3FAR1 mRNA transcript levels remain equal during fasting/re-feeding (right panel). 18S rRNA served as housekeeping gene to demonstrate equal amount of starting material. L declares Hyperladder IV.-rt samples represent negative controls to exclude genomic DNA contamination.

2.5 Discussion

In this study, we developed a rigorous and reproducible method for the isolation of duodenal I-cells and performed a targeted transcriptomic analysis to determine if I-cells expressed mRNA transcripts encoding GPCRs that have been ascribed pivotal roles in sensing of fatty acids, endocannabinoid peptides or lipid amide derivatives.

First, we validated the fidelity of the transgene expression in the CCK-eGFP mouse (Tg (CCK-EGFP) BJ203Gsat/Mmmh) duodenum. eGFP-tagged cells were observed to be diffuse, rare (less than 1%) and have the morphological characteristics of classic 'open type' EEC cells. We also observed that eGFP+ cells displayed a spectrum of different levels of eGFP fluorescence, probably due to differential activity of the CCK promoter. Immunohistochemistry verified that the majority (approximately 90%) of eGFP-tagged cells expressed CCK, thus confirming the reliability of this animal model for the study of I-cells. It is possible that the expression level of CCK in the ~10% of cells that expressed eGFP, but that were not stained by the CCK antisera, was very low and below the detection limit of the immunostaining method we employed. In agreement with other studies (Liou *et al*., 2011a, Liou *et al*., 2011b, Wang *et al*., 2011), FACS analysis showed that eGFP+ cells represented 0.3-0.7% of the total duodenal cell population.

Using FACS, we sorted populations of eGFP+ and eGFP- cells and successfully extracted RNA of high quality from these cells. RT-PCR for CCK mRNA, the characterizing marker of I-cells, and eGFP, the resident transgene, confirmed that sorted eGFP+ cells represented a highly enriched population of I-cells. RT-PCR for marker genes of possible contaminating enterocytes or goblet cells revealed that the

eGFP+ cell population, although not devoid of contamination, represented a highly enriched I-cell population with little contamination from enterocytes and goblet cells. These results instilled confidence that the subsequent amplification of PCR products from eGFP+ cDNA pools were not a result of contamination from enterocytes or goblet cells but representative of the I-cells transcriptome.

mRNA transcripts encoding the LCFA receptors GPR40/FFAR1 and GPR120/O3FAR1 have been reported to be present in EEC cells and specifically I-cells (Liou *et al*., 2011a, Edfalk *et al*., 2008). Our results showing the enrichment of Gpr40/Ffar1 and Gpr120/O3far1 mRNA transcripts in I-cells are confirmatory of our successful purification of I-cells using the method we developed. Presence of Gpr40/Ffar1 and Gpr120/O3far1 mRNA transcripts in I-cells support the concept that activation of these nutrient sensors triggers or modulates CCK release in response to LCFA (Liou *et al*., 2011a, Tanaka *et al*., 2008). GPR40/FFAR1 has been suggested to be responsible for mediating fatty-acid induced CCK secretion in native I-cells (Liou *et al*., 2011a). In addition, GPR120/O3FAR1 has been reported to be responsible for triggering fatty-acid induced CCK secretion in the I-cell surrogate cell line model, STC-1 cells (Tanaka *et al*., 2008). Our finding that Gpr120/O3far1 mRNA is present in non I-cells may indicate a function for this receptor in enterocytes, where it may function to co-ordination of lipid absorption. Gpr120/O3far1 mRNA transcript is also present in macrophages (Oh *et al*., 2010), so its presence in intestinal macrophages may also explain its detection in non I-cells cDNA preparation. Clearly, functional studies are needed on native I-cells to clarify the contribution of GPR40/FFAR1 and GPR120/O3FAR1 in the modulation of fatty-acid induced CCK secretion by I-cells.

In the present study we report for the first time that duodenal I-cells are highly enriched in mRNAs encoding the SCFA receptors GPR41/FFAR3 and GPR43/FFAR2. Previously, Samuel *et al* (Samuel *et al*., 2008) showed that Gpr41/Ffar3 mRNA was expressed in small intestine with highest expression in ileum and reported that Gpr41/Ffar3 mRNA was highly enriched in CCK-containing enteroendocrine cells isolated from small intestine. Our results refine this latter observation by showing that Gpr41/Ffar3 mRNA is present in duodenal I-cells. Additionally, we show that Gpr43/Ffar2 mRNA is highly enriched in I-cells.

Without definitive knowledge of the localization of GPR41/FFAR3 and GPR43/FFAR2 within the I-cells, in particular whether the receptors sense luminal ligands or basolateral (plasma) ligands, it is difficult to ascribe a possible function to GPR41/FFAR3 and GPR43/FFAR2. However, it has been shown that CCK is released only in response to LCFA, but not SCFA (McLaughlin *et al*., 1999, McLaughlin *et al*., 1998), so it is unlikely that GPR41/FFAR3 or GPR43/FFAR2 in I-cells have a critical role as nutrient sensors regulating CCK secretion. On the other hand, there is evidence that GPR41/FFAR3 mediates secretion of enteroendocrine hormone PYY in response to SCFA generated from fermentation of dietary fibre by gut microbiota (Samuel *et al*., 2008). Thus, GPR41/FFAR3 regulates intestinal transit time (PYY delays the digestion process), absorption of SCFA and hepatic lipogenesis and is suggested to play a critical role as a link that connects gut microflora and energy balance. GPR41/FFAR3 and GPR43/FFAR2 also mediate GLP-1 release upon stimulation by SCFA, regulating glucose tolerance. Interestingly, GLP-1 release seems to be dependent on a Gq-mediated signalling pathway indicating a potentially crucial role of GPR43/FFAR2. Additionally, a study by *Lin* et al reported that GPR41/FFAR3 is not involved in butyrate or propionate-induced GIP release and only partially mediates

GLP-1 release in response to butyrate (Lin *et al*., 2012) .These findings may suggest that GPR43/FFAR2 has an important role in SCFA-induced release of gut hormones.

It is intriguing that duodenal I-cells, like small intestinal L-cells (Tolhurst *et al*., 2012a), are highly enriched in Gpr41/Ffar3 and Gpr43/Ffar3 mRNA transcripts , although the concentration of SCFA in the duodenal micro-environment is very low in comparison with distal ileum/colon where, due to bacterial metabolism, SCFA are present in high concentrations. With these findings in mind, GRP41/FFAR3 and GPR43/FFAR2 in duodenal I-cells may not function as apical projecting gut lumen sensors, but sense circulating SCFA in plasma to modulate I-cell function. The availability of specific antisera targeting GPR41/FFAR3 and GPR43/FFAR2 may answer this intriguing question.

Another GPCR mRNA transcript enriched in duodenal I-cells is Gpr119. Gpr119 mRNA expression has been previously reported in L- (Reimann *et al*., 2008, Lauffer *et al*., 2009) and K-cells (Parker *et al*., 2009). GPR119 can be classified as an endocannabinoid receptor that is activated by fatty-acid derived ethanolamides, such as the anandamide-related peptide OEA, and mediates GLP-1 release from L-cells (Overton *et al*., 2008, Lauffer *et al*., 2009). It is an open question whether binding of OEA or other natural acylethanolamide to GPR119 expressed in I-cells results in CCK secretion. OEA has been reported as a satiety messenger that is mobilized from the intestinal epithelium in response to fat ingestion (Schwartz *et al*., 2008). This satiety pathway is CD36-dependent and CCK-independent. The presence of Gpr119 in I-cells may indicate a link between the two satiety pathways (CCK-dependent and CCK-independent) that are activated after fat ingestion.

The presence of Gpr119 mRNA in I-cells led us to demonstrate that the classic endocannabinoid receptor CB1 mRNA transcript is highly expressed in I-cells. CB1 is expressed in vagal afferents (Burdyga *et al*., 2004) and has a critical role in the short-term regulation of appetite. Post-prandially, CCK down-regulates the expression of the orexigenic-signalling associated cannabinoid receptor CB1 in vagal afferent neurons, to inhibit food intake. CB1 receptor expression is up-regulated during fasting, when CCK levels are low and intestinal endocannabinoids levels are increased, to promote stimulation of appetite. Despite the agreement about the expression of CB1 receptor in vagal afferent neurons, its expression in intestinal epithelial cells is controversial (Wright *et al*., 2005, D'Argenio *et al*., 2007). We found that I-cells are highly enriched in the CB1 mRNA transcript. We also revealed that CB1 receptor mRNA transcript levels in I-cells are regulated in a similar way as in vagal afferent neurons, they are up-regulated during energy restriction and down-regulated during energy intake periods. Our findings suggest that intestinal endocannabinoids may regulate I-cell function. In an elegant study, DiPatrizio *et. al* recently reported that intestinal endocannabinoids were elevated in response to a fatty meal (DiPatrizio *et al*., 2011). This response, which was dependent on the orosensory properties of *the* fatty meal, was mediated by vagal afferents and was found to stimulate fat intake by signalling via CB1 receptors (DiPatrizio *et al*., 2011). Based on these findings, it is suggested that endocannabinoids might inhibit CCK release from duodenal enteroendocrine cells, and thereby stimulate food intake (Di Marzo, 2011, DiPatrizio and Piomelli, 2015, DiPatrizio et al., 2013). Our finding that CB1 mRNA is present in I-cells supports this hypothesis and may provide a means by which the local endocannabinoid system can regulate satiety pathways possibly by modulating CCK release.

By comparing the results of the current study with that of others detailing expression profiles of duodenal/proximal small intestine EEC cells, it can be seen that the

expression profile of nutrient sensors in different types of EEC cells is very similar. Studies from Gribble, Reimann and colleagues have shown proximal intestinal L-cells to be highly enriched for mRNA transcripts encoding GPR40/FFAR1, GPR41/FFAR3, GPR119 and GPR120/O3FAR1 (Reimann *et al*., 2008). Duodenal K-cells are also highly enriched in mRNA transcripts encoding GPR40/FFAR1, GPR119 and GPR120/O3FAR1 (Parker *et al*., 2009). This common repertoire of nutrient sensor GPCRs may suggest that enteroendocrine cells localized in the same segment of the intestine share similar mechanisms of nutrient sensing. A recent paper from the Gribble/Reimann group reported an extensive overlap between the hormone content of upper small intestinal L- and K- indicating that these EEC subtypes may have a similar profile of receptors that recognize nutrients and regulate hormone secretion (Habib *et al*., 2012).

In summary, we have developed a protocol for the isolation of duodenal I-cells. We have employed this approach to sort I-cells and perform targeted transcriptomic analysis of mRNA transcripts that encode nutrient sensors and endocannabinoid receptors. RT-PCR analysis was performed directly on unamplified or amplified cDNA from FACS sorted cells, with similar results. Our data suggest that mRNA transcripts encoding the LCFA receptors GPR40/FFAR1 and GPR120/O3FAR1, SCFA receptors GPR41/FFAR3 and GPR43/FFAR2, lipid amides receptor GPR119 and endocannabinoid receptor CB1 are enriched in duodenal I-cells. The presence of these GPCRs transcripts supports the suggestion that they act as nutrient sensors regulating CCK release. The presence of endocannabinoid receptors mRNA transcripts in I-cells may indicate that the endocannabinoid system modulates nutrient sensing and influences CCK release. Functional studies are required to determine how activation of these nutrient sensors mediate CCK release and to understand the role of the intestinal endocannabinoid system in the regulation of nutrient sensing and CCK secretion from duodenal I-cells.

CHAPTER 3

Duodenal I-cells express multiple gut hormones including ghrelin

This chapter is a modified version of the article

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Author contributions: I generated all data depicted in this chapter except for figure 3.9. I wrote the first draft of the manuscript.

3.1 Abstract

Enteroendocrine (EEC) cells have a pivotal role in intestinal nutrient sensing and release hormones that orchestrate food digestion, control appetite and regulate energy balance. EEC cells are found scattered throughout the intestine and are classified based on the primary hormone they contain. I-cells represent a subpopulation of enteroendocrine cells that secrete cholecystokinin (CCK) and are mainly localized in the duodenum. In this study, we investigated the hormonal profile of murine FACS-sorted duodenal I-cells using semi-quantitative RT-PCR, mass spectrometry and immunostaining methods. Our results reveal that I-cells are enriched in mRNA transcripts encoding CCK and also other key gut hormones including neurotensin, gastric inhibitory polypeptide (GIP), secretin, peptide YY (PYY), proglucagon and ghrelin. Mass spectrometry analysis of FACS-purified I cells confirmed the presence of these hormones in I cells. Immunostaining highlighted that subsets of I-cells co-express CCK, ghrelin, GIP or PYY indicating that a proportion of I-cells contain multiple hormones. Our results suggest that the traditional "one cell-one hormone" classification does not hold true for subpopulations of duodenal I-cells. We show that a subpopulation of duodenal I-cells express GIP (the predominant peptide of K-cells), proglucagon/PYY (the predominant hormones of L-cells) or the orexigenic hormone ghrelin and we suggest that there is a significant overlap between I-cells and other subtypes of EEC cells.

3.2 Introduction

Enteroendocrine (EEC) cells represent less than 1% of intestinal epithelial cell population and are found intermittently scattered throughout the intestinal epithelium. Their primary role is to sense ingested nutrients in the gut lumen, integrate nutrient and systemic signals, and emit paracrine, endocrine and neural messages in order to co-ordinate effective digestion and control appetite (Sternini *et al*., 2008, Moran *et al*., 2008, Engelstoft *et al*., 2008).

Conventionally, the classification of EEC cells has been based on their hormonal content, morphology and localization in the gastrointestinal tract, and accordingly more than fifteen distinct classes of EEC cell have been identified (Rindi *et al*., 2004). Each EEC cell subtype was initially believed to secrete a single hormone and this defined the specific subtype; the so-called “one cell type - one hormone hypothesis”.

However, subsequent immunostaining studies have cast doubt on this supposition. For example, colonic L cells were reported to express both Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Eissele *et al*., 1992, Roth *et al*., 1992). Additionally, other studies revealed that subpopulations of proximal small intestinal L-cells express gastric inhibitory polypeptide (GIP) (Mortensen *et al*., 2003, Reimann *et al*., 2008, Theodorakis *et al*., 2006), neurotensin (NTS) and cholecystokinin (CCK) (Roth *et al*., 1992).

More recently, Samuel *et. al.* reported that CCK-containing cells isolated from all segments of the small intestine were enriched in mRNA transcripts encoding preproglucagon, neurotensin , secretin, PYY, tachykinin and GIP (Samuel *et al*.,

2008) . Furthermore, Habib et al reported a considerable overlap in the hormonal compliment of L- and K-cells isolated from both proximal and distal small intestine (Habib *et al*., 2012). Taken together these data, for all intents and purposes, refute the "one cell-one hormone" hypothesis and indicate that EEC cells probably express multiple hormones.

Cholecystokinin is the archetypal satiety hormone expressed in a subset of EEC cells that are classically termed I-cells. Expression of CCK is apparent in EEC cells along the entire length of the small intestine, although the highest density of CCK-expressing cells is found in the duodenum (Chandra and Liddle, 2007, Dockray, 2009a). Duodenal I-cells contain mRNA transcripts encoding several key G-protein coupled receptors (GPCRs) that collectively chemosense LCFA, SCFA, fatty acid-derivatives and endocannabinoid peptides (Sykaras *et al*., 2012). Functionally, I-cells are considered to sense nutrients in the small intestinal lumen and secrete CCK in response to them. CCK in turn slows gastric emptying, stimulates gallbladder emptying and the release of pancreatic enzymes that together promote the optimal digestion and absorption of ingested nutrients. In addition, CCK limits the urge to continue eating by instilling satiety (Dockray, 2009b).

