Cellular expression and Function of CCK in the Mouse Duodenum

A thesis submitted to the University of Manchester for the degree of PhD Physiology in The Faculty of Life Sciences

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Claire Demenis

Faculty of Life Sciences

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ABSTRACT
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Enteroendocrine cells (EECs) express key gastrointestinal (GI) hormones including cholecystokinin (CCK), gastric inhibitory peptide (GIP), peptide tyrosine tyrosine (PYY), glucagon-like peptide-1 (GLP-1) and ghrelin. EECs are characterised to contain the hormones derived from one precursor protein. Of these, CCK-cells are typically concentrated in the proximal small intestine and release CCK upon stimulation by nutrient ligands and in so doing signal to multiple tissues to co-ordinate and optimise digestive, absorptive functions, and instil hunger or satiety. The aims of this study were to establish whether EECs co-expressed CCK alongside other key GI peptides and to determine a paracrine role for CCK to increase FA uptake in intestinal cells.

These studies utilised an eGFP-CCK transgenic mouse model. Tissue sections from eGFP-CCK mice were paraffin embedded and immunostained against an array of targets. Firstly, an anti-GFP antiserum was employed to visualise eGFP-cells along the GI tract, and duodenal sections were dual stained for anti-GFP and an anti-proCCK antiserum to confirm eGFP-cells represented CCK-cells. A series of dual-immunostaining experiments ensued to probe duodenal eGFP-cells for a range of different hormonal targets and demonstrated that a significant number of eGFP-CCK-cells contained GIP (37%), PYY (45%), proglucagon (14%) and ghrelin (50%). Further dual-staining experiments were carried out to stain for CCK alongside PYY, GIP or ghrelin and enabled analysis of the intracellular localisation of co-expressing peptides, which indicated that these peptides were packaged in the same and also within distinctly separate vesicles. These data demonstrate CCK-cells can co-express more than one peptide and analysis of intracellular labelling indicates they may have the ability to co-release CCK alongside other peptides.

To investigate a potential paracrine-signalling pathway for CCK a FA uptake assay was performed using a fluorescent C12-fatty acid (FA) analogue (Bodipy-FA) that was analysed using fluorescent activated cell sorting (FACS). Single small intestinal cells of eGFP-CCK mice were prepared using an EDTA chemical/mechanical dissociation method. Cell samples were either non-treated (control) or pre-treated with a targeted compound prior to incubation with Bodipy-FA. Treatment of cells with oleoylethanolamide, glucagon-like peptide-2 (GLP-2) or CCK increased FA uptake 2 to 3-fold and this increase was demonstrated to be carrier-mediated. Experiments ensued employing CCK-cell ligands to implicate activity of CCK-cells in this process. Bombesin and L-amino acids induced a dynamic increase in FA uptake comparable to that achieved by pre-treatment with CCK. However, implementation of the protocol using cells from a CCK KO model achieved replicate data and therefore demonstrated these effects were not exclusive to CCK-cells.

In conclusion, data presented in this thesis establish that a spectrum of key gut hormones is expressed in individual EECs. Furthermore, a paracrine action of CCK-signalling is implicated to increase the absorptive ability of neighbouring enterocytes. These data suggest that CCK-cells have the ability to integrate nutrient signals and secrete a cocktail of hormones in response. These findings imply an increased complexity to the enteroendocrine system whereby GI peptides may work together to potentiate a desired response without requirement of signals from higher centres.

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Abbreviations

AA	Amino Acids
Ab	Antibody
ACS	Acyl-CoA Synthetase
Ag	Antigen
AN	Arcuate Nucleus
AgRP	Agouti-Related Peptide
Apo	Apolipoprotein
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BLP	Bombesin-like Peptide
bHLH	Basic Helix-Loop-Helix
Bodipy-FA	Bodipy Fatty Acids
Bp	Base Pairs
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CART	Cocaine-and-amphetamine-regulated transcript
CCK	Cholecystokinin
CCK-NS	Non-sulphated Cholecystokinin
CCK-R	Cholecystokinin receptor
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
DAG	Des-Acyl Ghrelin
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EEC	Enteroendocrine Cell
eGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
FA	Fatty Acids
FABP	Fatty Acid Binding Protein
FACS	Fluorescence-Activated Cell Sorting
FATP	Fatty Acid Transport Protein

FBS	Foetal bovine serum
FFA	Free Fatty Acids
GFP	Green Fluorescent Protein
GIP	Gastric-Inhibitory Peptide
GLP-1	Glucagon-Like Peptide-1
GLP-2	Glucagon-Like Peptide-2
GI	Gastrointestinal
GPCR	G-Protein Coupled Receptor
GRP	Gastrin Releasing Peptide
GTP	Guanosine Triphosphate
HBSS	Hank's Buffered Saline Solution
KO	Knockout
LCFA	Long Chain Fatty Acid
LI	Large Intestine
Math1	Mouse Atonal Homolog 1
mRNA	Messenger Ribonucleic Acid
MOPS	3-(N-morpholino)propanesulfonic acid
Ngn3	Neurogenin 3
NPY	Neuropeptide Y
OEA	Oleoylethanolamide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PLC	Phospholipase C
PM	Plasma Membrane
POMC	Pro-Opiomelanocortin
PPAR-α	Peroxisome Proliferator Activated Receptor-α
PYY	Peptide Tyrosine Tyrosine
R	Receptor
RT	Room temperature
SCFA	Short Chain Fatty Acid
S.E.M	Standard Error of the Mean
SI	Small Intestine

SSO	Sulfo-N-Succinimidyl Oleate
STC-1	Secretin-Tumour Cell line-1
T2DM	Type 2 Diabetes Mellitus
TF	Transcription Factor
TG	Triglyceride
VA	Vectorial Acylation
VDCC	Voltage Dependent Calcium Channel
VLCFA	Very Long Chain Fatty Acid
WT	Wild Type

Chapter One

Introduction

1.0. Overview

The mammalian digestive system has evolved to optimally digest and absorb nutrients. Ingested nutrients pass through a long tube, the gastrointestinal (GI) tract, the lumen of which is continuous with the outside world. Within the GI tract, food is broken down - digested - by mechanical force and chemical degradation, to elaborate nutrients in a form that can be taken up - absorbed - into the body. The processes of digestion and absorption have evolved to fulfil the demands dictated by the environmental niche occupied by the species under consideration and accordingly have become highly specialised. In addition to simply digesting and absorbing food, the GI tract has evolved to sense nutrients. The sensing of nutrients confers an evolutionary advantage in that it enables the organism to optimise and fine-tune the processes of digestion and absorption. The work detailed in this thesis addresses nutrient sensing and nutrient absorption. What follows is an introduction to the GI tract, covering the anatomy, in particular the types of cells that constitute the epithelium lining the tract, with particular emphasis on hormone secreting enteroendocrine cells. Nutrient detection, nutrient digestion and nutrient uptake will also be covered with particular focus on lipids. In addition, the distribution, structure and function of gastrointestinal peptide hormones will also be covered in depth.

1.1. The Gastrointestinal Tract

The gastrointestinal (GI) tract is part of the digestive system and begins at the mouth, and ends at the anus. Between these two structures lies, the pharynx, the oesophagus, stomach and the intestine. The intestine is divided into two major sections, the small and the large intestine. The work detailed in this thesis primarily concerns the small intestine.

The small intestine (SI) in humans is about 7 metres long and consists of a tube of smooth muscle lined on its inside with a single layer of specialised epithelial cells.

The SI is divided into three subsections; the duodenum, the jejunum and the ileum. The duodenum receives food from the stomach and is the predominant region of digestion and absorption of dietary nutrients. Next is the jejunum where some digestion takes places but is primarily specialised for the absorption of nutrients into the bloodstream. This is followed by the ileum, which is again specialised to absorb nutrients that were not absorbed by the jejunum.

Each region of the small intestine is structurally adapted with finger-like villi that protrude into the intestinal lumen to maximise the surface area available for exchange (Figure 1.1).

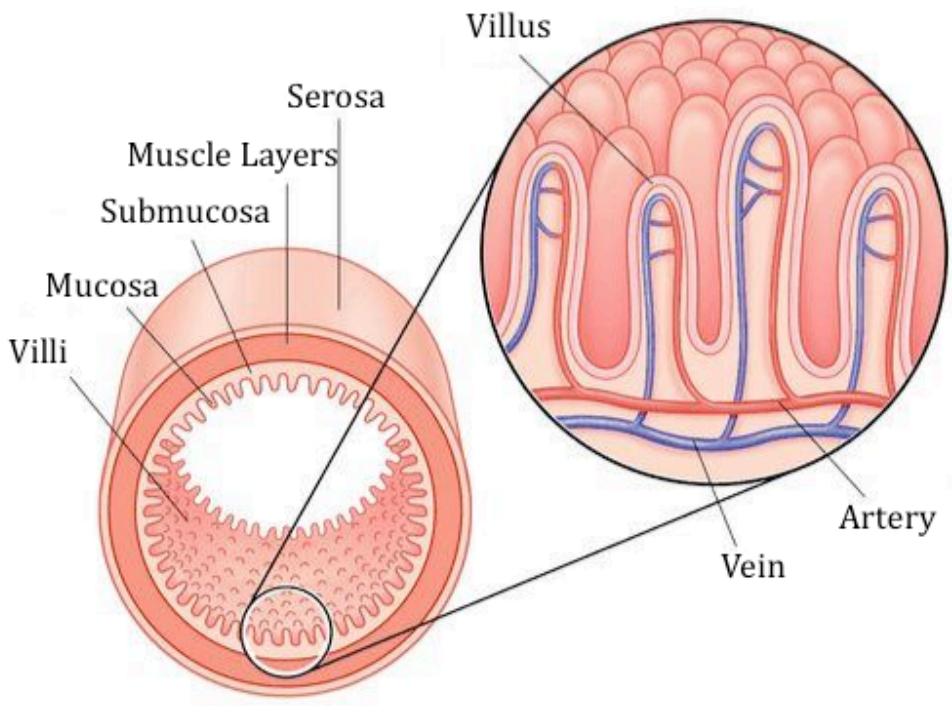


Figure 1.1. Cross-sectional representation of the structure of the GI tract.