Recently, the widespread use of the incretin mimetic drugs for the treatment of diabetes (Ahren, 2011) coupled with the discovery of the beneficial effects of bariatric surgery for the treatment of obesity and diabetes (Dixon *et al*., 2012, Mingrone *et al*., 2012) , has further fuelled interest in EEC cells. Given the efficacy of Roux-en-Y bariatric surgery and the fact that I-cells are concentrated in the by-passed intestine (duodenum), there is a need for a better characterization of I cells.

In this study, we focused on I-cells and we tried to determine if these cells solely express CCK or, like L- and K- cells, co-express a spectrum of hormones. To enable this we utilized a well characterized transgenic mouse model with fluorescent labelled CCK-expressing cells and employed semi-quantitative RT-PCR to profile mRNA transcripts encoding different gut hormones, and liquid-chromatography/mass-spectrometry (LC-MS/MS) and immunostaining to determine expression of gut peptides in I-cells.

Our findings show that I-cells express a range of gut hormones including GIP, NTS PYY, proglucagon and surprisingly the orexigenic hormone ghrelin. The overlap in expression of gut hormones indicates that, like L- and K- cells, I-cells are not a distinct subtype of EEC cells, but rather represent a heterogeneous population of hormone expressing cells.

3.3 Methods

3.3.1 Experimental animals

Adult male transgenic CCK-eGFP mice (Tg(CCK-EGFP)BJ203Gsat/Mmmh, generated by GENSAT project (Gong *et al*., 2003) were bred in-house and kept on a 12h light: dark cycle with ad libitum access to food and water for 8 to 16 weeks prior to experimentation. To obtain duodena, mice were anaesthetised with CO_2 and killed by cervical dislocation. All animal procedures used in this study were approved by the University of Manchester Ethical Review Process Committee, in accordance with the UK Home Office regulations and Animals Scientific Procedures Act 1986 (UK).

3.3.2 Isolation of duodenal epithelial cells

Duodena from four adult male CCK-eGFP mice were used for each sorting experiment. Mice were killed and duodena removed and dissociated to yield single-cell populations using a chemical/mechanical method, based on incubation of tissue at 37°C in CMF-HBSS medium containing 1mM EDTA as previously described in 2.3.4

3.3.3 Fractionation of duodenal villi and crypts

In order to isolate duodenal cell populations enriched in villi or crypts, mouse duodena were dissociated and fractionated using the method developed by Clevers and colleagues (Sato *et al*., 2009) with slight modifications. Briefly, after incubation of tissue in ice-cold PBS/2mM EDTA, the PBS/EDTA medium was removed and tissue fragments were resuspended vigorously in ice-cold PBS using a 10-ml serological

pipette over 1 min. Each resuspension step was followed by a 3-5 min break in which the tissue fragments were allowed to settle to the bottom of the tube by gravity. The supernatant was collected after the first 2 rounds of resuspension was enriched in villi and was used for FACS-sorting of villi eGFP+ and eGFP- cells. The sediment was further resuspended and the resulting supernatants (fractions 3-6) became gradually enriched in crypts. The supernatant collected after the last two rounds of resuspension (fractions 5 and 6) was highly-enriched in crypts. Pooled supernatants were spun at 150-200g for 3 min and resuspended in CMF-HBSS with 0.5 mM EDTA. Crypts were dissociated into single-cells by shaking (100 rpm) at 37°C for 20-25 minutes, followed by trituration using a pipette. This single-cell population was used for FACS-sorting of crypt eGFP+ and eGFP- cells.

3.3.4 FACS-sorting of eGFP+ and eGFP- cells

eGFP+ and eGFP- cells were sorted by FACS as follows: Background fluorescence and gating parameters were determined using single cell suspensions isolated from eGFP-negative mice or CD-1 mice (control sample). FACS-sorting was performed using a BD FACS Aria cell sorter (BD Biosciences) and Diva version 5/6 software, as previously described in 2.3.5. Dead cells were excluded using Sytox Red (Invitrogen, UK) using a 633nm laser and 660/20 bandpass.

3.3.5 RNA extraction, RNA amplification and semi-quantitative RT-PCR analysis

RNA extraction from sorted cells, RNA quality control, RNA/cDNA amplification, reverse transcription and semi-quantitative RT-PCR were performed as previously

described (2.3.6 and 2.3.7). Semi-quantitative RT-PCR analysis of gene expression from sorted cells was performed on amplified cDNA prepared from eGFP+ and eGFP- cells. Amplified cDNA was diluted 1/50 and 1 µl was used as template for each PCR reaction (unless otherwise stated). Non-amplified RNA diluted accordingly was used as a negative (-RT) control to exclude the possibility of PCR product amplification due to genomic DNA contamination. The housekeeping gene 18S rRNA was used to normalise the starting amount of cDNA template between eGFP+ and eGFP- samples.

RT-PCR analysis of gene transcripts in eGFP+ and eGFP- cells sorted from villi or crypts fractions was performed on unamplified cDNA. The appropriate negative (-RT) controls were generated by substituting dH_2O for reverse transcriptase. 18S rRNA was employed as the loading control. Amplified products of interest were verified by sequencing at University of Manchester Sequencing Core Facility. The PCR oligomers used are listed in Table 3.1. PCR reaction conditions for each target are listed in Table 3.2.

Target	Forward Primer (5→3)	Reverse Primer (5→3)	Amplicon size (bp)
CCK	CGCTGGAACTCGCCAAGCCA	GCGGCCAGAAGGAGCTTTGC	270
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151
Ghrl	AGCCACCAGCTAAACTGCAGCC	AGCTGGCGCCTCTTTGACCT	218
Nts	TGCAGCCTCATAAATAACGTGAA	TTTGCCAACAAGGTCGTCATC	75
Sct	CAAGACACTCAGACGGAATGTT	CTGGTCCTCTAAGGGCTTGGA	161
Pyy	ACGGTCGCAATGCTGCTAAT	GCTGCGGGGACATCTCTTTTT	185
Gip	ACTGGAAACACAACATCACCC	ACTGAGGCTCTTGGGCAAAG	113
Gcg	TGAATGAAGACAAACGCCACT	CCACTGCACAAAATCTTGGGC	92
Lgr5	GGACCAGATGCGATACCGC	CAGAGGCGATGTAGGAGACTG	194

Table 3.1 Oligomers used in the study
Sequences of primers used for semi-quantitative RT-PCR analysis and the expected size of PCR (base pairs-bp) amplicons are presented in this table.

Primer Pair	Denaturation	Annealing	Extension	Number of cycles
18S rRNA	94°C 30sec	58°C 30sec	72°C 30sec	14 Low (fig 3.2) 18 High (fig 3.2) 25 (fig 3.3) 22 (fig 3.8, left) 24 (fig 3.8, right)
Cck	94°C 45sec	60°C 45sec	72°C 45sec	22 Low (fig 3.2) 25 High (fig 3.2) 30 (fig 3.3) 30 (fig 3.8, left) 33 (fig 3.8, right)
Ghrl	94°C 30sec	60.5°C 30sec	72°C 30sec	28 Low (fig 3.2) 32 High (fig 3.2) 32 (fig 3.3) 33 (fig 3.8, left) 35 (fig 3.8, right)
Nts	94°C 30sec	57.5°C 30sec	72°C 30sec	30 Low 34 High
Sct	94°C 30sec	58°C 30sec	72°C 30sec	30 Low 34 High
Pyy	94°C 30sec	58°C 30sec	72°C 30sec	30 Low 34 High
Gcg	94°C 30sec	56.5°C 30sec	72°C 30sec	30 Low 34 High
Gip	94°C 30sec	58°C 30sec	72°C 30sec	30 Low 34 High
Lgr5	94°C 30sec	58°C 30sec	72°C 30sec	37

Table 3.2 PCR cycling parameters
Target names of primer pairs, PCR programme conditions and number of cycles are listed along with the figure number where results are shown. Initial denaturation was 95°C for 5 mins and final extension was 72°C for 10min, for all reactions.

Mouse small intestine was removed and flushed with ice-cold phosphate buffered saline (PBS). Samples, 1cm in length, were taken from middle of the duodenum and were fixed for 1hr with 4% paraformaldehyde-in PBS. Samples were dehydrated by passage through an ethanol gradient consisting of 70%, then 96%, then 99% for 2 hrs each. This was followed by 10 hrs incubation in xylene and embedding in paraffin (ThermoFisher, UK).

2-5µm sections were cut on a Leica RM2255 microtome (Leica, Germany) and mounted onto Superfrost Plus slides. Sections were deparaffinised in xylene and rehydrated using an ethanol gradient followed by water (99% 30mins, 96% 20mins, 70% 10mins, dH_2O 2 mins). Antigen retrieval was performed using TEG buffer (Tris, EGTA, dH_2O, pH 9) in a standardised microwave technique and left to cool to room temperature.

3.3.7 Double Immunostaining of eGFP mouse intestinal sections

Two methods were used to enable staining of sections with two antisera. For antisera raised in different species immunostaining was performed as described in 2.3.3 with mixtures of antibodies. For double staining experiments with antibodies raised in the same species a fab fragment technique was employed and the protocol was modified accordingly. Specifically, after incubation in the first secondary antibody, PBS washes were followed by incubation with 10% normal rabbit serum in PBS for 30mins. Slides

were washed three more times in PBS and replaced with 10% AffiniPure Fab Fragment Goat Anti-Rabbit IgG (Jackson ImmunoResearch, Product Code: 111-007-003) for 30mins which blocked any remaining free antigenic sites. Sections were washed again in PBS and 0.1%BSA, 0.2% gelatine, 0.05% saponin in PBS buffer and the second primary antibody in antibody buffer was applied and left o/n at 4^{o}C. The protocol was then followed as previously described (2.3.3). Controls included omission of fab fragments, omission of the second primary antibody and omission of both primary antibodies.

3.3.8 Bioimaging

Slides were visualised using an Olympus BX51 upright microscope using a 20X objective. Images were captured using a coolsnap ES camera (Photometrix) through MetaVue Softeware (Molecular Devices) and processed using ImageJ software.

3.4 Results

3.4.1 Duodenal I-cells contain mRNA transcripts encoding several gut hormones

To generate single-cells suitable for FACS analysis, a chemical and mechanical method was used to dissociate duodena. Using an optimized FACS analysis protocol, eGFP+ and eGFP- cell populations were prepared (Figure 3.1). The percentage of sorted eGFP+ cells on average constituted ~0.5% of the total dispersed epithelial cell population

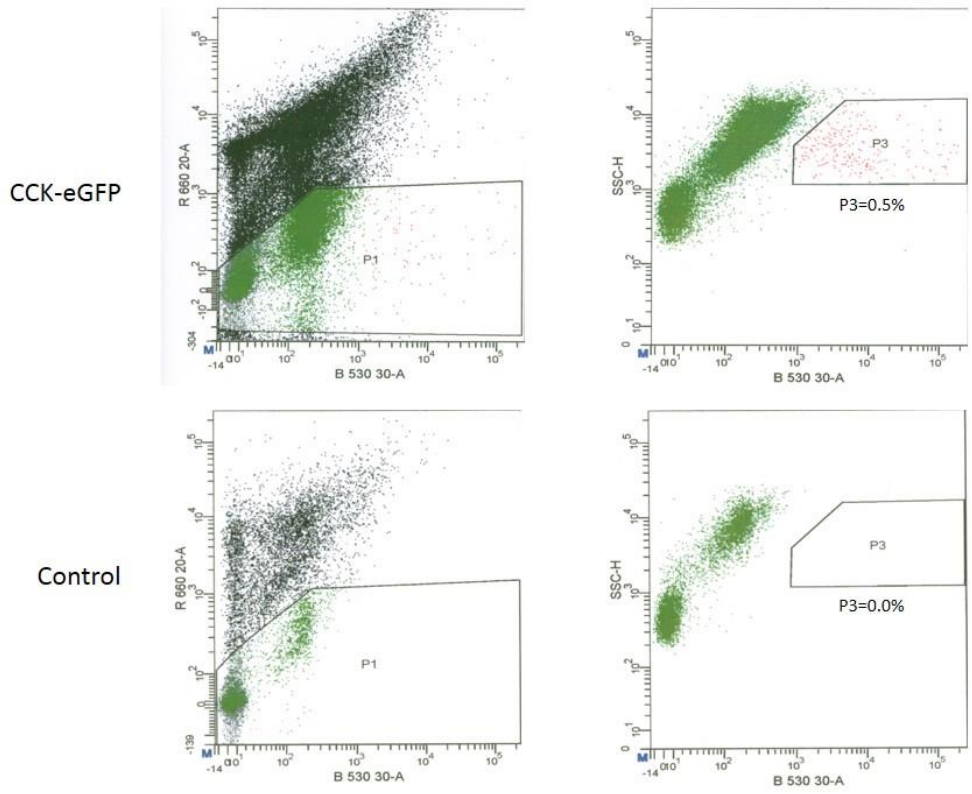

Figure 3.1 FACS-sorting of eGFP+ and eGFP-cells
Dissociated duodenal cells were subjected to FACS analysis. Cells prepared from WT mice were used as control (lower panels). Dissociated single cell populations were incubated with SytoxRed and analysed based on their eGFP (x-axis, left panel) and SytoxRed fluorescence (y-axis, left panel). SytoxRed positive cells (dead cells) were excluded from further analysis. Live cells (P1) were analysed based on their eGFP fluorescence intensity (x-axis, right panel) and side-scatter profile (y-axis, right panel). eGFP+ cells (P3) were present only in the sample prepared from transgenic mice and represented 0.5% of total cell population.Adapted from (Sykaras *et al. 2014)*

Subsequently, we performed semi-quantitative RT-PCR analysis on amplified cDNA prepared from sorted eGFP+ and eGFP- cells for mRNA transcripts encoding key gut hormones. As expected, eGFP+ cells were highly enriched in CCK mRNA transcripts and were also found to contain mRNA transcripts encoding GIP, PYY, NTS, secretin, proglucagon and unexpectedly ghrelin. In fact, CCK-containing cells were enriched in mRNA transcripts encoding NTS, GIP, secretin, PYY and ghrelin in comparison with eGFP- cells (figure 3.2). The enrichment of I-cells in mRNAs encoding multiple gut hormones was confirmed in independent experiments on amplified cDNA.