The GI tract consists of an outer protective membrane (serosa) with a thick smooth muscle layer beneath it. Inside this muscular tube are the submucosa and mucosa. The surface of the mucosa is structured with finger-like projections called villi. Image taken from http://tinyurl.com/nfd2u7m.

The epithelial monolayer lining the villi consists of specialised cells that work in concert to affect digestion and absorption of nutrients. Six main cell types make up the SI epithelium tract and these display 'region-specific' distribution. The six classes of cells are; pluripotent stem cells, enterocytes, goblet cells, tuft cells, paneth cells and enteroendocrine cells (EECs) (Roth et al., 1990).

Pluripotent stem cells are situated in the crypts of Lieberkuhn that are located beneath the base of the villi (Figure 1.2). Stem cells are the common precursor cell for the four other epithelial cell types listed above. Stem cells proliferate and differentiate into different cell types that migrate along the crypt-villi axis. The resultant populations of epithelial cells have a functional life-span of 3-7 days before they are sloughed off and are passed from the body in faeces (Gordon et al., 1992). Enterocytes represent the majority of the cells of the intestinal epithelium. These are the absorptive cells of the gut, responsible for the absorption of nutrients, water and electrolytes. The other three cell types are secretory cells: Paneth cells secrete anti-microbial compounds, that help maintain the GI barrier and in so doing are important for intestinal immunity and pathogen defence (Roth et al., 1990). Goblet cells secrete mucus that aids intestinal defence by trapping pathogens. In addition, mucus also lubricates the GI tract to assist the movement of chyme through the SI. The final cell type is EECs that secrete hormones or hormone-like substances upon detection of nutrients in the intestinal lumen and these cells and their actions will be the focus of this thesis.

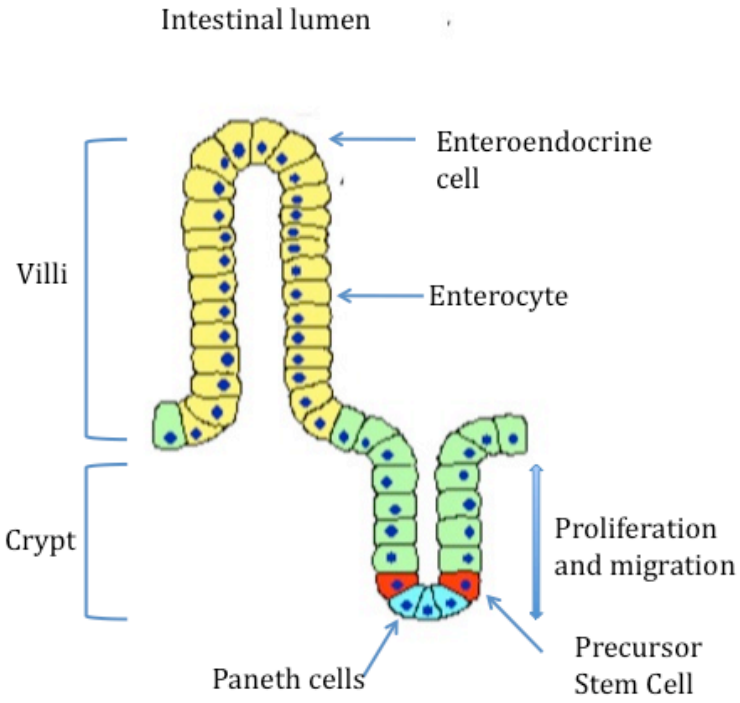


Figure 1.2. The cellular make-up of the small intestine.

Intestinal epithelial cells differentiate from precursor stem cells located in crypts. Nascent cells migrate up the crypt-villus axis and differentiate into enterocytes, paneth cells, goblet cells or enteroendocrine cells.

1.1.1. Enteroendocrine Cells (EEC)

The majority of enteroendocrine cells in the SI have a typical flask-shaped structure, and are termed 'open-type' cells. The narrow apical membrane faces toward the gut lumen and has many microvillus processes on the surface that greatly increase the surface area of the cell and thus exposure to the luminal contents (Liddle, 1997) (Figure 1.3).

Enteroendocrine cells represent only ~1% of the intestinal epithelia, yet together constitute the largest endocrine gland in the human body and are collectively known as the enteroendocrine system (Rindi et al., 2004).

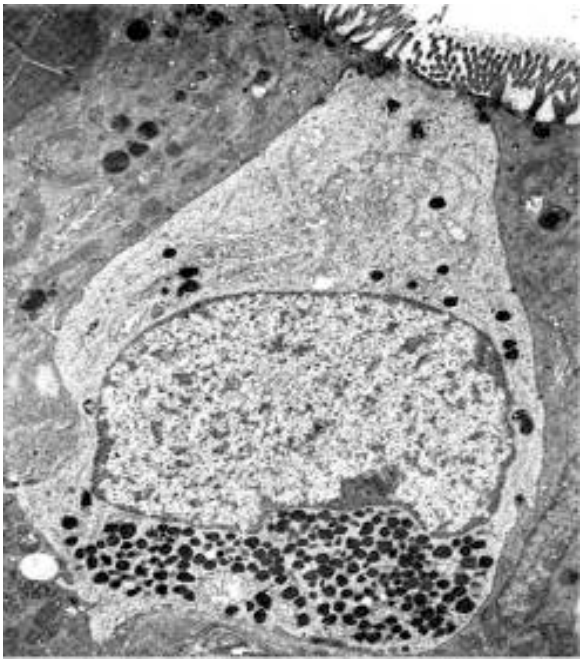


Figure 1.3. Electron micrograph of a typical 'open-type' enteroendocrine cell.

Electron micrograph showing the typical flask-shaped morphology of an open-type EEC. Microvillus processes on the narrow apical membrane and secretory granules (black circular structures) are concentrated at the basolateral membrane. Image taken from http://tinyurl.com/kknspl7.

In accordance to their specific roles, EECs exhibit clear distribution patterns throughout the GI tract: numerous EECs are found in the small intestine, particularly the duodenum and proximal jejunum, and are less frequently found in the large intestine (Roth et al., 1990). More than 14 EEC subtypes have been classified using the 'Wiesbaden' classification system (Creutzfeldt, 1970). The Wiesbaden system categorises EECs according to their specific hormonal content (Roth et al., 1990, Rindi et al., 2004). The Wiesbaden system is where the traditional 'one cell, one hormone' hypothesis originated (Table 1.1).

Recent publications in the field of EEC physiology have however raised question as to the continued validity of the 'one cell, one hormone' hypothesis. The first observation was the discovery that GLP-1 and PYY were both expressed in L-cells (Bottcher et al., 1986). Since then, several reports have been published showing co-localisation of a variety of hormones in cells previously thought to only express a single hormone type (Habib et al., 2013, Egerod et al., 2012). In addition, several novel gut peptides have been described within the tract raising the need for the introduction of a new classification system (Helander and Fendriks, 2012).

		Regional Distribution			
Cell Type	**Hormone**	**Pancreas**	**Stomach**	**SI**	**LI**
X/A	**Ghrelin**	**Yes**	**Yes**	**Yes**	**No**
Enterochromaffin	**Serotonin**	**Yes**	**Yes**	**Yes**	**Yes**
D	**Somatostatin**	**Yes**	**Yes**	**Yes**	**Yes**
L	**Peptide YY** **GLP-1**	**No**	**No**	**Yes**	**Yes**
α	**Glucagon**	**Yes**	**Yes**	**No**	**No**
Pancreatic polypeptide	**Pancreatic Polypeptide**	**Yes**	**No**	**No**	**No**
β	**Insulin**	**Yes**	**No**	**No**	**No**
Enterochromaffin-like cell	**Histamine**	**No**	**Yes**	**No**	**No**
G	**Gastrin**	**No**	**Yes**	**Yes**	**No**
I	**Cholecystokinin**	**No**	**No**	**Yes**	**No**
S	**Secretin**	**No**	**No**	**Yes**	**No**
K	**Gastrin Inhibitory Peptide (GIP)**	**No**	**No**	**Yes**	**No**
M	**Motilin**	**No**	**No**	**Yes**	**No**
N	**Neurotensin**	**No**	**No**	**Yes**	**No**

Table 1. 1. EEC subsets as defined by the Wiesbaden classification system.

Each EEC subtype is classified according to the specific hormone it contains. The distributions of 14 of the main EEC types within the pancreas, stomach, small intestine (SI) and large intestine (LI) are also noted. Table adapted from (Rindi et al., 2004).

1.1.2. Epithelial Cell Development

Intestinal epithelial cells are exclusively derived from stem cells that are characterised as expressing leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (Barker and Clevers, 2007). Lgr5+ cells can differentiate into any of the four epithelial cells types outlined above. Cell fate is influenced by specific cellular signals called transcription factors that drive terminal cell outcome in a particular direction. Cells migrate up the villus axis and differentiate into one of the 4 cell-types previously described. Two key transcription factors (TFs) have been characterised that direct differentiation into EECs: Mammalian Atoh Homolog 1 (Math1) and Neurogenin3 (Ngn3). Math1 initiates differentiation into one of the three secretory cell lineages and Ngn3, further steers the cell to develop into an EEC (Jenny et al., 2002, Mellitzer et al., 2010, Yang et al., 2001, Lopez-Diaz et al., 2007). The basic helix loop helix TF, NeuroD, then plays a key role in later stages of differentiation to direct cells into terminal cell fate as a mature EEC subtype (Figure 1.4).

Notch signalling has a strong influence on cell differentiation by instigating an inhibitory effect between adjacent cells. This prevents differentiation into the same cell type and helps to achieve cell variety amongst the epithelial population (Artavanis-Tsakonas et al., 1999). Absence of notch signalling leads to an increase in EEC number (Jensen et al., 2000). Once committed to an endocrine fate, cells require additional signals before they terminally differentiate into one of the various EEC subtypes (Roth et al., 1992).