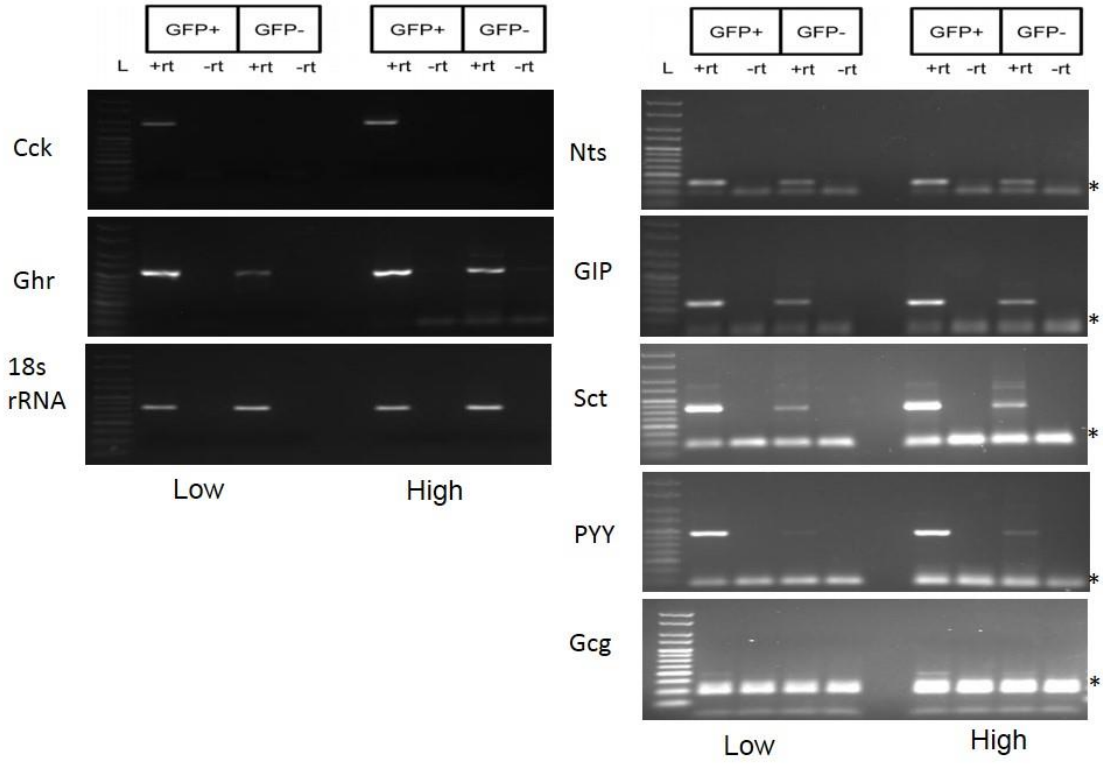

Figure 3.2 I-cells contain mRNA transcripts encoding multiple gut hormones
Semi-quantitative RT-PCR analysis revealed that I-cells contain mRNA transcripts encoding multiple gut hormones (Neurotensin, Secretin, GIP, PYY, preproglucagon and ghrelin). These mRNA transcripts are present in both I-cells (eGFP+ cells) and non I-cells (eGFP- cells) but enriched in I-cells. CCK mRNA transcript is exclusively expressed in I-cells verifying the I-cells isolation process. PCR products were amplified simultaneously, using different number of cycles (low-for lower number of cycles and high-for higher number of cycles) and amplified products were run on the same 2% agarose gel. 18S rRNA was used to demonstrate equal amount of starting material. L declares Hypeladder V, the asterisk declares primer dimers.

Additionally, Ghrl mRNA enrichment in I-cells was confirmed by semi-quantitative RT-PCR on non-amplified cDNA extracted from sorted cells (figure 3.3).

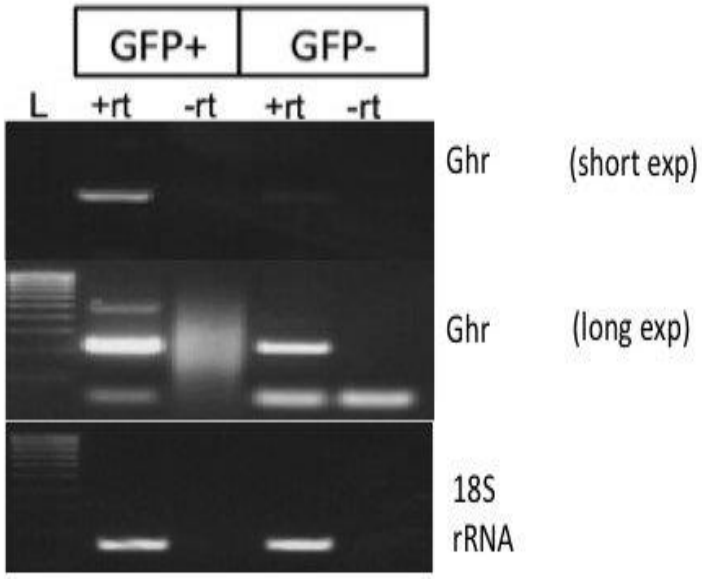

Figure 3.3 I-cells contain ghrelin (Ghrl) mRNA transcript
Semi-quantitative RT-PCR analysis of unamplified RNA extracted from sorted eGFP+ and eGFP- cells confirms that Ghrl mRNA transcript is expressed in I-cells (eGFP+) and non I-cells (eGFP-) but is enriched in I-cells. 18S rRNA was used as a loading control. A short and long exposure of the same gel/image is depicted.

3.4.2 Duodenal I-cells express multiple peptide hormones

To confirm our findings that I-cells contain transcripts encoding several gut hormones we performed proteomic profiling of sorted I-cells and non-I cells using LC-MS/MS. To confirm RT-PCR results, we performed a quantitative comparison between sorted I-cells and non-I cells samples using a label free quantification strategy. Analysis of the extracted ion chromatogram confirmed that selected gut peptides such as CCK, GIP, PYY, NTS, secretin, proglucagon and ghrelin were identified and present in relatively high abundances in I-cell samples In addition, we identified proteins associated with processing of preprohormones including prohormone convertase 1/3 and carboxypeptidase E. The identification of neuroendocrine markers chromogranin A,

secretogranin-1 (chromogranin B), secretogranin-2, secretogranin-3 and synaptophysin confirms the neuroendocrine character of I-cells.

To extend and reinforce our finding of the presence of key gut hormone transcripts in I-cells, we performed immunohistochemical experiments to localize hormones in duodenal sections using reliable antibodies against GIP, PYY, glucagon and ghrelin. Using this approach, we determined if the proteins encoded by hormone mRNAs are expressed in I-cells. Indeed, we found that ghrelin, GIP, PYY and glucagon were all expressed to some degree in I-cells. The percentage of eGFP+ cells staining positive for the hormones of interest were as follows: 50.1±1.7% of eGFP+ cells were positive for Ghrl, (1693 cells out of 3353 cells counted from 25 slides); 37.1±1.9% of eGFP+ cells were positive for GIP (214 cells out of 580 cells counted from 10 slides); 44.8±2.8% of eGFP+ cells were positive for PYY (173 cells out of 394 cells counted from 9 slides);14±2.1% of eGFP+ cells were positive for proglucagon (74 cells out of 624 cells counted from 10 slides). Unfortunately, we were unable to source dependable antibodies to enable us to detect NTS or secretin.

Co-localization of eGFP with PYY, GIP and proglucagon may be considered as predictable, since K- and L-cells localized in the upper small intestine express multiple hormones including CCK.

The finding that 50% of eGFP+ cells expressed ghrelin came as a complete surprise and we deemed it necessary to further confirm the coexpression of ghrelin in duodenal CCK containing cells and visually characterize the ghrelin expressing CCK cells. To do this we performed immunostaining experiments using two independent affinity purified ghrelin antisera, both of which we first validated as recognizing ghrelin by performing extensive control experiments. We co-stained sections with a CCK

specific antiserum and found that duodenal cells expressing CCK also expressed ghrelin. We identified three populations of cells expressing ghrelin in the duodenum. The majority of ghrelin/CCK positive cells had an “open-type” morphology with an apical membrane projecting to the gut lumen. However, there was a small number of open-type cells that expressed ghrelin, but that were not found to be CCK immunopositive. A further subset of cells were observed that were ghrelin-positive and CCK negative, however these had a “closed-type” morphology that was similar to gastric ghrelin-containing cells.

3.4.3 Both crypt-localized and villi-localized duodenal I-cells co-express multiple hormones

To extend our study, we aimed to investigate if the co-expression pattern of multiple gut hormones in I-cells was a feature of non-differentiated I-cells residing in crypts or whether multiple hormones are expressed in both crypt-based and fully differentiated villi-residing I-cells. To ascertain this we utilised semi-quantitative RT-PCR and immunostaining.

Therefore, we fractionated duodenal epithelial dissociated cell preparations (Figure 3.4) and prepared villi-enriched and crypt-enriched single-cell populations for FACS analysis (Figure 3.5).

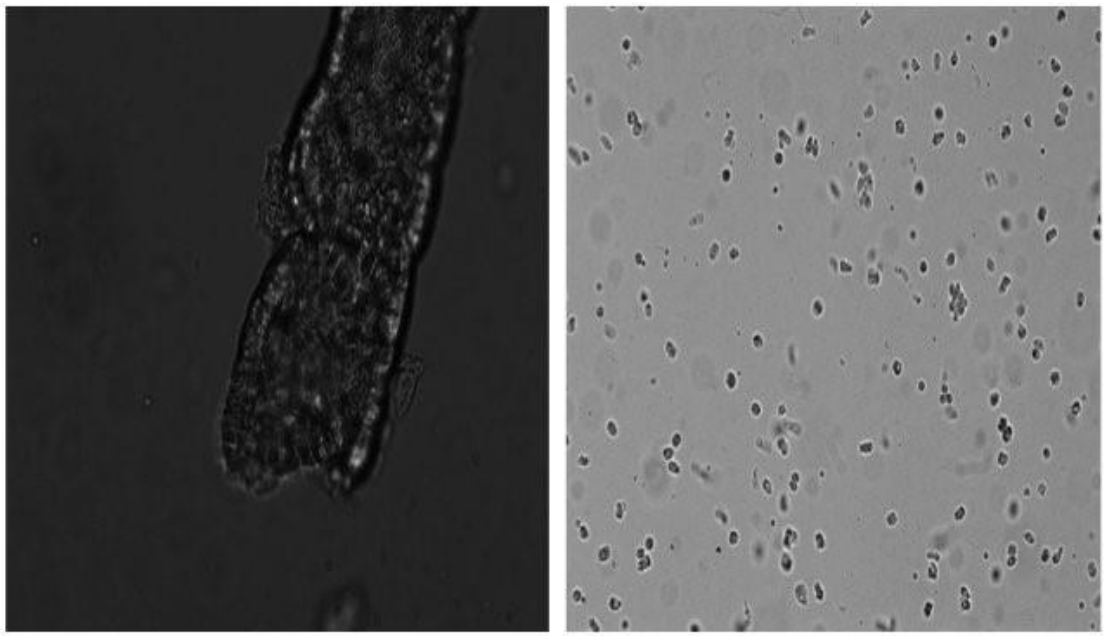

Figure 3.4 Fractionation of duodenal dissociated mucosal cells
Left panel:A representative image of fractions 5-6 that are highly enriched in intestinal crypts. A single intestinal crypt and few scattered cells (out of focus) are presented.
Right panel: A representative image of fractions 1-2 is presented. These fractions contain single cells, dissociated from the intestinal villi. A successful dissociation results in a population of single dispersed cells without presence of cell clumps. Fractions 1-2 do not contain crypts

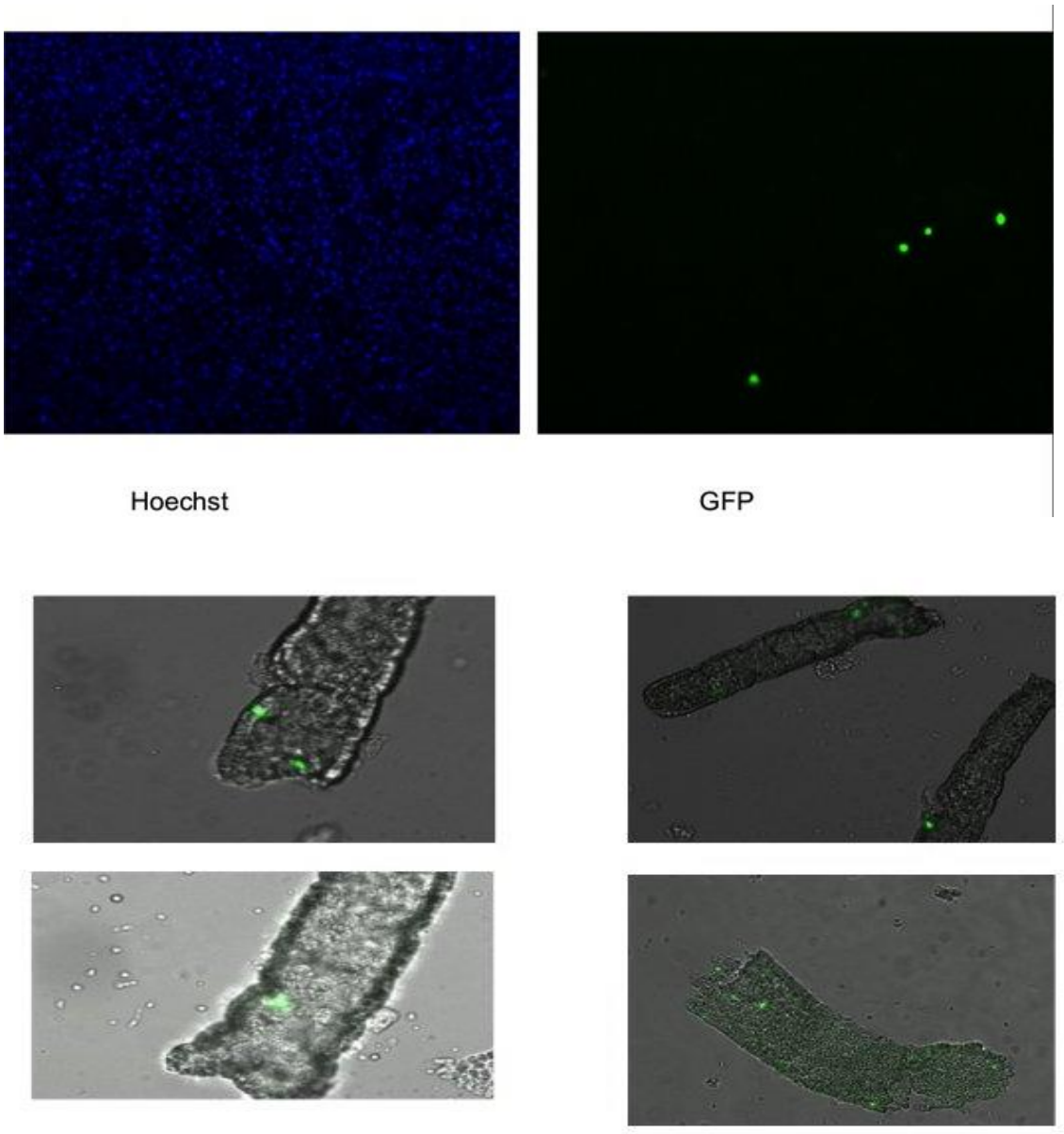

Figure 3.5 Imaging of dissociated villi-residing eGFP+ cells and crypt-based eGFP+ cells

Upper Panel: Imaging of eGFP+ cells that consist a very rare sub-population of dissociated duodenal villi single-cells. Left panel shows staining of cell nuclei with Hoechst (DAPI filter) whereas right panel shows eGFP fluorescence (GFP filter). eGFP+ cells display different levels of GFP fluorescence, even if they are isolated from the same animal

Lower Panel: Four representative images of eGFP+ cells that are localized in the intestinal crypts. Intestinal crypts were prepared following a dissociation-fractionation protocol. The final fractions are highly enriched in crypts with a minimum contamination of scattered villi single-cells. Each crypt contains one or more eGFP+ cells that are in contact with the intestinal epithelial cells. These crypts were subjected to a further dissociation/trituration protocol in order to prepare a single cell population of crypt-based cells. This method enables the FACS-sorting of crypt-residing eGFP+ cells.