Intestinal stem cells can be identified by the molecular markers Lgr5 or the polycomb group protein; Bmi1. Bmi1+ cells reside below position 4 in the crypts of proximal SI crypts (Sangiorgi and Capecchi, 2008) whereas Lgr5+ cells are typically found in crypt base columnar cells throughout the length of the intestine and are characterised as more rapidly proliferating and therefore contribute greatly to the regeneration of the intestinal epithelium (Yan et al., 2011).

As Bmi1+ and Lgr5+ cells appear to represent separate, spatially distinct, subtypes of intestinal stem cells they are difficult to use as an exclusive means of identification. An alternative means of identifying stem cells is to exploit their highly proliferative nature and employ a marker of proliferation such as Ki-67.

Ki-67 is a nuclear protein that is expressed during the interphase fraction of cell division and is therefore not expressed in resting cells (Schluter et al., 1993).

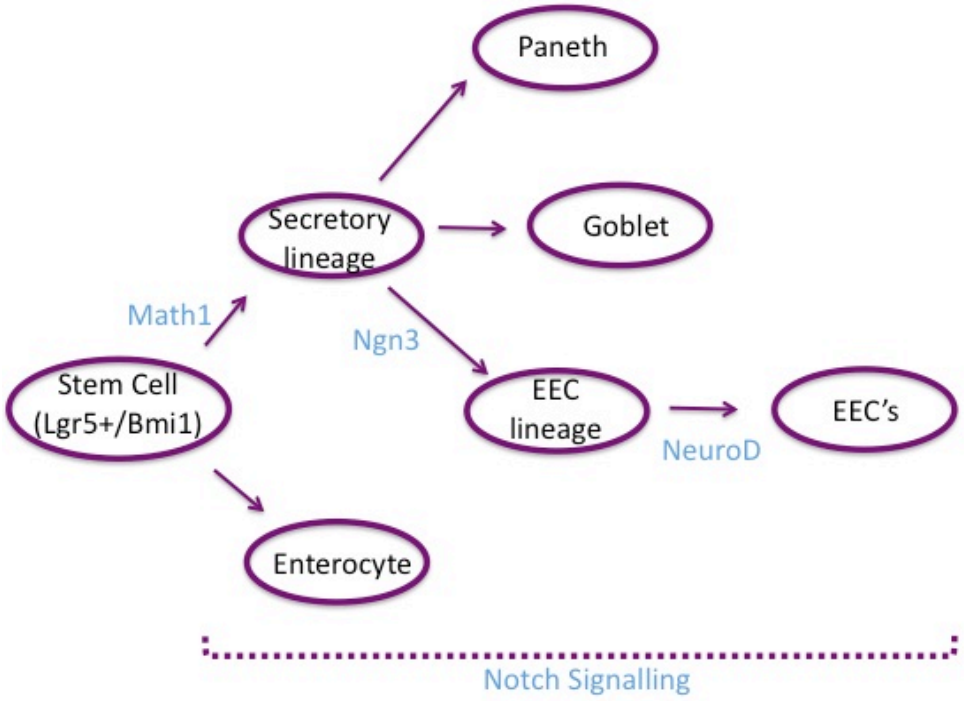


Figure 1.4. Schematic representation of intestinal cell differentiation.

Intestinal epithelial cells are differentiated from pluripotent stem cells to become one of the 4 main cell types of the intestinal epithelium. The transcription factor Math1 drives cells into a secretory lineage whereas Ngn3 commits cells to an EEC lineage. NeuroD is involved in the terminal differentiation into mature EEC's. From here additional TFs and signals determine terminal differentiation into an EEC subtype.

1.2. Nutrient Handling in the GI tract

1.2.1. Nutrient Digestion

Digestion of food begins at the mouth as soon as food enters the GI tract and continues as food transits down the GI tract. Continuing from the mouth, a variety of processes occur along the length of the GI tract, principally in the proximal SI, to ensure the efficient break down and absorption of dietary nutrients.
The GI tract is an intricate system demanded by the fact that the diet is composed of a complex mixture of nutrient types that each requires specific conditions for their breakdown and absorption. Enteroendocrine cells are key players in the detection system that has evolved to optimise nutrient handling by the intestine.
There are three main types of dietary macro-nutrients - carbohydrates, proteins and lipids.

Dietary carbohydrate is primarily constituted of starches and a smaller percentage as disaccharides and monosaccharides. Starch necessitates enzymatic breakdown that is initiated in the mouth by the salivary enzyme, α-amylase. These processes cause the hydrolysis of starch into smaller disaccharides such as maltose. The resultant by-products continue digestion in the SI where pancreatic amylase enzymes and disaccharidase enzymes that are located in the intestinal brush border, catalyse final hydrolysis reactions to produce monosaccharides. Monosaccharides are then freely absorbed via specialised transporter proteins located within the intestinal epithelium membrane (Mourad and Saade, 2011).

Ingested protein is hydrolysed into smaller peptides or amino acids that can be absorbed across the intestinal epithelium. The digestion of ingested protein begins in the stomach where hydrochloric acid and the gastric protease enzyme ‘pepsin’ act together to catalyse the cleavage of peptide bonds forming the protein scaffold, which breaks protein down into smaller molecules. When these molecules eventually leave the stomach and enter the SI, pancreatic protease enzymes such as trypsin and chymotrypsin continue digestion of protein hydrosylate into an eventual mixture of amino acids and mono-, di-, tri- or

oligopeptides, which are then ready to be absorbed by enterocytes (Mourad and Saade, 2011, Silk, 1980).

Lipids also require digestion prior to absorption, but their hydrophobic character presents several unique challenges. There are a variety of forms of dietary lipids including phospholipids, sterols and triglycerides (TGs) that are the dominant form of fat from the diet (Iqbal and Hussain, 2009). Lipids have a complex structure that is both hydrophobic and non-polar which make them insoluble in aqueous solutions and as such are difficult for the body to utilise. Lipid digestion begins in the mouth where the enzyme 'lingual lipase' begins the initial stages of digestion of TGs. The majority of lipid digestion occurs in the SI that receives TGs in the form of fine lipid droplets. The presence of fat within the SI stimulates the release of gut hormones which co-ordinate the following processes involved in lipid digestion. First to consider is bile; bile is the collective noun for a mixture of compounds that are synthesised by the liver, stored in the gall bladder and released into the proximal duodenum. The key components of bile are cholesterol, salts, electrolytes and bile acids. Bile acids emulsify lipid droplets, a process which is essential for lipid digestion due to its amphipathic, detergent-like properties that enable it to emulsify lipid, thus greatly increasing the exposure of lipids to digestive enzymes. Pancreatic juice is simultaneously released from the pancreas via the pancreatic duct into the duodenum. This contains bicarbonate, which neutralises the pH of the duodenal environment after receiving acidic chyme from the stomach. Pancreatic juice also contains a variety of digestive enzymes including pancreatic lipase that hydrolyse TGs into free fatty acids (FFA).

FFA encompass short chain fatty acids (SCFAs) that have a chain length of less than 6 carbon molecules (C<6), medium chain fatty acids (MCFAs) (C6-C11), long chain fatty acids (LCFA) (C12-C21) and very long chain fatty acids (VLCFAs) (C>22). FA of all chain length can be absorbed by enterocytes within the intestinal epithelium via diffusion or facilitated by membrane protein transporter systems such as FATP4, CD36 and FABPpm (Su and Abumrad, 2009), which will be discussed in detail later.

Once inside the enterocyte, FAs are sequestered by fatty acid binding proteins

(FABPs) that facilitate the intracellular transport of FAs. FAs and other products of TG hydrolysis are transported to the endoplasmic reticulum (ER) where they are processed and re-esterified back into complex TGs (Iqbal and Hussain, 2009, Abumrad and Davidson, 2012). TGs then bind with apolipoproteins and cholesterol to form lipoprotein particles called chylomicrons. These carry the lipid to the golgi complex to bind other apolipoproteins such as ApoA1 or ApoA4 which locate to the surface of the chylomicron and complete formation of chylomicrons (Iqbal and Hussain, 2009). Chylomicrons can then be exocytosed from the basolateral membrane of the enterocyte and released into the lymphatic system through which they can be transported around the body (Iqbal and Hussain, 2009, Mourad and Saade, 2011).

The lipid content of the typical western diet is around 35% of total energy intake and this is mostly in the form of triglycerides. The process of lipid handling in the body is very efficient and less than 5% of ingested lipids is lost in faeces (Stahl et al., 2001).

1.2.2. Nutrient handling in the Small intestine

Efficient assimilation of dietary nutrients is an evolutionarily selective advantage. Consequently, the intestine has evolved to optimise and control digestive and absorptive processes and EECs are crucial to these processes. This is highlighted by the finding that deletion of EEC cells in humans results in severe nutrient malabsorption (Cortina et al., 2007) and furthermore a lack of EECs in mice results in high fatality rate *in utero* and retarded growth in surviving animals (Mellitzer et al., 2010, Mellitzer and Gradwohl, 2011).

EECs co-ordinate and optimise nutrient digestion and absorption, and they do this by sensing dietary contents, integrating resultant signals with systemic inputs and relaying the prevailing signal via, neural and endocrine signals that are all geared to optimise the uptake of nutrients into the body (Figure 1.5).

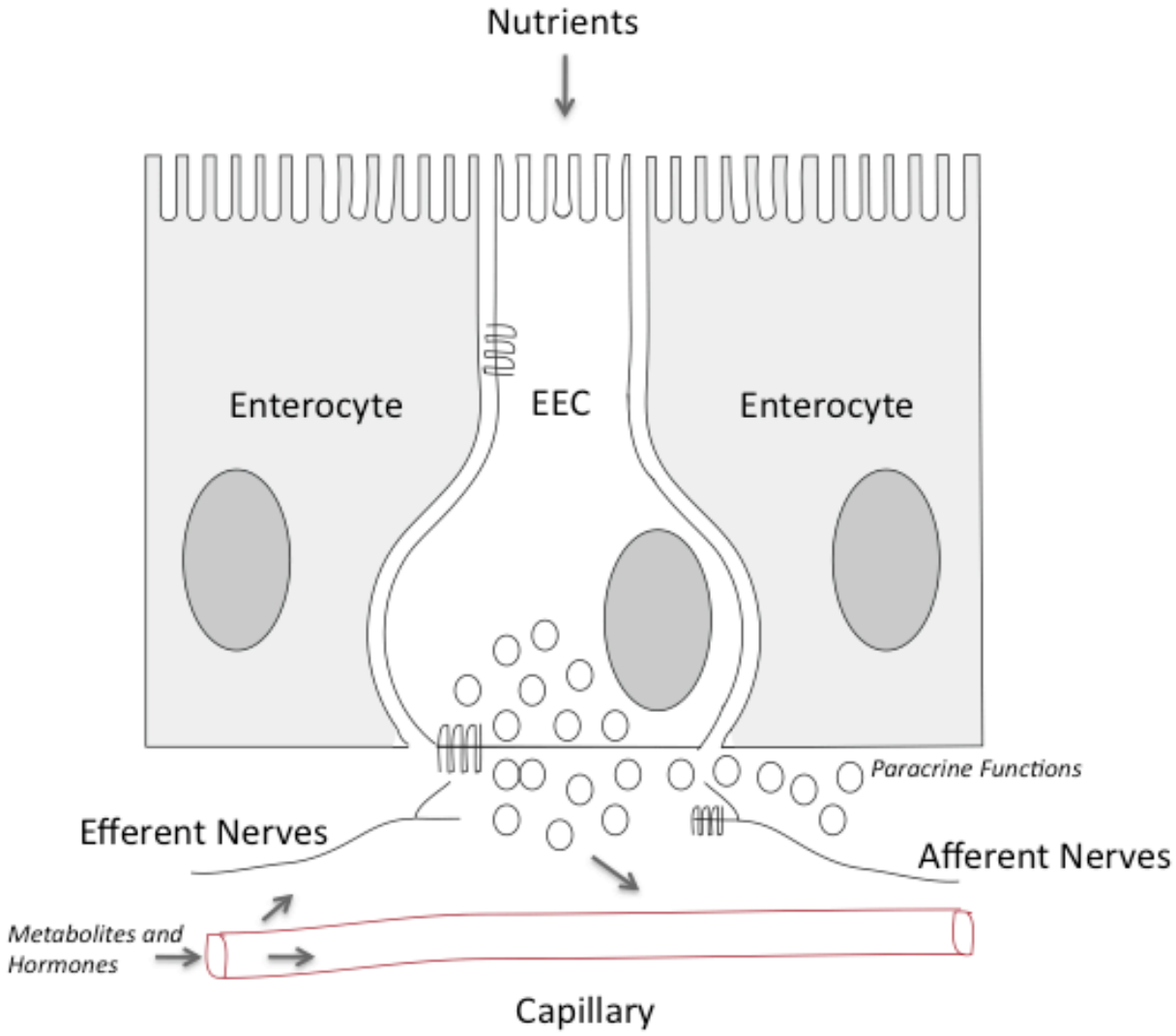


Figure 1. 5. Enteroendocrine cells within the intestinal epithelium.

The projection of microvillus processes into the intestinal lumen enables EEC's to come into close contact with nutrients in the intestinal lumen that stimulate EEC activity. EECs also receive neural signals from vagal efferent nerves and signals from the circulation such as hormones and metabolites. EECs are able to integrate these signals to elicit a response. EEC stimulation instigates the release of GI hormones that enter the blood supply at the capillary network within the villi or signal through receptors expressed on innervating afferent neurones that are in close proximity to the EEC. Adapted from (Helander and Fandriks, 2012).

1.2.3. Nutrient Sensing by EECs

Enteroendocrine cells are classifiable as chemosensory cells because they 'taste' or 'sense' nutrient molecules present in the intestinal lumen. By employing several different mechanisms, EECs are able to interpret information pertaining to nutrient type and concentration, as well as the presence of noxious entities such as toxins or infectious agents. Once sensed, the nutrient signals are transmitted into the cell where they are integrated into cellular and systemic signals. In this way, EEC cells act as integrators and processors of multiple inputs and this has led to EECs being termed 'the guts brain' by some (Bertrand, 2009).

A universal sensing mechanism employed by EECs is the interaction of nutrients with cell surface G-Protein Coupled Receptors (GPCRs). Activation of GPCRs, through binding of a specific ligand, induces a cascade of second messengers within the cell that ultimately leads to the release of bioactive hormone.

1.2.3.1. G-Protein Coupled Receptors

GPCRs have seven transmembrane domains with an extracellular N-terminus and intracellular C-terminus (Winzell and Ahren, 2007). Attached to the G-protein are three subunits; α, β, and γ. The α-subunit determines which intracellular signalling cascade is activated; examples of α-subunit types include Gαs, Gαi or Gαq. G-protein coupling to Gαs activate the adenylate cyclase pathway, which induces a rise in cellular cyclic adenosine monophosphate (cAMP), while coupling with Gαi inhibits this pathway thus decreases cAMP concentration. The third permutation coupling through Gαq activates the phospholipase C (PLC) cascade that hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacyl-glycerol, which respectively stimulate Ca^{2+} release from intracellular stores and activates protein kinase C (Prezeau et al., 2010, Neves et al., 2002).

There are many different types of GPCRs found in the GI epithelium; these vary in expression profile and ligand specificity. Expression analysis of purified EEC

populations has shown they are enriched in a wide array of GPCRs compared to neighbouring cells. Teleologically, the discovery of GPCRs in the GI tract and within EECs prompted the suggestion that EECs might have a chemosensory role. It is now known that an array of GPCRs are expressed on EECs and these respond to a multitude of specific ligands and relay signals of nutrient presence that once a threshold is reached induce the release of gut hormones in response to food intake (Engelstoft et al., 2008).

1.2.3.2. Fatty acid sensing GPCRs

Within the past two decades, free fatty acids (FFAs) have been indentified as ligands for some previously considered 'orphan' G-protein coupled receptors (Katsuma et al., 2005). These receptors include GPR40, -41, -43 and -120, and have each been demonstrated to be expressed by EECs of the SI (Edfalk et al., 2008, Sykaras et al., 2012). Of these, GPR40 and GPR120 are currently thought to be the principle GPCRs involved in sensing dietary FA within the GI tract, specifically these receptors detect the products of fat hydrolysis, FFAs, and not intact triglyceride (Little et al., 2007). More specifically, it is the detection of LCFA (≥C12 chain length) by EECs that induces the release of anorectic hormones such as CCK, PYY and GLP-1 (Feltrin et al., 2004, McLaughlin et al., 1998, McLaughlin et al., 1999). In contrast, GPR41 and GPR43 are sensors of SCFA. Expression of GPR41 on EECs appears to relay signals of SCFA produced by bacterial fermentation (Samuel et al., 2008). A precise role for GPR43 is yet to be validated although it has been shown to mediate the release of the GI peptide GLP-1 from colonic EECs in response to SCFAs, possibly in a manner similar to GPR41, implicating this receptor in sensing bacterial metabolites (Tolhurst et al., 2012).

1.2.3.3. Fat-derived compounds as ligands for intestinal GPCRs

A number of fat-derived compounds generated within the GI tract also have the ability to signal through GPCRs. These compounds contribute to the intricate signalling network involved in nutritional and energy status sensing mechanisms. Included here are the endocannabinoids and acyl-ethanolamines such as anandamide and oleoylethanolamide (OEA). These compounds are ligands for specific GPCRs including the cannabinoid receptors CB1 and CB2, GPR119 and the nuclear receptor PPAR-α. Importantly, cannabinoids are thought to have a major role in gut-brain signalling in that they influence appetite centres. Both of the cannabinoid receptors CB1 and GPR119 are expressed on EECs (Sykaras et al., 2012) and stimulation of GPR119 has been shown to mediate GI peptide secretion (Overton et al., 2008, Chu et al., 2008, Lauffer et al., 2009). CB1 receptors are highly expressed throughout the nervous system on central and peripheral neurones and also in the kidney, liver and intestine (Di Marzo, 2011). The role of CB1 in EECs remains unknown, however it has been hypothesised to exert a regulatory inhibitory function as CB1 activation is known to stimulate food intake (Di Marzo, 2011).

1.2.3.4. Amino Acid sensing by GPCRs

Amino acid sensing in the GI tract exists in the form of GPCR-dependent and GPCR–independent mechanisms. The hydrolysis of proteins along the GI tract results in a complex mixture of mono-, di-, tri-peptides and oligopeptides as well as amino acids. It is these products of hydrolysis that are detected by EECs. In this way EECs not only monitor the presence of a nutrient, but also discriminate the stage of degradation it has reached. A mixture of protein hydrosylates and amino acids are detected in the stomach by the protein hydrosylate-activated GPCR GPR93. GPR93 is expressed by enterocytes and EECs in the SI and GPR93 activation has been shown to induce CCK release in STC-1 cells (Choi et al., 2007b, Choi et al., 2007a). Additionally, the amino acid receptor GPRC6A, a family C

group 6 subtype A GPCR, is also expressed on EECs (Haid et al., 2012, Wellendorph et al., 2007). Amino acid signalling through GPRC6A has been shown to elicit hormone release from GLUTag cells (Brubaker et al., 1998, Reimann and Gribble, 2002, Oya et al., 2012).

The umami taste receptor T1R1/R3 and the calcium sensing receptor (CaSR) have also been implicated in amino acid induced hormone release from EECs. These receptors have individual specificities for amino acid structure with high selectivity for L-isoform amino acids. The taste receptor family is a heterodimeric family that comprises 3 members: T1R1, T1R2 and T1R3 that are expressed in the intestine. Of these, T1R1/R3 sense L-amino acids and are responsible for 'umami' taste perception, whereas T1R2/R3 are sweet sensors (Daly et al., 2013). T1R1/R3 is expressed in the intestinal epithelium (Bezencon et al., 2007) and stimulation of T1R1/R3 by amino acids has been shown to induce hormone release from STC-1 cells (Dyer et al., 2005) and more recently amino acid stimulated CCK release has been demonstrated from primary EECs (Daly et al., 2013). Stimulation of T1R1/R3 is exclusive for L-amino acids, and more specifically the aromatic amino acid L-phenylalanine. The dietary-derived amino acids leucine and glutamate are also able to potently stimulate this receptor (Daly et al., 2013).