Semi-quantitative RT-PCR for stem cell marker Lgr5 (Leucine-rich repeat-containing G-protein coupled receptor 5) transcript was used as a control target to verify the successful fractionation of crypt/villi preparations.

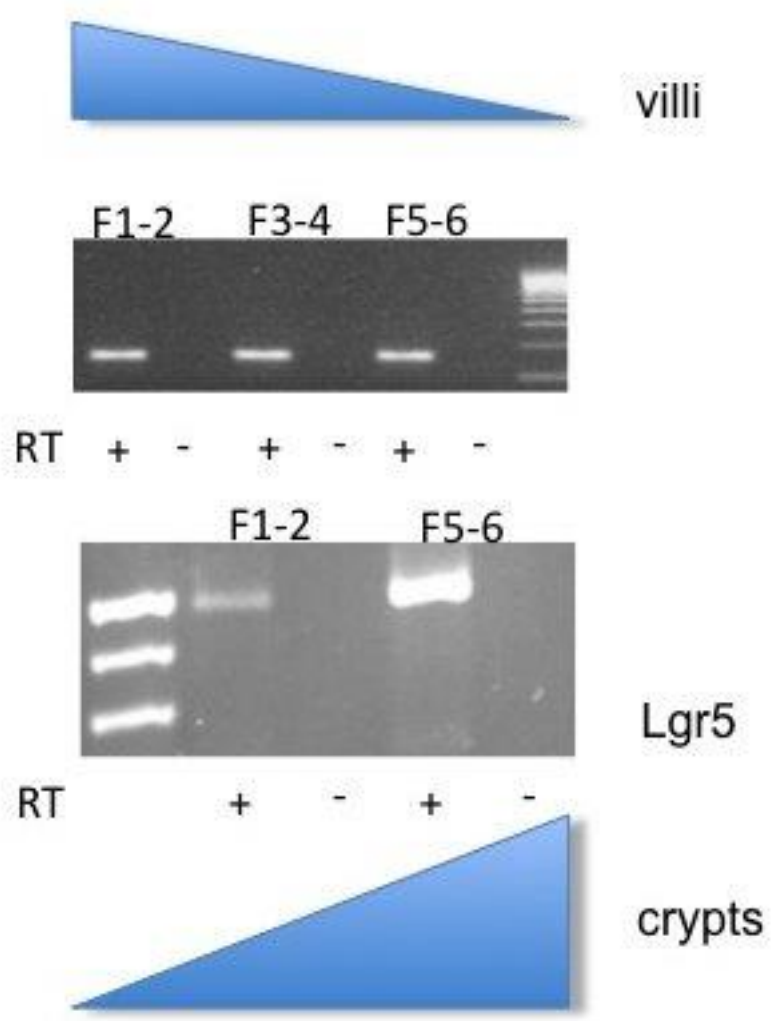

Figure 3.6 Validation of villi/crypt fractionation protocol
Upper panel: Semi-quantitative RT-PCR for 18S rRNA was used to demonstrate that equal amounts of starting cDNA material were prepared from different fractions.
Lower panel: Semi-quantitative RT-PCR for intestinal crypt marker (stem-cell marker) Lgr5 reveals that Fractions 5-6 are highly enriched in Lgr5 mRNA transcript in comparison with Fractions 1-2, confirming that fractions 5-6 are highly enriched in crypts and fractions 1-2 mainly contain villi-residing cells. RT +/- declares the presence or absence of reverse transcriptase.

We then used FACS sorting to isolate villi-based and crypt-based eGFP+ and eGFP- cells. eGFP+ cells represented 0.9% of total cell population in the villi-enriched fraction and 0.4% of total cell population in the crypt-enriched fraction (Figure 3.7).

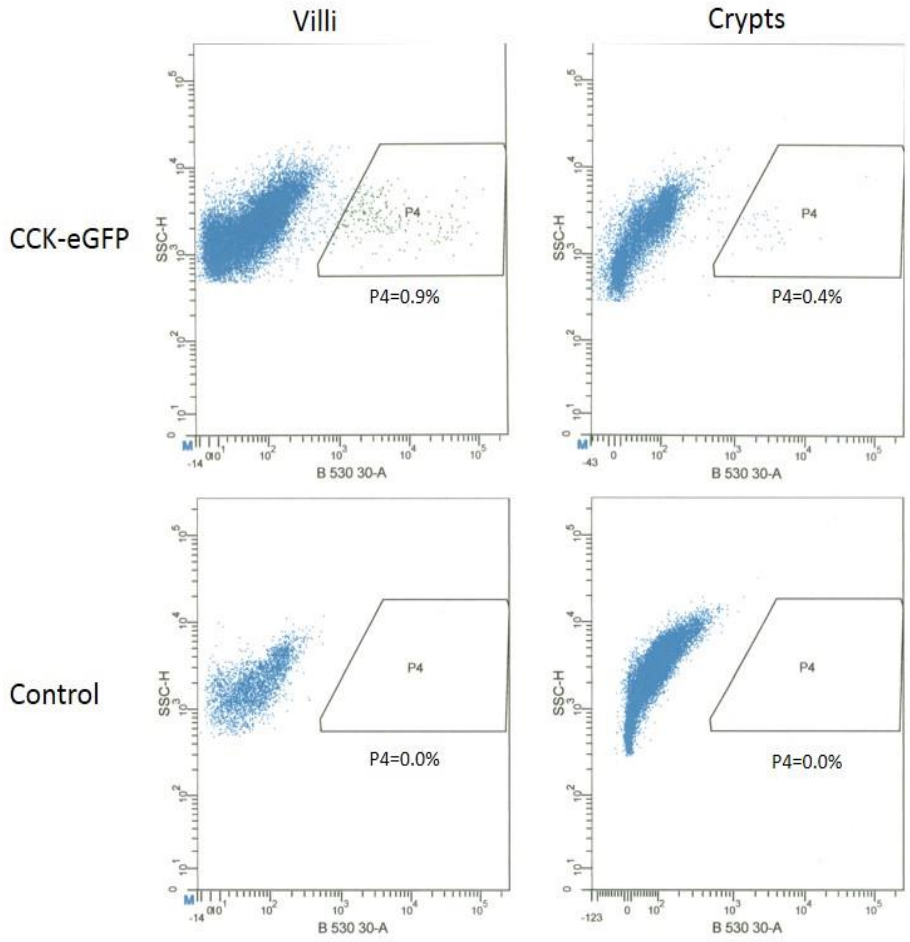

Figure 3.7 FACS-sorting of villi-residing and crypt-residing I-cells
Duodenal dissociated single-cells prepared from villi (left panels) or crypts (right panels) were subjected to FACS analysis. In parallel, cells prepared from non-transgenic mice were analyzed serving as a control in order to setup gating parameters. Dead cells were excluded from analysis after staining with SytoxRed. Live cells were analyzed based on their eGFP intensity (x-axis) and side scatter profile (y-axis) and eGFP+ cells (P4) were sorted. eGFP+ cells are present only in the samples prepared from CCK-eGFP mice but not in samples prepared from control mice. 0.9% of villi cells were eGFP+ (representing a population of 1800 cells) whereas 0.4% of crypt cells were eGFP+ (representing a population of 5600 cells). Adapted from (Sykaras *et al. 2014)*

The higher number of sorted crypt-based cells (despite their lower percentage) is a reflection of the high viability of isolated crypt-cells in comparison with isolated villi-cells. High viability of isolated crypt-based single cells resulted in a much higher number of analyzed cells (in comparison with villi cells) and therefore in a higher number of sorted eGFP+ cells.

Semi-quantitative RT-PCR analysis confirmed that sorted I-cells from both crypt and villi fractions were highly enriched in CCK mRNA transcript and importantly that both crypt-derived and villi-derived I-cells were enriched in Ghrl mRNA transcript in comparison with eGFP- cells (Figure 3.8). The finding that both immature (crypt-residing) and terminally differentiated I-cells (villi-residing) contained mRNA transcript encoding ghrelin suggest that the multi-hormone expression profile was not a characteristic restricted only to crypt-based immature I-cells.

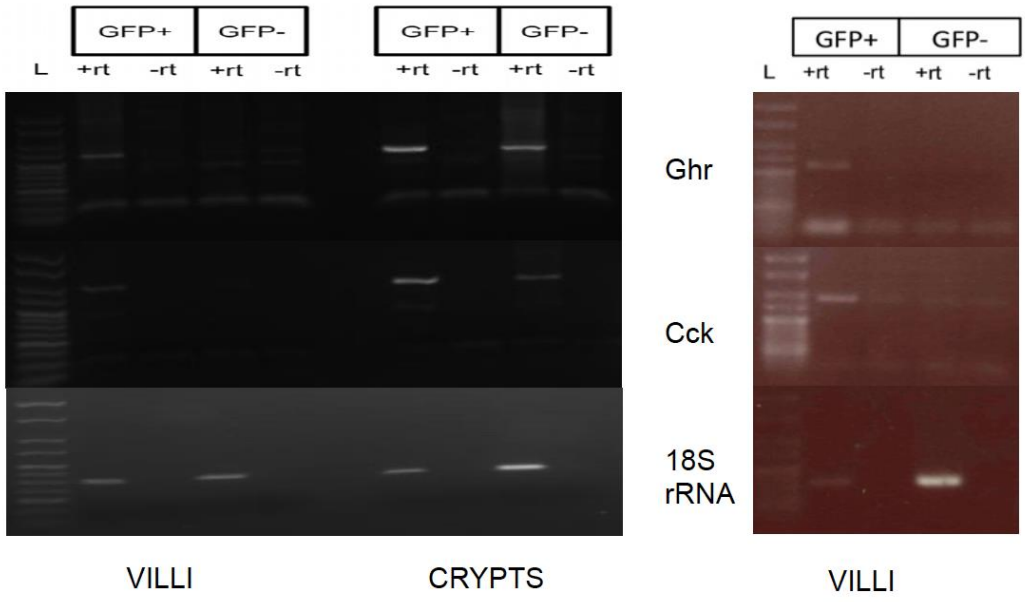

Figure 3.8 Both crypt-based and villi-based I-cells are enriched in Ghrl mRNA transcript. Semi-quantitative RT-PCR analysis was performed on unamplified RNA from sorted villi-residing and crypt-residing eGFP+ and eGFP- cells. PCR products were analyzed on the same gel (left panel). The RT-PCR analysis for villi eGFP+ and eGFP- cells was repeated using more cycles (33 instead of 30 for CCK and 35 instead of 33 for ghrelin) and amplified products were analyzed on a separate gel (right panel). As expected, both crypt eGFP+ cells (left panel) and villi eGFP+ cells (left and right panel) are highly enriched in CCK mRNA transcript. Similarly with CCK, Ghrl mRNA transcript is enriched in sorted eGFP+ cells residing in villi (left and right panel) or crypts. L declares Hyperladder V, -rt samples were used as a negative control to exclude amplification due to genomic DNA contamination. Adapted from (Sykaras *et al. 2014)*

Additionally, we performed double-immunostaining experiments with antisera against CCK and ghrelin, GIP or PYY. A co-expression pattern was observed for I-cells resident in crypts and villi (Figure 3.9). These findings suggest that a subset of I-cells express several gut hormones throughout their life and regardless of their localization. These I-cells that co-express CCK and other gut peptides are distributed in both intestinal crypts and villi.

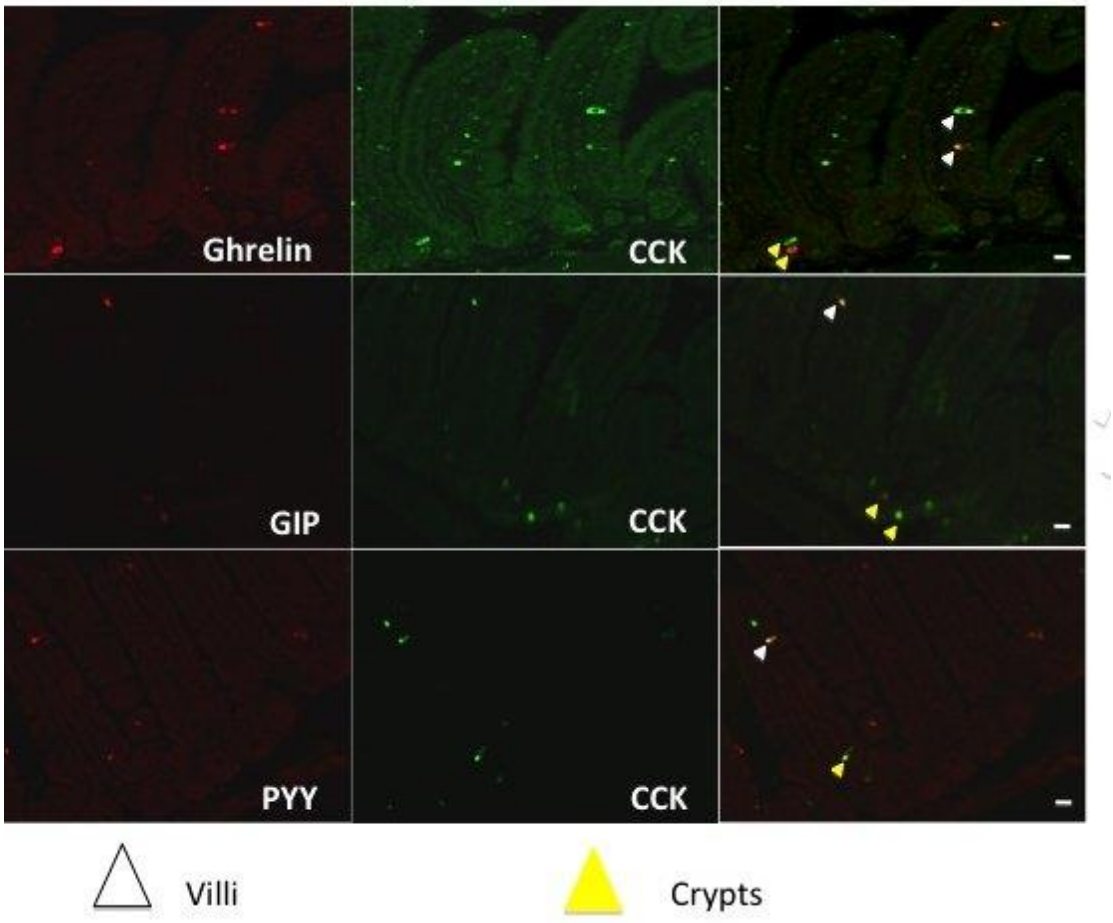

Figure 3.9 Co-localization of CCK with ghrelin, PYY and GIP in duodenal epithelium sections
Dual immunostaining was performed in duodenal sections of CCK-eGFP mice with antibodies against CCK (green channel) and ghrelin, GIP or PYY (red channel) respectively. A sub-population of CCK-positive cells co-expressed ghrelin, GIP or PYY with CCK. Dual labelled cells are not only restricted to intestinal crypts (yellow triangle) but are also observed in villi (white triangle). Images were acquires with a 20X objective. Courtesy of Claire Demenis and Craig P.Smith.

3.5 Discussion

The aim of this study was to describe the hormonal profile of CCK- containing duodenal cells, classically termed I-cells (according to the Wiesbaden classification of enteroendocrine cells). Furthermore, we aimed to determine if immature I-cells that reside in the intestinal crypts and terminally differentiated I-cells that are localized in the intestinal villi contained hormones other than CCK.