CaSR is highly expressed in a number of EEC subtypes; namely D-cells (somatostatin), G-cells (gastrin), S-cells (secretin) and I-cells (CCK) (Liou et al., 2011c, Haid et al., 2012). The classical sensing mechanism for CaSR in the intestine is the sensing of extracellular Ca^{2+} however, CaSR also has the ability to sense amino acids which act as an allosteric modulator for the sensitivity of CaSR to extracellular Ca^{2+} (Conigrave et al., 2007, Hira et al., 2008, Saidak et al., 2009). Amino acid signalling through CaSR has been shown to induce hormone release in the STC-1 cell line and in primary I-cells, and this was validated by an absence of stimulation when performed in CaSR KO mice (Liou et al., 2011c, Conigrave et al., 2007, Hira et al., 2008, Wang et al., 2011).

1.2.3.5 Carbohydrate sensing by GPCRs

The detection of carbohydrate in the intestine can occur through activation of membrane GPCRs, or alternately carbohydrate signalling can occur intracellularly following its uptake.

The taste receptor family (mentioned previously) is the primary GPCR involved in glucose sensing in the gut. The heterodimer T1R2/R3 is a sweet sensor for natural sugars, sweet proteins and artificial sweeteners (Daly et al., 2013). Cell line models have demonstrated that ligand binding T1R2/R3 activates the G-protein gustducin that leads to a rise in intracellular Ca^{2+} concentration and the release of incretin hormones (Jang et al., 2007). However, analysis of EECs in primary intestinal cell cultures, found no enrichment of taste receptors in EECs and also an absence of an effect of artificial sweeteners upon hormone release or Ca^{2+} signalling in EECs (Reimann et al., 2008). This suggests that taste receptors may not act as the glucose sensor in EECs (Reimann et al., 2012).

1.2.3.6. Non-GPCR mediated nutrient detection

Interestingly, alternative detection mechanisms, independent of GPCRs, have been hypothesised to exist for some nutrients. Nutrients can cross from the lumen into the intestinal epithelium by diffusion or carrier mediated transport, which is conducted by solute carrier transporters (SLC). SLCs facilitate the movement of nutrients across the membrane from the intestinal lumen. Once inside the cell, nutrients can activate signalling mechanisms that lead to hormone release. This can be through solute-coupled charge movement causing a cell to depolarise and induce an action potential. Alternatively, intracellular signalling can occur as a direct result of nutrient metabolism, which can raise concentrations of intracellular metabolites such as ATP (Liu et al., 1999, Bertrand, 2009, Tolhurst et al., 2009). EECs are electrically excitable thus membrane depolarisation has an instantaneous effect on hormone release (Reimann et al., 2008, Rogers et al., 2011).

The mechanism of intracellular glucose sensing, first began to gain acceptance

when reports demonstrated that the sodium-glucose transporter (SGLT1) mediated glucose uptake and subsequently induced release of GI peptides (Reimann et al., 2008). In this instance glucose uptake via SGLT1 is coupled with a positively charged Na^+ and thus causes action potential firing within the cell that results in opening of voltage dependent Ca^{2+} channels (VDCC) situated in the plasma membrane and causes an influx of extracellular Ca^{2+}. In this way, cell depolarisation initiates exocytosis of secretory vesicles from the cell (Reimann et al., 2008, Mace et al., 2012).

A similar mechanism has been proposed for peptides. The amino acids glutamine and asparagine instigate a similar response as their uptake is coupled to Na^+ uptake that changes cellular polarity causing Ca^{2+} influx and hormone release (Tolhurst et al., 2011). Additionally, the proton-coupled oligopeptide transporter (PepT1) plays an indirect role in amino acid sensing in EECs (Liou et al., 2011a). PepT1 mediates uptake of di- and tri-peptides into enterocytes and EECs in the intestinal epithelium. PepT1 co-transports oligopeptides alongside positively charged protons, in this way PepT1 activity is dependent on pH (Matsumura et al., 2005). Therefore, PepT1 activity in EECs results in cell depolarisation in a manner like that described for SGLT1. Electrical activity within the EEC opens VDCCs and intracellular Ca^{2+} concentrations rise and triggers the release of GI peptides (Figure 1.6). It is in this way, that PepT1 was shown to elicit amino acid stimulated peptide release from STC-1 cells (Matsumura et al., 2005). This activity has since been demonstrated in I-cells (Liou et al., 2011a).

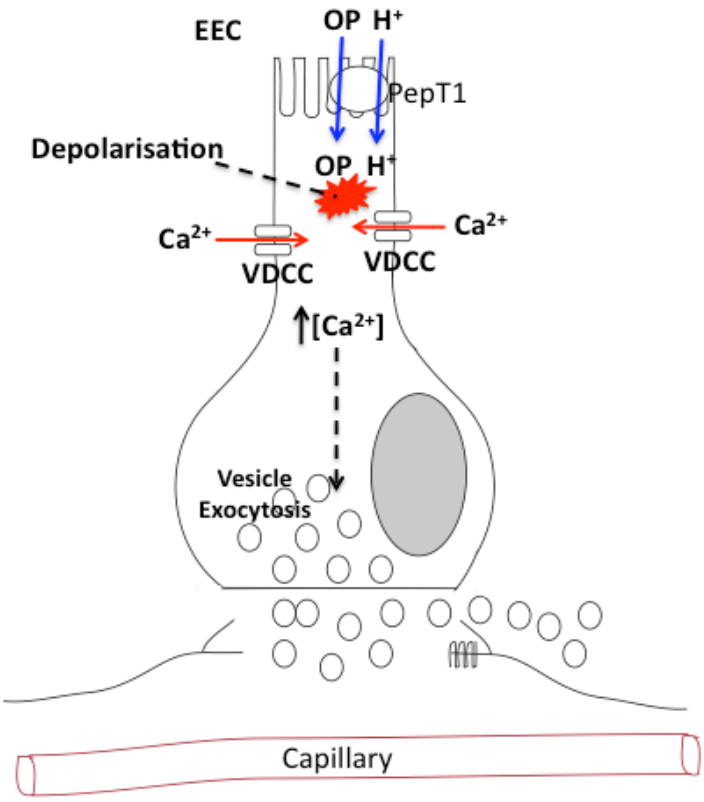


Figure 1.6. Uptake by PepT1 causes EEC depolarisation and peptide release.

EEC expression of PepT1 executes the co-transport of oligopeptides (OP) and protons (H^+). The uptake of protons brings a positive charge into the cell, which initiates cell depolarisation. Depolarisation and action potential firing causes voltage dependent Ca^{2+} channels (VDCC) to open and results in a subsequent influx of Ca^{2+}. This raises intracellular Ca^{2+} concentrations $[Ca^{2+}]$ to a threshold that instigates exocytosis of secretory granules and therefore the release of GI peptide from the EEC. This is an example of an intracellular nutrient-signalling pathway.

1.2.4. Systemic Signals influencing EEC activity

EECs are subjected to a plethora of incoming signals from nutrient ligands within the intestinal lumen. EECs receive and integrate these signals and, depending on the prevailing outcome of this signal, processing elicits the subsequent release of GI peptides. However, nutrient signals are not the only signals that contribute towards EEC functioning. Systemic signals are generated in local or distant tissues in the body and communicate the requirements and internal status of the body. These include information about nutritional status, as well as signals from gut micro flora and immunological signals. Superimposed on all these inputs are communication loops between EECs themselves.

1.2.4.1. EECs and nutritional status

The nutritional status of local cells and the body as a whole can be relayed through neuromodulatory compounds that constitute part of the endocannabinoid system. These include the derivatives of FA digestion, OEA and 2-oleoyl glycerol as well as chemical cannabinoids (Lauffer et al., 2009). The expression of receptors GPR119 and CB1 on EECs was discussed previously (section 1.2.3.3.). Firstly, OEA, the endogenous ligand of GPR119, is synthesised within a cell exclusively upon availability of dietary fat. OEA can induce GI peptide release and therefore accentuates the response of EECs in the presence of dietary FAs. OEA also exerts satiety through signalling via vagal nerve afferents independently of GI peptide activity (Sarro-Ramirez et al., 2013, Fu et al., 2003). The expression of CB1 on EECs, in particular CCK containing I-cells, is believed to have an inhibitory action upon peptide release (Sykaras et al., 2012). In this way EEC activity may be subject to modulation by local and peripheral cannabinoid release.

Long-term changes in nutritional status or energy exposure also affect EEC activity in terms of cell number, distribution and relative expression of hormones. For example, obesity can induce a complete remodelling of the enteroendocrine

system and can cause a complete change in concentrations of circulating gut hormones (Murphy and Bloom, 2006, Moran-Ramos et al., 2012). Currently, it is difficult to characterise these changes experimentally although the astounding efficiency of bariatric surgery for the treatment of obesity and Type 2 diabetes highlights the GI tracts major role in health and disease (Moran et al., 2008).

Bariatric surgery is a procedure whereby the GI tract is surgically remodelled to either reduce the capacity of the stomach to limit food intake or to restrict nutrient exposure to regions of the GI tract thereby potentially limiting nutrient signalling and activity of EECs. Importantly, all bariatric surgery procedures have been reported to cause significant changes in the secretion profile of GI hormones. The modulation of gut hormones is so significant that in the majority of patients T2DM is resolved and substantial weight loss is achieved (Sjostrom et al., 2012, Mingrone et al., 2012). These effects are attributable to vast changes in the secretion profile for both proximal and distal gut hormones that result in a decrease in appetite and improved glucose tolerance. This is exerted through a reduced secretion profile of the orexigenic (appetite-stimulating) hormone ghrelin coupled with an increased secretion of anorexigenic (appetite-inhibiting) hormones, of which is particularly significant for GLP-1 and PYY which are released from the distal intestine (Tschop and DiMarchi, 2011, le Roux et al., 2006, Olivan et al., 2009, Peterli et al., 2009). Similar changes in the profile of circulating GI peptides can be achieved through prolonged diet improvement yet in these instance patients are susceptible to weight gain when the diet is ceased (Sumithran et al., 2011, Tschop and DiMarchi, 2011).

It is postulated that the altered circulatory profile of gut hormones following bariatric surgery or prolonged diet is at least partly attributable to changes in EEC activity including an increase in EEC number (Ockander et al., 2003). This was demonstrated for CCK-cells, which increased in number following gastric bypass and following surgery-induced bypass of the proximal SI (Ockander et al., 2003). Alternatively, in cases of diet-induced weight loss, the on-set of changes is comparably slow to that which occurs following surgery. Furthermore, diet-induced weight-loss is highly associated with relapse as the enteroendocrine system remains susceptible for the patient to revert back to an obese state (Anderson et al., 2001). In this way bariatric procedures offers an unparalleled

efficiency for weight loss and treatment of metabolic diseases (Mingrone et al., 2012, Sjostrom et al., 2012).

1.2.4.2. EECs and resident gut microflora

EEC activity is influenced by activity of gut microbiota. Within the GI tract there are 1000s of different species of bacteria and other microorganisms that together constitute the gut microbiota. These microbiota can modulate intestinal barrier function and hormone secretion through production of metabolic by-products that are sensed by the host. Therefore gut microbiota can influence EEC activity (Nicholson et al., 2012), (Cani et al., 2009).

Gut microbiota can influence the development (Bates et al., 2006), as well as the distribution and activity of EECs (Uribe et al., 1994). What is more, bacterial fermentation within the gut generates SCFAs as metabolic bi-products. These are detected by the SCFA receptors; GPR41 and GPR43 that are expressed on EECs (Samuel et al., 2008, Sykaras et al., 2012) and consequently can elicit release of GI hormones (Tolhurst et al., 2012). These demonstrate a link between intestinal microbes, digestion and nutritional status (Samuel et al., 2008).

1.2.4.3. EECs and intestinal immunity

EECs are subject to signals from the immune system of the host. EECs play a role in the intestinal immune response and as such are considered 'innate immunity sensors'.

Intestinal pathology can influence EEC activity. A model of GI infection using *Trichinella Spiralis* observed a significant reduction of food intake due to an increase in circulating CCK concentrations and CCK-cell number (McDermott et al., 2006). Furthermore, a model of ileitis (inflammation of the ileum) demonstrated a resultant increase in the number of cells expressing somatostatin, neurotensin and serotonin (O'Hara et al., 2004). An additional link between the host immunity and EEC activity was depicted in T-cell receptor α-chain KO mice that developed colonic inflammation as a result of this deletion.

Analysis of the intestinal EEC population of T-cell receptor α-chain KO mice saw a decrease in the number of CCK, serotonin and neurotensin cells (Rubin et al., 2000).

These examples of intestinal immunity induced a direct modulation of EEC activity. In this way gut secretion is altered as well as changes in EEC number and circulating GI peptide profiles (McDermott et al., 2006, O'Hara et al., 2004, Rubin et al., 2000). This enables the enteroendocrine system to exert a protective role by altering gut motility and secretions (Moran et al., 2008). This aids the host firstly through reduced energy expenditure, but also limits intestinal absorption whilst promoting expulsion which protects the host from possible noxious compounds imparted by pathogens (McDermott et al., 2006).

1.2.4.4. Intra-EEC signalling

The final EEC regulatory signal to consider is the hormonal communication-loops that exist between EECs of the GI tract. This involves both paracrine and neuronal signalling pathways that influence EEC activity according to the activity of other EEC subtypes. In this manner, the release of one GI peptide feeds into the signals being processed by neighbouring or distant EECs enabling communication throughout the enteroendocrine system (Roberge et al., 1996, Roberge and Brubaker, 1993, Damholt et al., 1998). In this way, gastric inhibitory peptide (GIP) and gastrin releasing-peptide (GRP) are separately able to stimulate the release of GLP-1 from L-cells (Roberge et al., 1996, Roberge and Brubaker, 1993, Damholt et al., 1998). This is also depicted in LCFA-stimulated CCK secretion that has been shown to stimulate the release of GLP-1 and PYY from distal SI L-cells (Beglinger et al., 2010, Degen et al., 2007).

Intra-EEC signalling importantly also provides a means for peptides to signal between different portions of the GI tract. This enables EECs to prepare for on-coming nutrients. A key example of this is demonstrated through glucose-stimulated GIP release in the duodenum, which initiates the release of glucagon like peptide-1 (GLP-1) and GLP-2 in the distal SI (Buchan, 1999, Kellett et al.,

2008). This connection is also known as the proximal-distal signalling loop within EECs of the SI (Gribble, 2012).

It is probable that communication between EECs also involves a neural component given the characteristic connections between EECs and vagal innervation (Rocca and Brubaker, 1999). Released GI peptide can bind and activate specific receptors on vagal afferents. It is postulated that this can elicit a signal, which can stimulate alternate EEC to release peptides. Release of secretin (Li et al., 1995) and GRP (Knuhtsen et al., 1984) has been demonstrated to be mediated in this way.

The ability of an EEC to respond acutely to demands is an important characteristic. Modulation of the enteroendocrine system via signals of nutritional status, gut microflora, immune status and GI peptide releases demonstrates that an intricate network is in place. Integration of these signals helps to co-ordinate nutrient handling within the body and this is exerted through EEC activity. With this in mind, the ultimate end-point for this system is to optimise nutrient uptake according to demands and this is demonstrated by altering the activity of nutrient transporters and carrier proteins within the plasma membrane of enterocytes. The methods in which cells absorb specific nutrient types will be discussed below.

1.2.5. Intestinal Nutrient absorption

Digested nutrients require absorption into enterocytes prior to entry into the systemic circulation. Some nutrient types such as FAs can freely transverse the plasma membrane however others necessitate help from membrane proteins such as SLCs on the apical membrane. These proteins mediate nutrient transport across the apical plasma membrane into enterocytes from the intestinal lumen. Thereafter, nutrients are either utilised by the cell or transported across the cell and traverse the basolateral membrane via carrier-mediated transport or

through a channel protein and enter the bloodstream for transportation around the body to target tissues.

Dietary carbohydrates are exclusively absorbed in the form of monosaccharides. Glucose and galactose enter the cell via SGLT1 and fructose via glucose transporter 5 (GLUT5). These molecules are then exported from the cell across the basolateral membrane via GLUT2 transporters (Reimann et al., 2008, Mourad and Saade, 2011). Signals from GI peptides can modulate the expression of these SLCs. Signalling by glucagon, GLP-1, GLP2 or GIP can increase enterocyte glucose absorption by inducing an up-regulation of SGLT1 expression in the plasma membrane (Margolskee et al., 2007, Cheeseman, 1997, Dube and Brubaker, 2007, Debnam and Sharp, 1993)

As briefly described in section 1.2.1 the products of proteins digestion (mono-, di-tri- or oligopeptides and amino acids), are taken up by enterocytes. Amino acid transporter proteins exist that have specificity for different amino acid structure. Additionally the oligopeptide symporter PEPT1, that was discussed in section 1.2.3.6, enables uptake of peptides into the cell (Mourad and Saade, 2011).

For the purpose of this thesis the uptake of FAs by enterocytes will be discussed in detail. The properties of FAs enable them to passively enter a cell via simple diffusion across the plasma membrane. In the past, simple diffusion was believed to be the sole form of FA entry into a cell, diffusing down a concentration gradient until equilibrium was reached (Schwenk et al., 2010). It is now known that protein-facilitated FA transporters play a significant role in the process of FA uptake. Several FA transporters have been characterised and these are generally more specific for LCFA, with a carbon chain length of 12 or more, and have K_m for FA in the low nM range (Su and Abumrad, 2009). These transporter proteins help to increase the efficiency and speed of FA uptake and also to facilitate transport of longer chain FAs that characteristically diffuse across membranes less effectively than shorter FAs.

Efficient absorption of FAs is important as FAs provide a rich source of metabolic energy within the body, are an essential component of cellular structures such as

cell plasma membranes, and also act as signalling messengers (Hirasawa et al., 2005).

1.2.5.1. Membrane proteins involved in intestinal fat uptake

Transporter proteins influence cellular FA uptake. The membrane proteins known to be involved in intestinal FA uptake will now be described.

1.2.5.2. The Fatty Acid Transport Protein (FATP) family

The fatty acid transport protein family to date consists of six family members (FATP1-6) that are also known as 'very long chain acyl-CoA synthetases'. FATPs are ~63kDa transmembrane proteins that have only one membrane spanning domain with an extracellular N-terminus that is shorter than the intracellular C-terminus (Lewis et al., 2001, Niot et al., 2009). The FATP family displays a degree of tissue specific expression. Highest FATP1 expression is found in adipose tissue, skeletal muscle and heart, FATP2 in kidney and liver and FATP3 in lung, liver and pancreas. FATP4 has the broadest pattern of distribution in many key organs in the body yet is the dominant LCFA transporter in the SI. Moreover, FATP5 and -6 have specific expression within the liver and heart respectively. Of particular relevance are FATP2 and FATP4 which are the only FATP family members that have been identified as expressed in the SI (Hirai et al., 2007, Falcon et al., 2010, Kazantzis and Stahl, 2012).