Previously, we have demonstrated that the CCK-eGFP transgenic mouse model permits faithful delineation of CCK containing duodenal cells. Using FACS, we were able to obtain relatively pure populations of I-cells and demonstrated that these cells have the molecular characteristics of nutrient sensing endocrine I-cells.

In the current study, semi-quantitative RT-PCR analysis of gut peptides gene expression revealed that I-cells are enriched in mRNA transcripts encoding multiple gut hormones such as neurotensin, secretin, GIP, proglucagon (GLP-1), PYY and ghrelin, in comparison with non I-cells. These data were reinforced by mass spectrometry analysis of FACS-sorted I-cells and by comparison of these cells to non-I-cells (representing mainly duodenal enterocytes). Protein profiling confirmed that PYY, GIP, proglucagon, NTS, secretin and ghrelin are co-expressed with CCK in duodenal I-cells. In agreement with the ascribed function of I-cells, bioinformatic analysis of the proteins identified in I-cells by mass spectrometry identified I-cells as neuroendocrine secretory cells.

Our results confirm the findings of Samuel *et al* that small intestinal CCK-containing cells contain mRNA transcripts of several gut hormones and refine this observation specifically for duodenal CCK-expressing cells that represent the typical I-cells. They also agree with the findings of Schwartz and colleagues who used the CCK-eGFP mouse model to profile transcripts and reported the enrichment of mRNA transcripts encoding secretin, GIP, ghrelin, proglucagon, NTS and PYY in eGFP-CCK cells collected from different segments of small intestine (Egerod *et al*., 2012).

To determine the distribution of I-cells that expressed other gut hormones we performed immunostaining for key gut hormones for which we could source trustworthy antisera. The proportion of I-cells co-stained with other gut hormones varied depending on the hormone under investigation but indicated that subpopulations of I-cells express at least two gut peptides hormones. It is tempting to conclude that a given I-cell contains more than two peptide hormones, but without multiplex immunostaining or mass spec analysis of cells labelled for two hormones this notion remains unconfirmed. These data suggest that duodenal I-cells are not a distinct EEC cell type committed to express/secrete only CCK. Rather, I-cells represent a class of EEC cells that predominantly express CCK in addition to at least one other gut hormone that primarily characterize other subtypes of EEC cells such as K-, L-, N-, S- and X/A cells.

The finding of multiple hormones in EEC cells is not without precedent: Previous targeted EEC cell ablation studies have reported that specific-ablation of GIP-positive cells (Althage *et al*., 2008, Pedersen *et al*., 2013), PYY-expressing cells (Sam *et al*., 2012) or secretin-positive cells (Rindi *et al*., 1999) resulted in significant reductions in CCK-positive cells, indicating an overlap between different EEC cells subtypes.

Importantly, recent elegant studies have reported a similar blurring of the boundaries between EEC cell subtypes. A study from Gribble and Reimann group reported that upper small intestinal K- cells contained transcripts encoding CCK, secretin, proglucagon and PYY as well as GIP (Habib *et al.*, 2012). In addition, upper small intestinal L-cells were found to contain mRNA transcripts encoding proglucagon, PYY, GIP, CCK, NTS and secretin. Furthermore, almost all L-cells were reported to co-express CCK and ~40% of CCK-positive cells co-expressed Venus, the transgenic fluorescent marker used to delineate L-cells. In comparison, ~80% of fluorescent-labelled K-cells co-expressed CCK. These results clearly illustrate that there is an extensive overlap of the expressed hormone repertoire of cells traditionally classified as L-, K-, and I- cells.

This suggestion is supported by studies that have targeted specific transcription factors that regulate the differentiation of enteroendocrine cell lineage. For example, the deletion of transcription factor Arx was shown to result in the loss of CCK-, GLP-1/Glucagon and secretin expressing EEC cells indicating that Arx is crucial for the development of all these EEC cell subtypes (Du *et al*., 2012a, Beucher *et al*., 2012). Moreover, transcription factor NeuroD1 has been shown to be critical for the development of both CCK and secretin enteroendocrine cell lineages (Naya *et al*., 1997). Together these studies show that several EEC cell subtypes share pathways that regulate their terminal differentiation and that this feature supports the co-expression of several gut hormones in I-cells.

Transcript analysis and immunostaining also revealed that I-cells contain multiple hormones during their differentiation (crypt I-cells) and when mature (terminally

differentiated villi I-cells). This finding is in agreement with Egerod *et al.*, who also observed co-expression of CCK with other gut peptides in crypt CCK-containing cells and villi-residing differentiated CCK-containing cells. Our results and those of Egerod *et. al*. are in contrast with the with the model proposed by Wank and colleagues (Sei *et al*., 2011) according to which only crypt based differentiating immature CCK-eGFP+ cells contain multiple hormones.

Probably the most surprising finding of the current work was that I-cells express Ghrl mRNA and ghrelin peptide. Notably, the subpopulation of I-cells that express ghrelin constitutes approximately 50% of all I-cells. Ghrelin/CCK-positive cells were of the classic 'open type' morphology suggesting that they sense luminal nutrients and secrete hormones in response. We also identified a population of ghrelin-positive cells that were not CCK-positive and that were located at the basolateral pole of the duodenal epithelia, but did not conform to the "open-type" morphology. These cells may represent 'closed type' ghrelin cells, that are morphologically similar with ghrelin-positive cells in stomach (Sakata *et al*., 2002). Our findings agree in part with those published by Egerod *et al* who reported that I-cell contain Ghrl mRNA transcript, but did not express ghrelin peptide. The use of different antisera and different immunostaining protocols may account for these discrepancies.

Taken together the findings of the current study and other recent studies (Svendsen *et al.*, 2014, Cho *et al.*, 2014b, Cho *et al.*, 2014a) suggest that there is considerable overlap in the hormone content of small intestinal EEC cells. Given their apparent common developmental lineage, small intestinal EEC cells may represent a more complex class of secretory cells than was previously suggested by the "one-cell - one hormone classification". In addition, given the spectrum of hormone content we

observed for the pairs of hormones we stained, the boundaries between different subtypes of EEC cells are rather obscure. Clearly, it may be time to re-evaluate the EEC classification system in light of recent data.

In summary, we show that duodenal I-cells contain mRNA transcripts encoding multiple gut hormones and we also report co-localization of CCK with ghrelin. Our findings suggest that enteroendocrine cells expressing CCK, traditionally classified as I-cells, in fact express a repertoire of hormones and in so doing may be capable of mounting differential responses to ingested nutrients.

CHAPTER 4

Generation of an antibody against human proCCK

Author contributions: I performed all analyses and generated all data presented in this chapter except for figure 4.8. Claire Demenis contributed to molecular cloning and western blot experiments presented in figures 4.3-4.4 and performed the immunostaining depicted in figure 4.8.

4.1 Aim

FACS sorting of human I-cells has not been achieved so far. There are several limitations that prevent researchers from performing this task. The lack of an I-cell specific surface-plasma membrane marker is the main limitation that makes the direct isolation of human I-cells impossible. An alternative option would be to use an antibody against GPR40 or other GPCRs that are expressed in enteroendocrine cells in order to sort them. In this case, the population of sorted EEC cells would represent different subtypes, including I-cells. Again, the lack of reliable antibodies against nutrient sensing GPCRs is a major drawback.

Given the current lack of a suitable cell surface marker that can be used for this approach, we considered the option of using an intracellular protein as a marker for I-cells sorting. CCK, the hormone that is expressed predominantly and specifically in I-cells, would be the ideal marker that can be used for this approach. The use of an intracellular protein as a marker for FACS sorting of human cells is a quite technically challenging approach but has been described in the past for the isolation of primary cells (Moerch *et al*., 2007, Pechhold *et al*., 2009), resulting in purified cell populations that can be used for subsequent transcriptomic analyses.

4.2 Selection of epitope

The ideal antibody that can be used for human I-cells FACS sorting would recognize the intracellular proCCK peptide (amino acid sequence 21-115 of human preproCCK). Amino acids 1-20 of preproCCK represent the signal peptide that is cleaved, thus they cannot be selected as an epitope. The currently used anti- human CCK antibodies are

generated against C-term epitopes (amino-acids 97-104) so that they can detect circulating CCK-8, CCK-22 and CCK-33 forms in plasma. The majority of these antibodies directed against human proCCK C-term epitopes cross-react with gastrin, due to the high homology between the C-term region of CCK and gastrin.

Our aim is to develop an antibody that will recognize the unprocessed form of CCK with high specificity. Thus, we selected an epitope that is close to the N-term region of the peptide in order to recognize the unprocessed proCCK and the processed full length CCK-83 but not the circulating forms CCK-33, CCK-22 and CCK-8 (Figure 4.1). Additionally, the selection of this epitope instilled us confidence that the antibody would recognize human CCK but not human gastrin. The selected epitope is a 19 amino acid peptide (RAEEAPRRQLRVSQRTDGE) that corresponds to amino acids 35-53 of human preproCCK. This amino acid sequence does not exist in human gastrin.

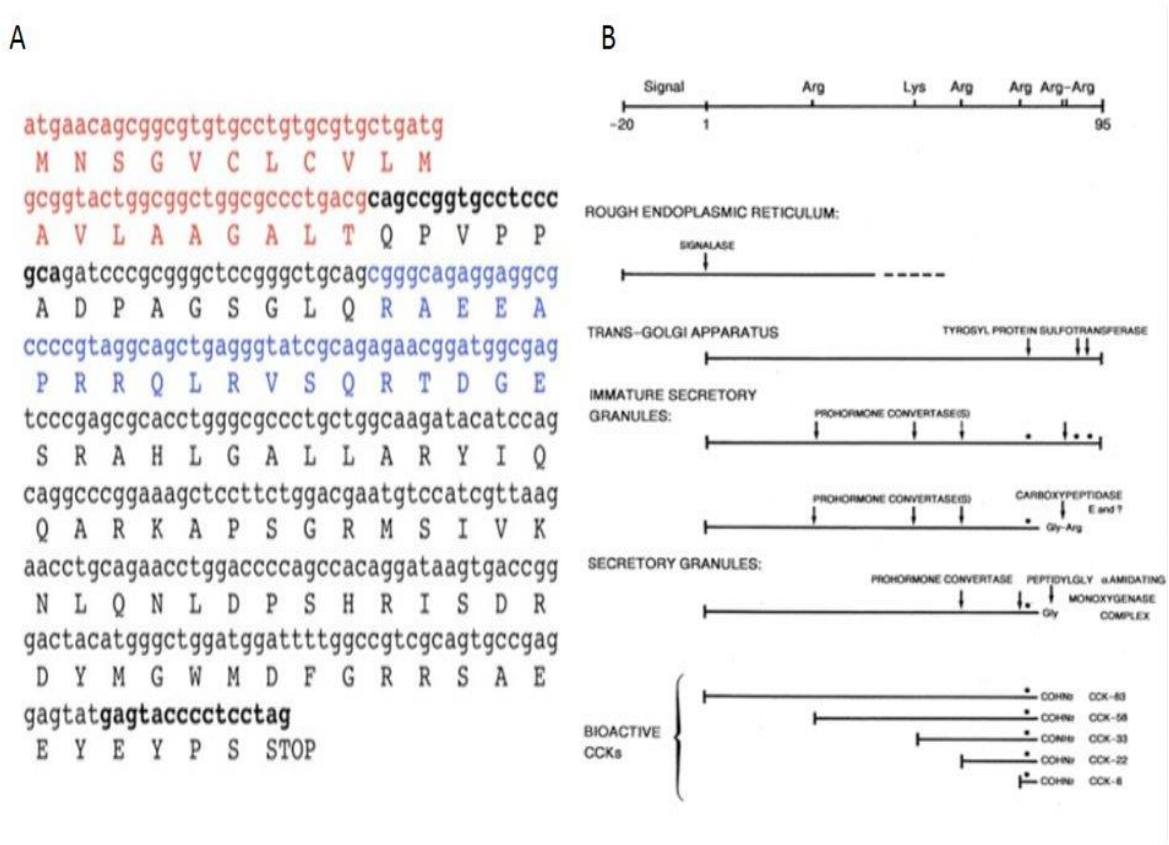

Figure 4.1 The selected epitope for the generation of anti-human preproCCK antibody.
A**.**The human CCK cDNA nucleotide and amino acid sequence. Red indicates the signal peptide (first 20 amino acids of human proCCK). Blue represents the selected targeted. The pro-CCK forward and reverse primers are indicated in bold
B. PreproCCK undergoes a series of post-translational modifications that include the cleavage of signal peptide, the cleavage of far C-term region with parallel amidation of the remaining C-term amino acid and a series of cleavages by prohormone convertases that produce the circulating forms of CCK. Panel B is adapted from (Rehfeld *et al*, 2001)

To analyse the antigenic propensity of the selected peptide and to identify other more antigenic peptides in human proCCK, we used a software program (http://imed.med.ucm.es/Tools/antigenic.pl) that predicts antigenic peptides based on the Kolanskar and Tongaonkar method (Kolaskar and Tongaonkar, 1990). Based on results of the antigenic plot, there are 2 main antigenic determinants/domains in the protein. The first one (amino acids 4-27) cannot be used because it is within the signal peptide (amino acids 1-20). The second one (amino acids 54-70) was avoided because it is included in both unprocessed and processed/circulating forms of CCK

(CCK-33). The preferred epitope has an antigenic propensity similar with the antigenic propensity of full preproCCK and does not contain any amino acid sequence of very low antigenic propensity, thus it was finally selected as the target for the production of a polyclonal antibody (Figure 4.1).

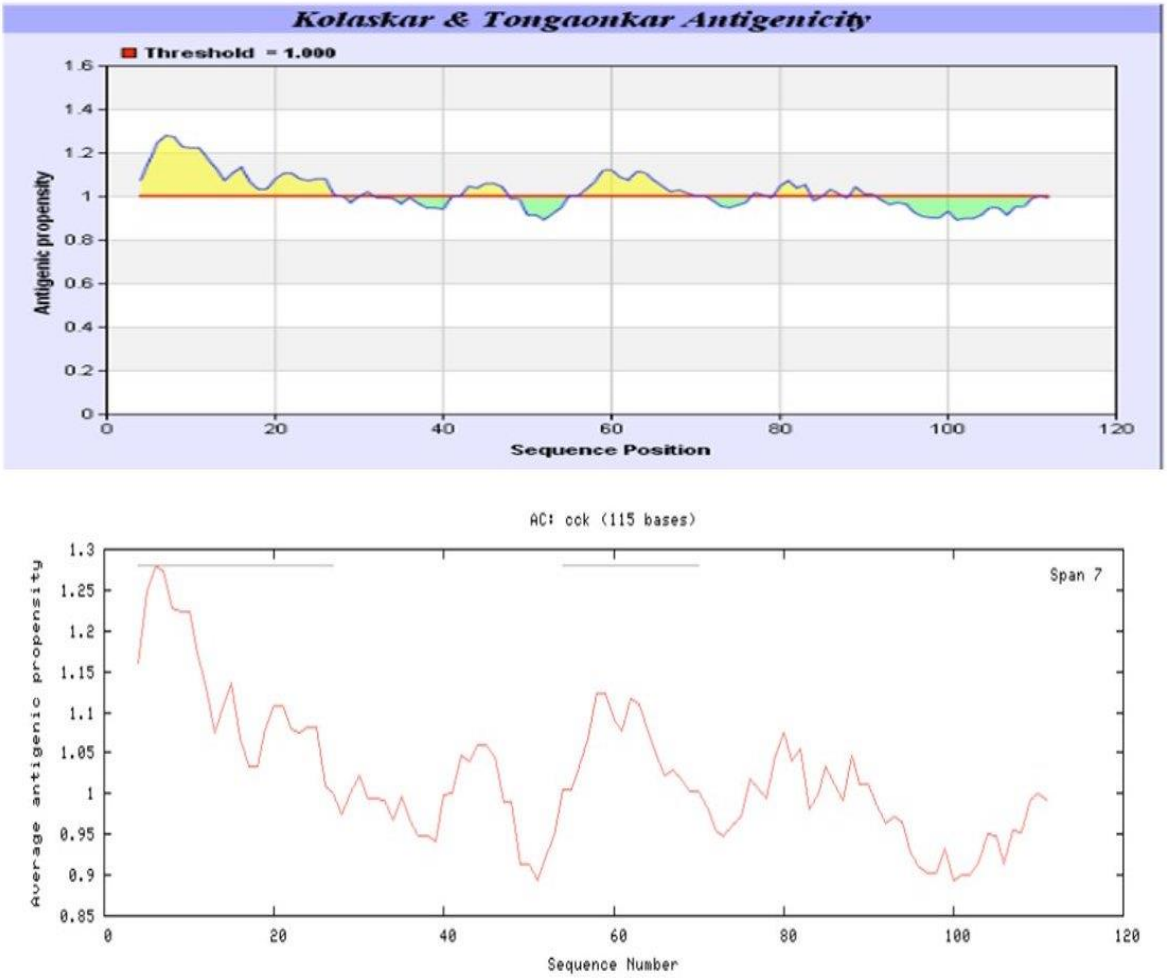

Figure 4.2.The antigenic propensity map of human preproCCK protein.
Threshold is 1.00 so every sequence with antigenic propensity higher than 1.00 is considered as potentially antigenic and can be used as an epitope (yellow). Peptide sequences with antigenic propensity lower than 1.00 (green) are predicted to cause a weak response and are not selected as antigens.

4.3 Production and purification of the antibody

The production of the antibody against the selected epitope was performed by Perbio-Thermo Scientific UK. 2 rabbits were immunized with KLH (Keyhole Limpet Hemacyanin, a carrier protein that is used to enhance the antigenic properties of the peptide)-conjugated peptide and a 70-days protocol was followed. 10ml of pre-immune serum and 100 ml of anti-sera (terminal bleed) were received and the antibody was affinity purified.

The isoelectric point of the antigenic peptide was calculated 10.23 by using GenScript Peptide Property Calculator https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi therefore Affigel-10 (Biorad) was selected for the affinity purification. Specifically, 5ml of Affigel-10 were washed with 50ml dry DMSO (dimethyl sulfoxide) solution (5-10 washes with gentle stirring). Then, 1-2 mg of the antigenic peptide were dissolved in 10ml dry DMSO solution containing 100 μl triethylamine and incubated with the Affigel-10 resin for 12-16 hours at room temperature on a rotating platform. Then, 500μl ethanolamine were added to the mix in order to block any remaining active carboxylates and the incubation continued for 1 hour. Then, the buffer (containing the unbound peptide) was removed and the resin was washed 3 times with 10ml dry DMSO solution. After resuspension, Affigel-10 beads were washed 3 times with 10ml 1M acetic acid and 3 times with 10ml dH_2O each time. Finally, Affigel-10 with bound peptide was resuspend in 10ml dH_2O and transferred in a column to settle. Then, the beads were washed 3 times with 10ml PBS each time.

Crude serum was diluted 1:1 with 10X PBS and 6ml of the diluted serum were applied to the column to settle for 10-15 minutes. Then, the sample passed through the

column, and the flow through fraction was collected and passed again through the column. This step was repeated another two times. After the completion of the binding step, the column was washed 3 times with 10ml 5X PBS. The bound antibody was eluted from the column by using 20ml 100mM citrate (pH 2.5) and was collected in 20 fractions of 1 ml each. Each fraction was collected in a tube containing 0.5ml of 1M Tris pH 8.5. IgG concentration in each fraction was measured by Nanodrop (by measuring OD at 280nm) and the first four fractions (1-4) were found to contain antibody. These fractions were dialyzed in 1X PBS for 16 hours at 4^{o}C by using a dialysis membrane (Sigma). Finally, 10 ml of the "dialysis antibody solution" were concentrated by centrifugation at 1000g using a Centricon Ultracel YM-10 filter device (Millipore). The final volume of the concentrated purified antibody was 500μl and was stored at -20 ^{o}C.

4.4 Validation and Characterization of the custom made anti-human proCCK antibody

We performed a series of pilot experiments to investigate if the antibody can reliably detect human proCCK.

a) We transfected HEK293T cells with a plasmid encoding human proCCK tagged with mRFP (red fluorescence protein) (mRFP_CCK fusion protein) and performed western blot using anti-CCK antibody to confirm that it recognizes specifically proCCK.

b) We performed pilot FACS experiments to investigate if the antibody can be used for flow cytometry applications. In these experiments, we tried to use anti-CCK antibody to identify/sort cells transfected with plasmid expressing human proCCK.

c) We performed immunohistochemistry in sections prepared from human duodenal biopsies to identify if the antibody can specifically stain human I-cells.

4.4.1 Western blot application

Preparation of pcDNA3.1 (+) mRFP_CCK plasmid

We constructed a plasmid encoding mRFP_proCCK fusion protein that contain a N-terminus mRFP tag. This plasmid is designed to express mRFP fluorescent protein fused to the N-terminus of a truncated form of hCCK (human proCCK 21-115 amino acids). This truncated form of hCCK does not contain amino acids 1-20 (signal peptide) to ensure that the expressed fusion protein will be localized in the cytoplasm and will not be secreted (Figure 4.1).

pcDNA3.1 (+) _mRFP was used as the template vector and was cut using the restriction enzymes KpnI (Roche, Switzerland) and EcoRI (Roche, Switzerland) in a digestion reaction (1µl DNA, 2µl NEB Buffer 1, 0.5µl KpnI, 0.5µl EcoRI with 16µl H_20 to make a 20µl total reaction volume). Human proCCK insert (amino acids 21-115) was prepared from a pCMV-SPORT6 plasmid encoding human CCK (GenBank Accession No: NM_007929). This plasmid (Image Clone ID: 5199880, MGC: 1171870, GenBank Accession No. BI757432) was purchased from GeneService, UK.

A diagnostic digest with KpnI enzyme confirmed the identity of the human CCK plasmid. Then, we performed PCR and amplified the cDNA sequence that encodes the amino-acid sequence 21-115 of human proCCK. PCR was performed by using a forward primer (5'-GGTACCCAGCCGGTGCCTCCCGCA-3') that contains a KpnI cleavage site (shown in red) and a reverse primer (5'-GAATTCCTAGGCGGGGTACTC-3') that contains an EcoRI cleavage site (shown in red) and encodes a stop codon (shown in blue) (Figure 4.1). KpnI and EcoRI restriction enzymes were selected to insert hCCK fragment into the multiple cloning site of pcDNA3.1 (+) _mRFP because they do not cut inside the human proCCK fragment sequence.

50ng of plasmid DNA was used as template for each PCR reaction with 2.5µl 10X NH_4 buffer, 2µl dNTPs (2.5mM), 1.5µl $MgCl_2$, (1.5mM), 1µl DMSO, 0.4µl Biotaq. (Bioline, UK) and H_2O up to 25µl.

PCR cycling parameters were:

95°C 5 min (Initial Denaturation)

94°C 1 min

56°C 1 min (Amplification for 35 cycles)

72°C 1 min

72°C 10 minutes (final extension)

4°C Hold

The amplified PCR product was a 300bp fragment that was subsequently purified using a Qiagen PCR purification kit (Qiagen, UK) and subjected to a double restriction digestion with KpnI and Eco RI restriction enzymes (in a 20 µl digestion reaction containing 1µl DNA, 2 µl NEB Buffer I, 0.5µl KpnI, 0.5µl EcoRI and 16µl H_20).

Vector and insert ligation reaction was performed by using a 1:6 vector to insert ratio (50ng of vector DNA + 300ng of insert DNA). The reaction mixture contained 1X DNA Dilution buffer up to 10 µl, 10 µl T4 DNA ligation buffer and 1 µl T4 DNA ligase (Roche Rapid Ligation kit) and was incubated at 25 °C for 20 minutes. Next, competent DH5α E.Coli cells were transformed with the ligation product. A control transformation reaction where cut empty vector was used instead of the ligation product was set up in parallel.

Positively transformed colonies were screened by using colony PCR: Each colony was picked up and diluted into 50µl H_2O and 2µl of this mini culture was used as a template for PCR. The PCR reaction mixture was the same as described above.PCR was performed with an mRFP specific forward primer (5'-

CTACAAGGTGAAGCTCGCC-3') and pro-CCK reverse primer (5'-GAATTCCTAGGCGGGGTACTC-3') to check successful ligation.

PCR cycling parameters were:

95°C 5 min (Initial denaturation)
94°C 1 min
56°C 1 min (Amplification for 35 cycles)
72°C 1 min
72°C 10 min (Final extension)
Hold at 4°C.

20µL of positive mini-cultures were added to 5ml LB nutrient broth (Invitrogen, UK) and cultured overnight shaking at 225rpm at 37°C. 2ml of cultures was purified using the Qiagen miniprep kit according to manufacturer's instructions (Qiagen, UK).
Plasmid DNA sequenced by The University of Manchester DNA sequencing facility to confirm the correct orientation and sequence of the insert.

Transient expression of pcDNA3.1 (+) mRFP_hCCK in HEK293T cells-Methods

Cell culture

HEK293T-cells (a kind gift from Blanche Schwappach, University of Manchester, UK) were cultured in a 37°C/5% CO_2 incubator. Cultured cells were maintained in Dulbecco's modified eagle medium (DMEM) (GIBCO, Invitrogen, UK) with high glucose (4.5g/L) and L-glutamine, supplemented with 10% foetal bovine serum and 1% streptomycin and penicillin (Invitrogen, UK). Cells were seeded into a 6-well plate (5×10^5 cells/well) in order to be 80% confluent at the time of transfection. In parallel, cells were grown on coverslips in a 6-well plate. The cells were transiently transfected with mRFP_hCCK expression plasmid or empty vector pcDNA3.1 (+) by using

FugeneHD transfection reagent, according to the manufacturer's instructions. Medium was changed 24 hours after transfection.

Immunofluorescence

48 hours after transfection, transfected cells grown on coverslips were visualised using an *Olympus BX51* upright microscope using a 20X objective. Specific band pass filter sets for *DAPI and Texas red* were used to prevent bleed through from one channel to the next. Images were analysed using ImageJ (http://rsb.info.nih.gov/ij).

Immunoblotting

48 hours after transfection, media was removed from the wells and the cells were washed once and then collected in 1ml ice cold PBS. Scraped cells were pelleted by centrifugation at 1000g for 10minutes at 4^{o}C. Cell pellets were resuspended in 150µl 1% TritonX lysis buffer (PBS (pH7.4), 1.5mM$CaCl_2$, 1.5mM $MgCl_2$, 1 %(v/v) Triton-X and one tablet of protease inhibitor cocktail per 10ml buffer) and lysed by passing 10-15 times through a 23-gauge needle. Whole cell lysates were collected by centrifugation at 1000g for 5mins at 4^{o}C. An equivalent proportion of each cell lysate was resuspended in 4X lysis buffer (0.313M Tris HCl pH6.8, 10% SDS, 0.05% bromophenol blue, 50% glycerol, 0.4M DTT) containing ß-mercaptoethanol (2.5%). Samples were heated to 95^{o}C for 5 minutes to denature proteins and were separated by electrophoresis on a 10% SDS-PAGE resolving gel. A broad-range prestained protein marker (7-175kDa) (NEB, UK) was used as a protein molecular weight standard. Gels were run at 100V for 100 minutes and proteins were transferred onto a Protron nitrocellulose membrane (Whatman, GE Healthcare) by electroblotting at 400A for 1 hour. After transfer, we used Ponceau staining to confirm successful transfer. Membranes were then incubated in 5% fat-free dry milk solution for 3 hours at room temperature (blocking step) and then washed in TBS-Tween20 0.1% for 15 minutes (3 times X 5 min each).Membranes were incubated overnight with a rabbit anti-mouse anti-mRFP antibody (Medical and Biological Laboratories Ltd, Japan,

PM005 Lot.040) diluted to 1:1000 in 2.5% non-fat dry milk; with unpurified antibody (serum from a rabbit immunized with hCCK antigen peptide diluted 4 times with TBS-Tween); or with a series if dilutions of purified anti-hCCK antibody (1:250, 1:1000, 1:2000, 1:10000) in 2.5% non-fat dry milk. After incubation with primary antibodies, membranes were washed in TBS-Tween20 0.1% three times for 5 minutes each and incubated in secondary antibody Horseradish-Peroxidase-conjugated goat anti-rabbit IgG (DAKO, UK) diluted to 1:2000, in TBS-Tween20 0.1% for 35 minutes at room temperature. After the incubation with the secondary antibody, membranes were washed in TBS-Tween three times for 5 minutes each and incubated with (ECL) enhanced chemiluminescence substrate (Biological Industries, UK)) for 4 minutes in the dark and exposed using Fujifilm LAS-1000 Intelligent Dark Box (exposure time varied from 20 seconds to 1 minute).

Transient expression of pcDNA3.1 (+) mRFP_hCCK in HEK293T cells-Confirmation with immunofluorescence

Transfected cells were stained with nuclei staining reagent Hoechsst33342 (Invitrogen) and visualised using an Olympus Widefield light microscope. Mock-transfected cells displayed no red fluorescence whereas cells transfected with the expression plasmid were observed to express mRFP. mRFP expression proves that these cells had been successfully transfected with mRFP_hCCK plasmid. (Figure 4.3).

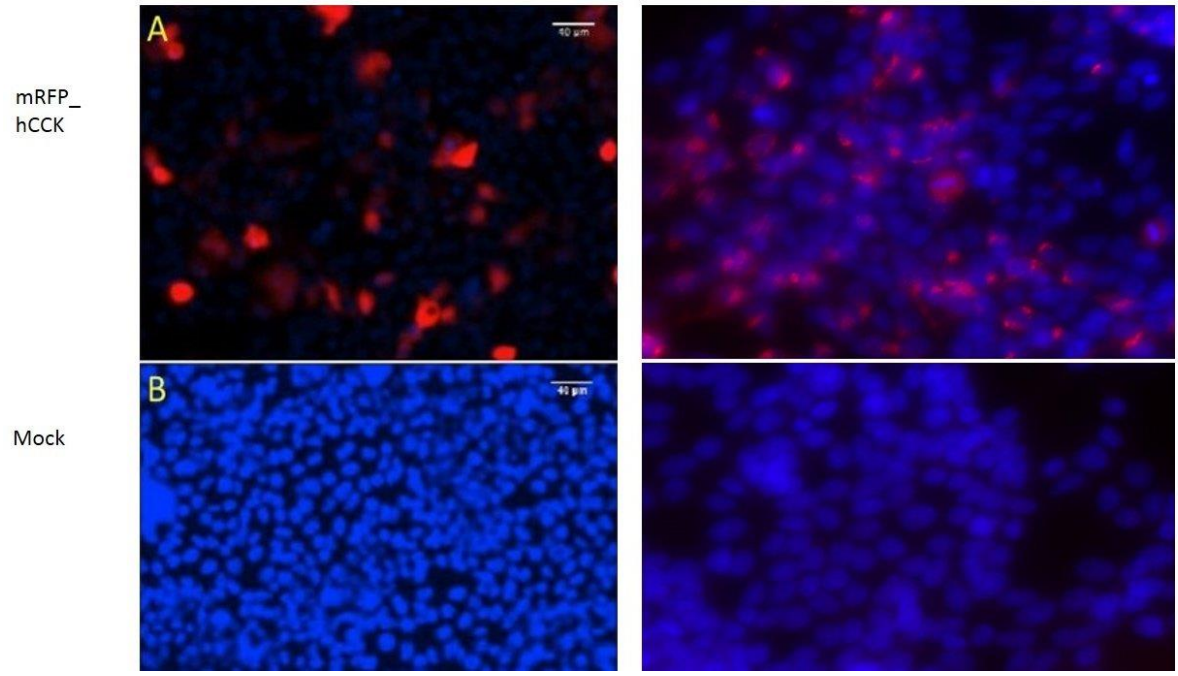

Figure 4.3 HEK293T cells transfected with a pcDNA3.1 (+) mRFP_hCCK plasmid.
A.Positively transfected HEK293T cells display red fluorescence. Hoechst 33342 has stained nuclei, shown in blue.
B.Negative control- Mock-transfection of HEK293T cells transfected with pcDNA3.1 (+) vector. Cells do not display any red fluorescence.
Left and right panels represent two different transfection experiments. Left panel images were captured with 10X magnification, whereas right panel images were captured with 20X magnification

Characterisation of antiserum generated against hCCK.