Although expression of FATP2 has been reported in the intestine a functional role of FATP2 in this tissue is currently obscure. FATP4 however has a more defined function and acts as a functional LCFA transporter in the SI (Kazantzis and Stahl, 2012), as well as in the skin, heart and adipose tissue (Nassir and Abumrad, 2009, Shim et al., 2009). FATP4 is especially enriched in the apical membrane of small intestinal enterocytes whilst smaller amounts are located within the ER, sub-apical membranes and in vesicles close to the apical membrane (Stahl et al., 1999, Stahl et al., 2001). The membrane-associated mechanism of FATP4 is unclear as FATP proteins do not possess a LCFA-binding site (Niot et al., 2009). Indeed,

whether FATP4 proteins mediate transmembrane translocation of FAs across the plasma membrane remains a much-debated topic. However, FATP4 has enzymatic 'acyl-CoA synthetase' (ACS) activity, with a preference for LCFAs and VLCFAs (Figure 1.7). Through its ACS activity it is possible that FATP4 can increase LCFA uptake through 'vectorial' acylation (VA) (Black et al., 2009). This process enables cells to rapidly metabolise incoming FAs, and trap them as fatty acyl-CoA products. This helps to effectively keep intracellular FA concentrations low and thus maintains the driving force for FA diffusion into the cell (Klein et al., 1971). The intracellular localisation of FATP4 supports this proposed function (Milger et al., 2006). Furthermore, localisation of FATP4 to the ER suggests a possible involvement of FATP4 ACS activity in the re-esterification of TGs, therefore promoting chylomicron formation and favouring FAs to leave the cell from the basolateral membrane thus further decreasing intracellular FA concentrations (Niot et al., 2009).

1.2.5.3. The Fatty Acid Translocase CD36

Another principal intestinal FA transporter is the class B scavenger type 1 receptor 'CD36', also known as fatty acid translocase (Lynes et al., 2011). CD36 is a 75-88kDa, 472 amino acid, heavily glycosylated transmembrane protein with two transmembrane domains located near the N- and C-terminal tails which results in a hairpin configuration (Niot et al., 2009, Goldberg et al., 2009).

CD36 has broad ligand specificity; most notably this includes LCFAs and cholesterol. CD36 expression is ubiquitous, including the heart, skeletal muscle, capillary endothelium, adipose tissue and the taste buds of the tongue (Fukuwatari et al., 1997, Nassir et al., 2007). Of particular importance to the current work is that CD36 is expressed in the brush border of the small intestine with highest expression in the proximal SI (Nassir et al., 2007) depicted in Figure 1.7. This expression pattern corresponds with the relative contributions of the proximal and distal intestine to FA absorption (Lynes et al., 2011).

CD36 undergoes many post-translational modifications. These include glycosylation, ubiquitination, phosphorylation and palmitoylation, all of which

influence cellular trafficking of CD36 and its expression in the plasma membrane (Glatz et al., 2010).

CD36 enhances intestinal FA uptake by acting as a FA translocase across the plasma membrane. CD36 possesses an extracellular FA-binding domain, which enables it to bind LCFAs and subsequently internalise its ligand. In addition, CD36 can bind extracellular LCFAs and simply anchor them close to the plasma membrane. This enhances FA uptake firstly by maintaining a steep inward diffusion gradient and secondly as it enhances exposure of FA to other transport channels or for VA by FATP4 (Schwartz et al., 2008, Glatz et al., 2010, Lynes et al., 2011). This is depicted in Figure 1.7.

The various roles of CD36 have largely been established through use of sulfo-succinimidyl esters of LCFAs, specifically sulfo-N-succinimidyl oleate (SSO) that effectively binds CD36 and subsequently blocks the extracellular FA-binding domain at the residue lysine-164 (Glatz et al., 2010, Kuda et al., 2013). Binding of SSO to CD36 irreversibly inhibits both the capacity of CD36 to anchor FA and the ability of CD36 to translocate FAs without affecting other aspects of cellular transport (Coort et al., 2002, Kuda et al., 2013).

In addition to its role in SI FA uptake, CD36 acts as a 'chemosensor' in the mouth for the detection of dietary lipids. CD36 is highly expressed within the tongue taste buds where it signals the chemical perception of LCFA within the oral cavity, subsequently relaying the perception of a 'fatty taste'. Oral FA signalling via CD36 also acts to instigate the initial phases of FA digestion through stimulating the release of lingual lipase enzymes (Fukuwatari et al., 1997, Laugerette et al., 2005, Degrace-Passilly and Besnard, 2012).

1.2.5.4. The Plasma Membrane Associated 'Fatty Acid Binding Protein' (FABPpm)

FABPpm is expressed in the SI with highest expression in the jejunum and ileum; sites that correlate with intestinal lipid absorption (Stremmel et al., 1985a, Niot et al., 2009). FABPpm has been shown to have a role in intestinal FA uptake

(Stremmel et al., 1985a, Kampf et al., 2007) yet the detailed mechanism of FABPpm upon FA transport is unknown. FABPpm adheres to the plasma membrane through a specific N-terminal peptide therefore it has been postulated that FABPpm may exert a FA-anchoring role similar to that of CD36 (Berk et al., 1990). The localisation and hypothesised function of FABPpm is depicted in Figure 1.7.

1.2.5.5. Peroxisome Proliferator-Activated Receptor- α (PPAR-α)

PPAR-α is a nuclear receptor that is implicated in regulating many cellular processes including the absorption, storage and utilisation of dietary fat (Bookout et al., 2006, Lefebvre et al., 2006, Evans et al., 2004). PPAR-α exerts these actions by altering the expression of a large number of target genes including CD36, FATPs and FABPs, which all contribute to FA uptake in enterocytes.

PPAR-α is activated by FAs and FA-derived compounds including the ethanolamide lipid, oleoylethanolamide (OEA), which is generated by enterocytes in the presence of dietary FAs (Figure 1.7) (Schwartz et al., 2008, Mandard et al., 2004, Rakhshandehroo et al., 2010). OEA is a fatty acid amide that has been shown to increase FA uptake by up-regulating the expression of CD36 within the plasma membrane. Up-regulation of CD36 is mediated, at least in part, by the activation of PPAR-α (Fu et al., 2003, Yang et al., 2007, Schwartz et al., 2008). This creates a feed-forward effect because CD36 then provides the FAs, commonly oleic acid, that is essential for further synthesis of OEA (Schwartz et al., 2008, Guijarro et al., 2010).

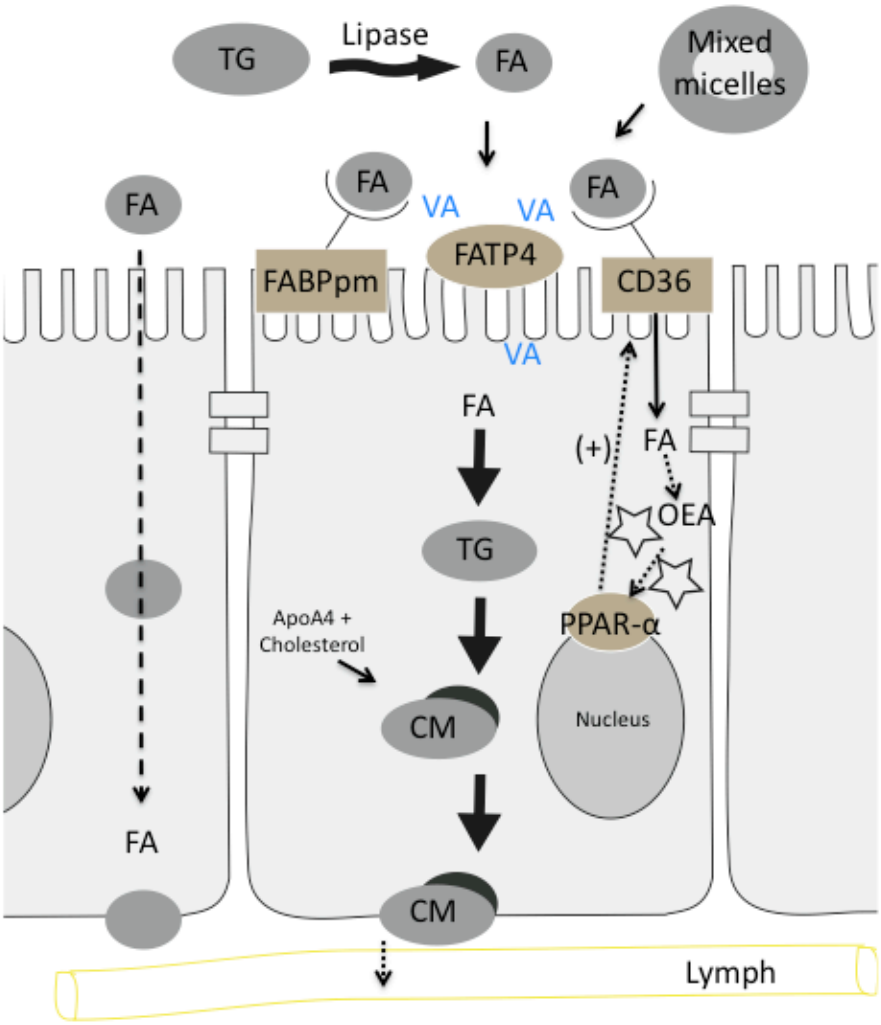


Figure 1.7. An overview of FA handling in the intestinal epithelium.

Within the intestinal lumen, triglycerides (TG) are hydrolysed into fatty acids (FA) by lipase enzymes. FA and mixed micelles of FA enter enterocytes via diffusion or via carrier-protein mediated transporters. The membrane proteins CD36, FABPpm and FATP4 facilitate the translocation of FA across the plasma membrane into intestinal cells. CD36 acts as an FA translocase and both CD36 and FABPpm act as an anchor to bring FAs close to the plasma membrane. The primary actions of FATP4 occur in a process of vectorial acylation (VA) that uses its acyl-CoA synthetase properties to produce FA derivatives. The anchoring abilities of CD36 and FABPpm aid this process by holding FAs close to the plasma membrane. Inside the cell, the majority of FAs are re-esterified back into TGs whereby they bind apolipoproteins such as ApoA4 and cholesterol to form chylomicrons (CM). Chylomicrons can be exocytosed across the basolateral membrane and enter the lymphatic system. Alternatively FAs can pass freely across the cell (Masson et al., 2010).
Dietary FA uptake initiates cellular synthesis of OEA. This binds the nuclear receptor PPAR-α within the cell and modulates transcriptional activity. This includes up-regulation of CD36. Figure adapted from (Masson et al., 2010).