After the confirmation that HEK293T cells had been successfully transfected with mRFP_hCCK plasmid, cell lysates were prepared from plasmid transfected and mock-transfected cells and were analysed on a 10% SDS-PAGE gel (loaded in triplicates). To ensure appropriate equal loading, we used coomassie blue dye staining (Figure 4.4). Coomassie staining verified that approximately equal amounts of protein have been loaded in each lane.

The other lanes (duplicates) was transferred onto duplicate nitrocellulose membrane filters that were incubated with a rabbit anti-mouse anti-mRFP antibody and an unpurified anti-hCCK serum from rabbit immunised with the hCCK antigen peptide (Figure 4.4).

anti-mRFP antibody mainly detected a band that corresponds to a molecular weight of approximately 40kDa and additional bands of smaller molecular weight. The molecular weight (MW) of the expressed fusion mRFP_hCCK protein is 39kDa (28kDa of mRFP and 10.8kDa hCCK). Therefore, the main detected band mRFP_hCCK that is recognized by the anti-mRFP antibody. The presence of lower MW bands suggest that CCK may be processed in the HEK293T cells. A potential cleavage of CCK-8 and CCK-33 fragments from the C-term of the fusion protein would result in these lower MW bands. mRFP is present in the N-terminus of the hCCK sequence, therefore cleavage of CCK does not affect the anti-mRFP antibody binding.

The unpurified anti-hCCK antiserum detected the same bands (suggesting that it successfully detects human proCCK) but also background bands in the mock-transfected sample lane. The presence of these non-specific bands was expected because we used crude antiserum and not the purified antibody. The anti-human CCK antiserum also detected smaller molecular weight bands. This antiserum was generated against the N-term human proCCK sequence, therefore it should detect mRFP_hCCK even after cleavage of the molecular forms CCK-8 and CCK-33 (Figure 4.4).

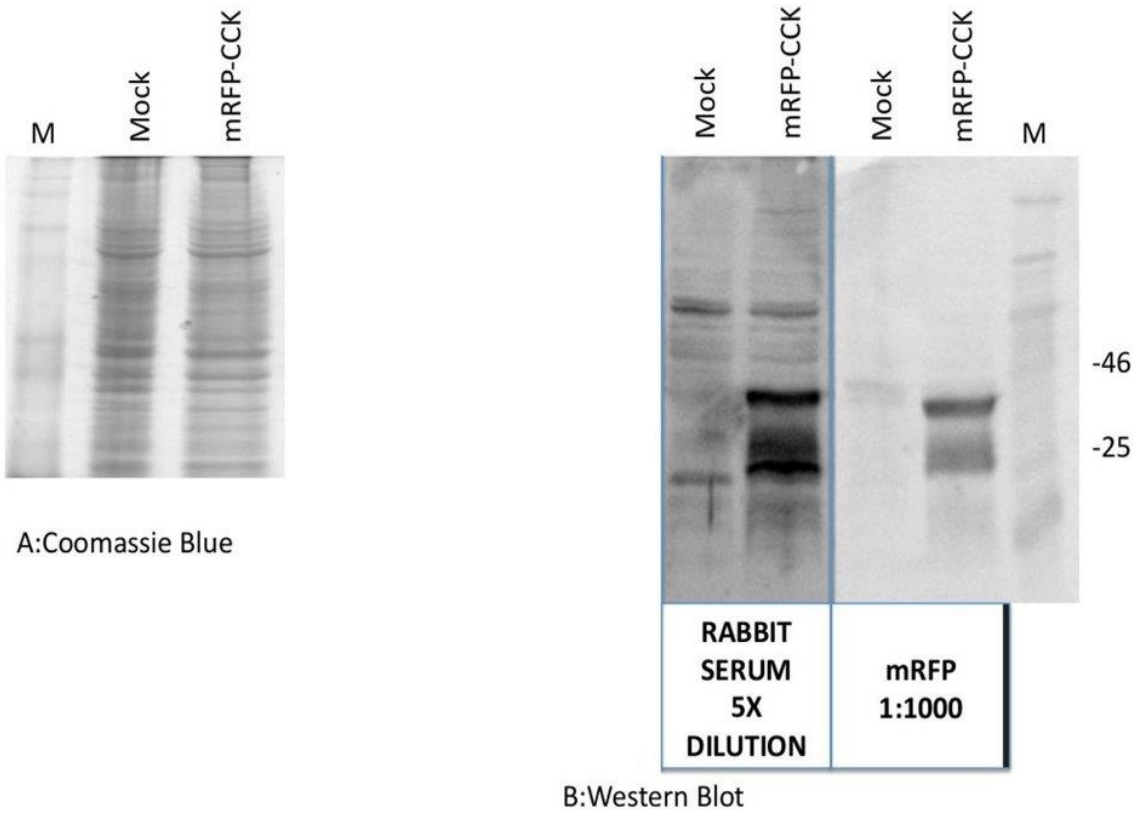

Figure 4.4 Antiserum raised against the selected hCCK epitope recognizes human proCCK.
A. Coomassie blue stain verifies equal loading of the cell lysates samples prepared from mRFP_hCCK transfected and mock-transfected cells. B. Immunoblot of cell lysates prepared from mRFP_hCCK transfected cells and mock transfected cells. Samples were run on the same gel as duplicates. Filters were incubated with anti-mRFP antibody and diluted unpurified serum from rabbit immunized with hCCK antigen. Prominent bands of 39kDa MW are present in each of the lanes representing transfected cells indicating that the raised antibody can detect hCCK. Image was obtained using a Fujifilm LAS-1000 Intelligent Dark Box (20 second exposure). M declares prestain protein ladder.

Subsequently, we tested the purified antibody against hCCK to confirm its sensitivity and specificity. In this experiment, we subjected to immunoblot analysis cell lysates prepared from cells transfected with mRFP_hCCK plasmid, mRFP plasmid or mock-

transfected cells as negative control. We ran 5 replicates and performed western blot with anti-mRFP antibody or 4 different dilutions of anti-hCCK antibody (dilution range from 1:250, 1:1000, 1:2000 up to 1:10000). As expected, mRFP antibody recognized both mRFP protein (approximate molecular weight 28kDa) and mRFP_CCK fusion protein (and additional bands that possibly correspond to processed forms of mRFP_CCK fusion protein). hCCK antibody successfully detects the mRFP_CCK protein (full length and processed forms) but not mRFP protein only or any protein in the mock transfected cells. High concentrations of hCCK antibody (dilutions 1:250, 1:1000, 1:2000) produce a rather non-specific pattern and recognize additional bands that do not correspond to the target protein. The optimum concentration of the antibody is found in the high dilution (1:10000) that produces a clear recognition pattern of mRFP_hCCK fusion protein (full length and processed forms) (Figure 4.5)

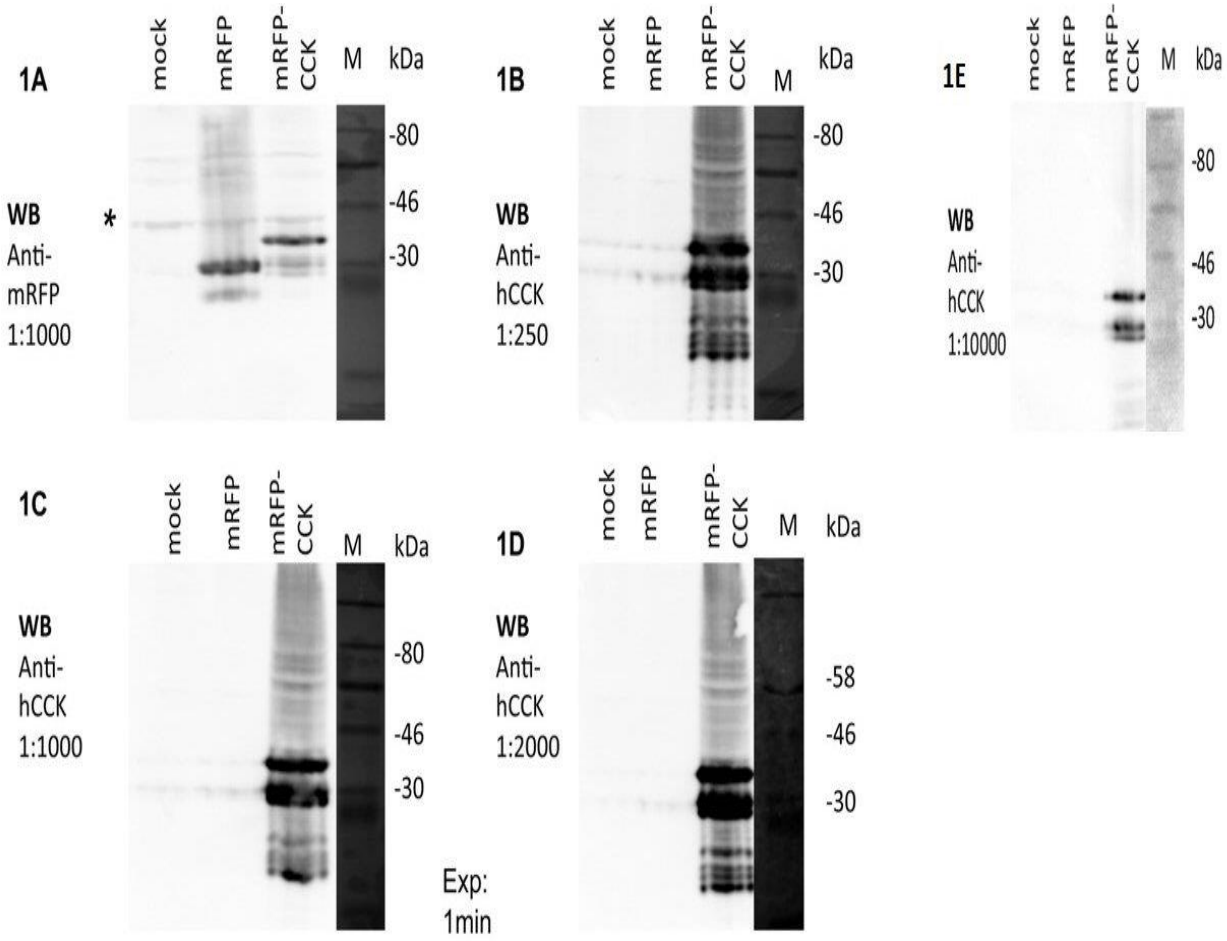

Figure 4.5 Purified anti-human proCCK antibody successfully recognizes human proCCK

Immunoblot of lysates prepared from mock transfected, mRFP transfected or mRFP_hCCK transfected cells with anti-mRFP and anti hCCK antibodies. 1A represents immunoblot with anti-mRFP antibody that recognizes both mRFP and mRFP_hCCK proteins. The shift in the molecular weight of recognized bands is approximately the molecular weight of hCCK. Asterisk declares non-specific bands. 1B-1E represent immunoblots with different dilutions of anti-hCCK antibody and show that the antibody can recognize the mRFP-CCK fusion protein but not mRFP alone. 1:10000 dilution (1E) recognizes CCK without cross-reaction with other proteins. M declares prestained protein ladder.

4.4.2 Pilot FACS analysis experiments

After demonstrating that our custom made hCCK antibody can be used in immunoblot experiments for the recognition of hCCK, we proceeded to perform pilot FACS analysis experiments to verify if the antibody can detect intracellular hCCK so it can

be employed in the flow cytometry sorting of human CCK-containing cells. For this purpose, we investigated if we can use the antibody to sort cells transfected with mRFP_hCCK.

HEK293T cells were transfected with mRFP_CCK plasmid, mRFP plasmid or empty vector (mock transfected). 48 hours after transfection, transfected cells were washed with ice cold PBS and then collected and pelleted by centrifugation at 1000g for 10 minutes at 4^{o}C. Supernatant was discarded and cell pellets were resuspended in 1ml of methanol and stored at -20C for 30 min. Methanol fixed and permeabilized cells were incubated with anti-hCCK antibody (1:500 dilution) for 16 hours at 4^{o}C in the dark. After incubation with primary antibody, cells were washed with PXS (3 times for 5 minutes each) and incubates with FITC-conjugated anti-rabbit secondary antibody (1:500 dilution) for 1 hour on ice in the dark. Finally, cells were washed in FACS buffer (PBS+3% FCS) and were subjected to FACS analysis. FACS analysis was performed by using a BD Biosciences FACS Aria cell sorter with Diva software version 5.

Cells were analysed based on their fluorescence intensity using a 488nm laser (530/30 excitation bandpass filter for FITC and 617/25 excitation bandpass filter for mRFP). Firstly, we analysed separately FITC and mRFP fluorescent intensity and then we combined both analyses in one plot. The resulting FACS plots were split in 4 quadrants:

Q1 represents the mRFP+ve/FITC –ve cells (expected for mRFP transfected cells)

Q2 represents the mRFP+ve/FITC+ve cells (expected for mRFP_hCCK cells if the antibody can detect intracellular CCK)

Q3 represents the mRFP-ve/FITC-ve cells (expected for mock transfected cells)

In the first experiment that we performed, we achieved a very high transfection efficiency. Therefore, above 95% of mRFP transfected cells and 90% of mRFP_hCCK

transfected cells were highly fluorescent for mRFP. From the mRFP+ve cells that were stained with hCCK antibody, only 1.4% was found to be FITC-fluorescent indicating a low non-specific staining. Unfortunately, from the 92% of mRFP_hCCK transfected cells that are highly fluorescent for mRFP there is a very low percentage (5.1%) of mRFP+ve/FITC+ve cells that were stained with the hCCK antibody (Figure 9). Based on this experiment, it seems that the hCCK antibody cannot be used effectively for flow cytometry applications.

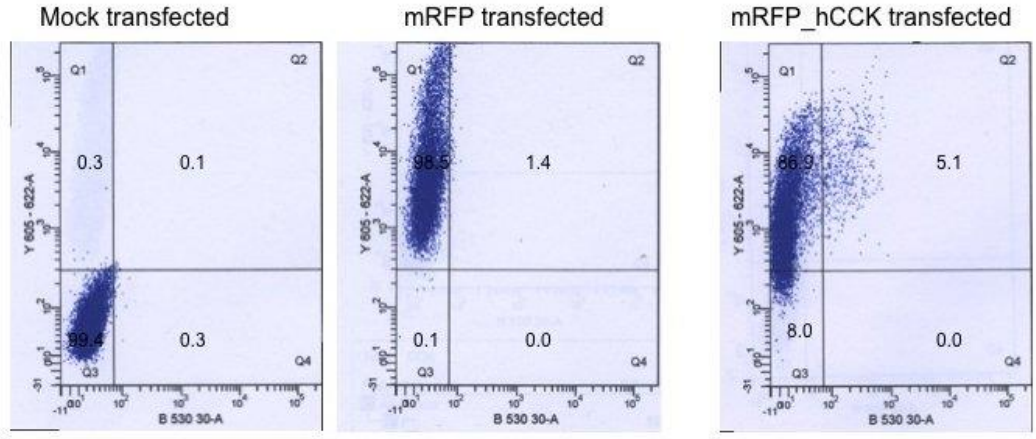

Figure 4.6 FACS analysis of mock-transfected, mRFP transfected and mRFP_hCCK transfected cells incubated with anti-hCCK primary antibody (1:500) and FITC-labelled secondary antibody.
Cells were analysed based on their FITC fluorescence (x axis, 530nm) and RFP fluorescence (y axis, 605 nm). Mock transfected cells were expected to display no fluorescence (Q3), whereas mRFP transfected cells were expected to be RFP+ve (Q1) and mRFP_hCCK cells were expected to be RFP+ve/FITC+ve (Q2) if the antibody recognizes CCK. The population of RFP+ve/FITC+ve mRFP_CCK transfected cells is 5.1% of total mRFP+ cells indicating that the antibody could not recognize CCK.

We repeated the experiment, following the same protocol but applying a higher concentration of antibody (dilution 1:250). Similarly with the first experiment, the transfection efficiency for mRFP was very high. Additionally, there was only a very low percentage of mRFP+ve cells that were non-specifically stained for CCK. A major

difference with the first experiment was that the efficiency of transfection with mRFP_CCK antibody was lower than expected (35.5% of cells were mRFP+ve). In this experiment, 9.4% of this cell population were also FITC-positive and represented cells stained with hCCK antibody. Therefore, approximately 25% of all CCK-containing cells were positively stained with hCCK antibody (Figure 4.7).

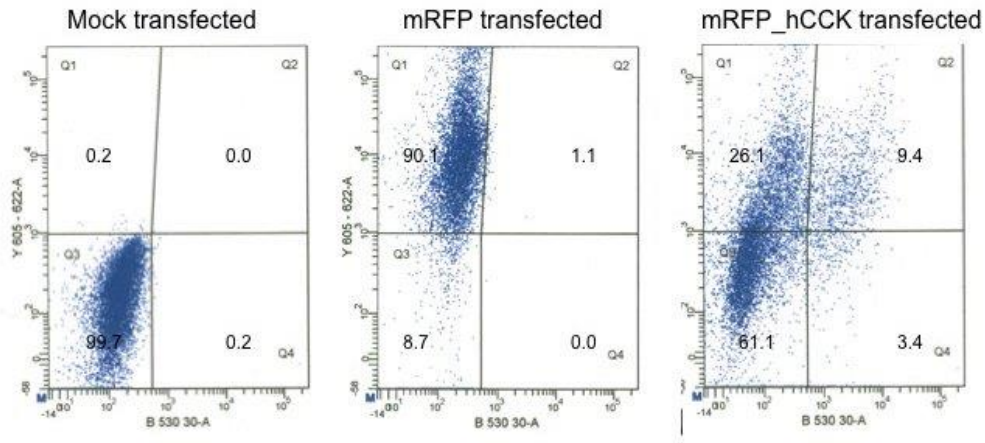

Figure 4.7 FACS analysis of mock-transfected, mRFP transfected and mRFP_hCCK transfected cells incubated with anti-hCCK primary antibody (1:500) and FITC-labelled secondary antibody.
The analysis and the generation of FACS plots was performed as described in Figure 4.6 legend. In this experiment, the transfection efficiency for mRFP_hCCK was low and totally 35.5% of cells were mRFP+ve. 9.4% of them were stained with the anti-hCCK antibody showing that the antibody can recognize approximately 25% of CCK-expressing cells.

These results indicate that the efficiency of the anti- human proCCK antibody in flow cytometry application is quite low and suggest that a series of optimization experiments should be performed in order to conclude if the antibody is suitable as a tool for intracellular FACS sorting and develop a specific protocol for its use. Even if the antibody will be proved useful for the sorting of CCK containing HEK293 cells, the different characteristics between a cell line and primary intestinal epithelial cells

indicate that a further characterization of the antibody and the development of new protocols for cell dissociation and flow cytometry staining will be required for the successful employment of the technique for the isolation/purification of human I-cells.

4.4.3 Immunohistochemistry

We used the anti-hCCK antibody to perform immunohistochemistry in paraffin-embedded sections prepared from human duodenum biopsies (a gift from Professor John McLaughlin, University of Manchester). The applied dilution of the antibody was 1:500 and Alexa 594 goat anti-rabbit secondary antibody was used to detect signal. The anti-hCCK antibody stains rare, scattered cells that have the typical morphology of enteroendocrine cells, displaying higher intensity in the basolateral membrane where the secretory vesicles containing CCK are localized. This antibody staining pattern is specific, without non-specific staining of epithelial cells (Figure 4.8)

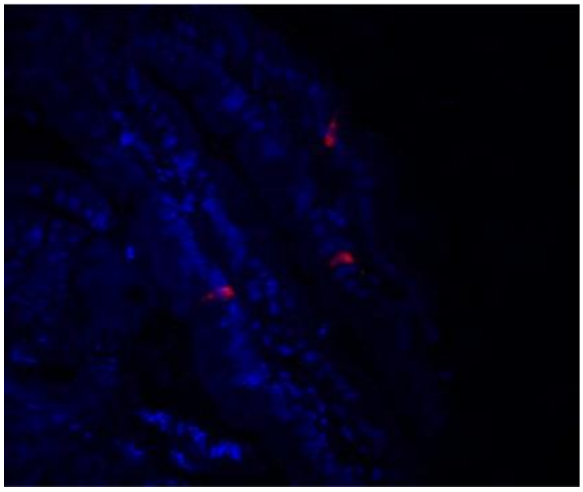

Figure 4.8 Immunohistochemistry of human duodenum section with anti-hCCK antibody.
The antibody stains specifically very few flask-shaped cells that have a narrow apical and a broad basolateral membrane. These cells have the typical morphology of enteroendocrine cells and may represent human I-cells. Courtesy of Claire Demenis and Craig P. Smith

CHAPTER 5

General discussion

The enteroendocrine system “tastes” nutrients that are present in the gut lumen after a meal and integrates information from the luminal environment (that can be considered as the outside world) to the epithelium of the gastrointestinal tract (Moran *et al*., 2008). There are different subsets of EEC cells that release a variety of peptides (Rindi *et al*., 2004), although they may share a common mechanism of response to nutrients. This chemosensing mechanism triggers the release of gut hormones from the EEC cells (Engelstoft *et al*., 2008). Secreted gut hormones signal to the brain via a vagus nerve-mediated pathway or act directly to their target cells in an endocrine or paracrine way. I-cells respond to nutrients by releasing CCK that transmits a potent anorectic signal to the brain. Additionally, CCK co-ordinates digestion by promoting the release of digestive secretions (bile, pancreatic enzymes) and by delaying the delivery of nutrients from the stomach to the duodenum. Despite of their importance, native I-cells have not been studied so far.

We used a transgenic mouse model with fluorescent-tagged CCK-containing cells in order to perform a molecular characterization of duodenal I-cells. We restricted our interest to the duodenal CCK-containing cells because they represent the typical I-cells that are mainly localized in the upper small intestine. Duodenal I-cells constitute a subpopulation of EEC cells that are in early contact with the ingested nutrients, after their entrance to the lumen of the small intestine.

Our first aim was to validate the experimental animal model and we showed that the eGFP-tagged cells truly represent CCK-containing cells. Then, we developed successfully a method for the isolation of relatively pure populations of native I-cells, with minimum contamination from enterocytes. Using this protocol, we can isolate routinely 5000-15000 I-cells (from 4 mice). In parallel, we tried to set up cultures of

purified I-cells but they were dying within one hour from their isolation. We also attempted to set up cultures of mixed dissociated duodenal cell populations (that contain I-cells) but we could not maintain them due to the very low viability of the cultured cells. The lack of a robust and reliable assay for CCK measurement did not allow us to perform functional experiments in order to assess the response of native I-cells to a wide range of nutrients that have been assumed to stimulate I-cells and trigger CCK release. These limitations oriented our study to the molecular characterization of the native I-cells. Thus, we used semi-quantitative RT-PCR for a targeted analysis of I-cells transcriptome and immunostaining/mass spectrometry to check the expression of peptides/hormones in I-cells.

The first main finding of our study is that duodenal I-cells contain mRNA transcripts encoding LCFA receptors, SCFA receptors, fatty acid derivative peptides and endocannabinoid receptor CB1. The presence of LCFA receptor mRNA transcripts in I-cells is in line with previous observations in enteroendocrine cell lines and other native enteroendocrine cell subtypes (L- and K- cells) (Liou *et al*., 2011a, Reimann *et al*., 2008, Parker *et al*., 2009). It also suggests that GPR40/FFAR1 and GPR120/O3FAR1 are the chemosensors that mediate CCK release in response to LCFA, an effect that is described in both cell line models and humans (McLaughlin *et al*., 1998, McLaughlin *et al*., 1999). It remains to be determined which receptor has a critical role in this dietary lipid-induced CCK secretion.

In contrast with LCFA receptors that mediate CCK release, the presence of mRNA transcripts encoding SCFA receptors GPR41/FFAR3 and GPR43/FFAR2 in I-cells has no clear physiological role, at least in the nutrient sensing process. SCFA receptors have been proposed to mediate GLP-1 and PYY release from distal intestinal and

colonic L-cells (Samuel *et al*., 2008, Tolhurst *et al*., 2012a). It is unlikely that they have a similar role in duodenal I-cells because SCFA do not stimulate CCK release. Additionally, the intraluminal concentration of SCFA in the duodenal microenvironment is very low in contrast with their high levels in the distal small intestine and the colon, where they are generated by the gut microbiota. It is tempting to speculate that SCFA receptors in I-cells may be localized in the basolateral membrane and sense plasma, and not luminal, SCFA.

An interesting finding of our study is that duodenal I-cells contain mRNA transcripts encoding GPR119 and CB1 receptor. GPR119 may act as a classic fat chemosensor that triggers hormone release in response to 2-monoacylglycerols that are products of fat digestion in the intestinal lumen (Hansen *et al*., 2012). GPR119 has also the ability to sense fatty acid derivatives and endogenous lipid metabolites, like oleoylethanolamide, that are generated in the enterocytes (Schwartz *et al*., 2008). Expression of GPR119 in I-cells may indicate that these lipid amides act in a paracrine way to transmit signals from the intestinal epithelium to I-cells and relay a CCK-mediated anorectic signal to the brain.

The presence of CB1 receptor in duodenal I-cells raises the possibility that the intestinal endocannabinoid system may target these cells and control CCK release at this level. Intestinal endocannabinoid peptides are upregulated during energy restriction and promote food intake via the activation of CB1 receptor localized in the vagal afferent neurons (Izzo *et al*., 2009). A parallel suppression of CCK release from I-cells may contribute indirectly to this orexigenic response. Endocannabinoid peptides, released by intestinal epithelial cells, are also selectively upregulated after a fatty meal and signal to the brain in order to re-inforce fat intake (DiPatrizio *et al*.,

2011). The activation of CB1 receptor expressed in duodenal I-cells by the local endocannabinoid peptides may inhibit CCK secretion (Di Marzo, 2011), an effect that attenuates the satiety signal to the brain and promotes further fat intake.

Our second main finding is that duodenal I-cells express multiple gut hormones including GIP, proglucagon, PYY, neurotensin, secretin and ghrelin. We used semi-quantitative RT-PCR to reveal that I-cells are enriched in mRNA transcripts encoding these gut hormones. Mass spectrometry analysis of sorted I-cells confirmed that they express these peptides. We also performed immunostaining experiments and identified subpopulation of I-cells that co-express CCK with GIP, proglucagon, PYY and ghrelin. The subpopulations of I-cells that co-express CCK with other gut hormones are localized in both the intestinal crypts (immature I-cells) and the villi (terminally differentiated I-cells).

It is remarkable that a similar study performed for duodenal K- and L- cells reported that almost all of them co-express CCK with GIP and GLP-1 respectively (Habib *et al*., 2012). There is a significant overlap between duodenal I-, K-, and L- cells with the majority of them expressing CCK that represent a “common hormone” for these duodenal EEC cell subsets. Our data suggest that I-cells also overlap with neurotensin, secretin and ghrelin containing cells. Interestingly, a subpopulation of I-cells appears to co-express the principal anorectic peptide CCK with the orexigenic hormone ghrelin. The extensive overlap of CCK-containing cells with other duodenal EEC cells subtypes and the presence of CCK mRNA/protein in almost all the duodenal native EEC cells may indicate that CCK is the predominant gut hormone expressed in the (EEC cells) of the proximal small intestine. On the other hand, the

predominant gut hormones of distal small intestine are the L-cell derived peptides GLP-1 and PYY.

Further studies are needed in order to identify what is the percentage of I-cells that co-express CCK with other gut hormones and if this co-expression pattern is restricted to 2-3 peptides or is broader and includes all the gut hormones expressed in the duodenum. Another outstanding question is if duodenal I-cells have the capacity to co-release two or more hormones, under physiological stimulation. It is particularly interesting to investigate if CCK and ghrelin are co-secreted (and under which physiological stimuli) or if they present totally distinct release patterns. Functional studies are required to determine what are the stimuli that trigger ghrelin release from I-cells and how the chemosensing machinery tailors the response of I-cells to release CCK or/and ghrelin to the energy status of the organism.

The overlap between different subtypes of EEC cells is not restricted only to these localized in the duodenum. Distal small intestinal CCK-containing cells co-express CCK with other gut hormones (Habib *et al*., 2012, Egerod *et al*., 2012) and the ileal EEC cells co-express multiple hormones, with L-cell derived peptides being predominant. The traditional classification of EEC cells is based on the “one cell-one hormone” model that does not seem to reflect the peptide content of native EEC cells and specifically I-cells. This classification may be true for subpopulations of EEC cells that express only one predominant hormone but needs to be revised in the light of recent studies.

The characterization of native EEC cells indicates that different subsets may share a common gene expression profile of receptors that mediate the response to nutrients and also a common expression pattern of gut hormones that they secrete upon stimulation. This overlap may indicate the plasticity of EEC cells that enables them to

respond to a wide range of rapidly changing metabolic signals. It is crucial a) to elucidate the pathways that couple the sensing of nutrients to the release of hormones and b) to identify the mechanisms that govern the adaptive response of the enteroendocrine system to different environmental factors. These are the key questions that we have to answer in order to develop new gut-based therapeutic tools that will target the endocrine cells and mimic the metabolic benefits of bariatric surgery to fight “diabesity”.

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