1.3. The Gut-Brain Axis

The 'gut-brain axis' broadly encompasses the communication system that connects the GI tract with the brain. Hormonal and neuronal signals provide a communicative link between many tissues of the GI tract with the brain. These tissues include the liver, intestine, stomach, pancreas and adipose tissue, which together produce regulatory peptides that transmit information regarding energy status and nutrient availability as well as the chemical, mechanical and nutritive properties of food. As previously discussed, EECs of the GI tract secrete hormonal signals in response to intra-luminal nutrient stimuli. Alongside these, there are additional neural signals that are generated by 'mechanoreceptors' of the stomach and SI that provide information regarding distension of the gut. Neural and peptide signals are transmitted to the brain via vagal afferent neurones that feed to, and activate, areas of the brainstem. Integration of peptide signals occurs through expression of specific receptors on nerve fibres that are situated in close proximity to EECs. Receptors for nutrients, such as SCFAs, or derivatives of digestion, such as OEA, are also expressed on these vagal afferent neurones. Receptor-ligand interaction on these afferent neurones relays signals directly to the brain regions (Lal et al., 2001, Capasso and Izzo, 2008). Alternatively, peptides or nutrients can cross into the brain through the semi-permeable blood brain barrier (BBB), enabling these potent signals to directly interact with areas of the brainstem and brain including the arcuate nucleus (AN) of the hypothalamus (Figure 1.8.).

Two types of neuronal populations exist in the AN, the orexigenic, agouti-related peptide neurones (AgRP) and the anorexigenic pro-opiomelanocortin (POMC) containing neurones activation of which stimulates or inhibits appetite, respectively (Figure 1.8.). The hypothalamus integrates incoming signals and transmits signals for further processing in higher brain areas where they can initiate activation of relevant effector mechanisms, including those primarily affecting satiety or GI functions.

Through these pathways, under the control of the central nervous system (CNS), the GI tract has a central control over food intake.

The intrinsic innervation of the GI tract is termed the enteric nervous system and consists of the neural network of neurones and glial cells that are embedded in the lining of the GI tract and are distributed along its length. In this way, signals are relayed from the brain to the GI tract via efferent nerve fibers. Efferent innervation of the GI tract ultimately controls multiple GI functions to optimise nutrient handling. This includes modulation of gastric emptying, intestinal peristalsis, gall bladder contraction and the release of pancreatic juice into the proximal SI to enhance nutrient digestion and absorption (Dockray, 2009). This completes the gut-brain communication loop.

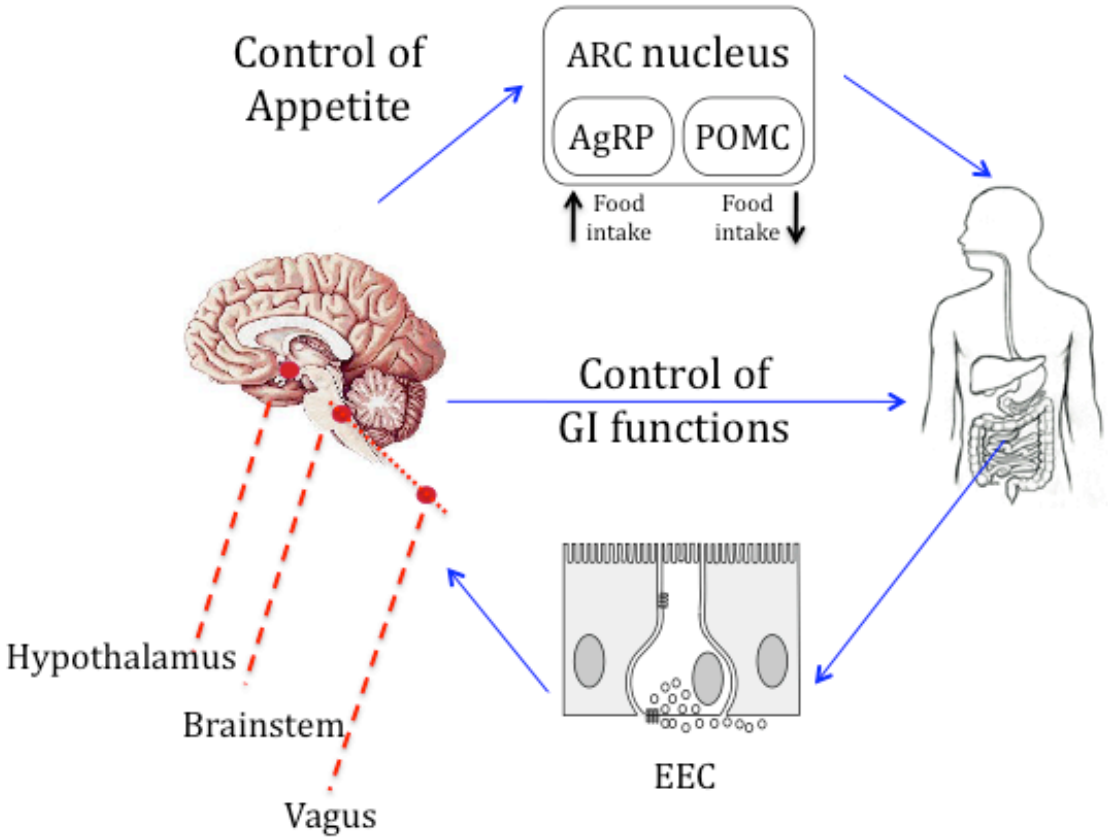


Figure 1.8. The gut-brain axis in the regulation of appetite and GI functions.

Food ingestion stimulates the release of GI peptides from EECs in the GI tract. GI peptides signal to the brainstem and hypothalamus in the brain directly or via the vagus nerve. These brain centres integrate signals to modulate control of food intake and GI functioning. Food intake is specifically modulated by the arcuate (ARC) nucleus of the hypothalamus. ARC contains two distinct neurone populations; the orexigenic Agouti related Peptide (AgRP) neurones and anorexigenic Pro-opiomelanocortin (POMC) neurones that relay signals to increase or decrease food intake respectively. Adapted from (Sam et al., 2012).

This thesis focuses on the gut hormones that are involved in gut-to-brain signalling. More than 30 peptides genes have been identified within chemosensory cells of the GI tract. These are primarily anorexigenic in nature - hormones that are released after food intake and act to induce satiety and inhibit food intake. Anorexigenic hormones include cholecystokinin (CCK), glucagon-like-peptide (GLP-1), peptide tyrosine tyrosine (PYY), gastric-inhibitory peptide (GIP) and secretin (Table 1.2). By contrast, an orexigenic hormone, ghrelin exists which has been the focus of much research. This is released preprandially and stimulates appetite and feeding behaviour.

Satiety control is only one role of gut hormones. Gut hormones also work in concert to control digestive and absorptive processes by influencing GI functions such as GI motility, release of digestive enzymes and bile and control the rate of the delivery of nutrients from the stomach. What follows is a description of the key GI hormones that are involved in gut-brain signalling.

Peptide	Structure	Anorectic?	Reference
ProCCK	95 AA	Yes	(Liddle et al., 1985)
Des-acyl Ghrelin (DAG)	28 AA	Yes	(Asakawa et al., 2005)
Gastric Inhibitory peptide (GIP)	42 AA	Yes	(Dupre et al., 1973)
Gastrin	34 AA	Yes	(Grossman, 1970)
Ghrelin	28 AA	No	(Asakawa et al., 2005)
Glucagon	29 AA	Yes	(Drucker, 1998)
Glucagon-like peptide 1 (GLP-1)	30 AA	Yes	(Drucker, 1998)
Glucagon-like peptide 2 (GLP-2)	33 AA	Yes	(Drucker, 1998)
Insulin	51 AA	Yes	(Grodsky, 1970)
Motilin	22 AA	Yes	(Itoh, 1997)
Neurotensin	13 AA	Yes	(Mustain et al., 2011)
Obestatin	23 AA	Yes	(Zhang et al., 2005)
Oxyntomodulin	37 AA	Yes	(Drucker, 1998)
Pancreatic Polypeptide	36 AA	Yes	(O'Brien et al., 1993)
Peptide Tyrosine Tyrosine (PYY)	36 AA	Yes/No	(Batterham and Bloom, 2003)
Secretin	27 AA	Yes	(Bayliss and Starling, 1902)

Table 1.2. Regulatory peptides of the GI tract.

The name and amino acid size is stated. The majority of listed peptides exert an anorectic function except ghrelin, which is an orexigenic, appetite-stimulating peptide.

1.4. Enteroendocrine gastrointestinal hormones

More than 30 different hormones genes and 100 different forms of bioactive peptides are expressed and produced within the GI tract (Rehfeld, 1998). The principle GI hormones will now be described:

1.4.1. Cholecystokinin

Cholecystokinin (CCK) was the first satiety hormone to be identified (Gibbs et al., 1997b). It is a potent anorexigenic hormone, secreted postprandially from I-cells. CCK will be covered extensively later in this thesis.

1.4.2. Peptide tyrosine tyrosine (PYY)

Peptide tyrosine tyrosine is a 36 amino acid peptide in its mature form. Its name is derived from its characteristic tyrosine (Y) residues at the C- and N-terminals. PYY is produced and secreted by enteroendocrine L-cells that are found in the distal small intestine and colon. More specifically PYY is found in ~45% of L-cells in the distal ileum and ~70% of L-cells in the colon (Habib et al., 2012).

PYY is typically an anorectic hormone. Binding of PYY to YR receptors in the hypothalamus induces satiety (Batterham and Bloom, 2003). As is characteristic of all of the anorectic hormones, circulating levels of PYY are low during fasting and increase postprandially in proportion to caloric intake (Adrian et al., 1985). PYY has a variety of additional roles in the GI tract; it regulates energy expenditure, delays gastric emptying and induces the release of gastric acid, pancreatic juice and bile (Talsania et al., 2005). It has also been proposed that PYY is involved in the 'ileal-brake' phenomenon that inhibits intestinal motility rates in response to the presence of food (Pironi et al., 1993).

PYY release is stimulated by dietary nutrients, particularly FA and glucose (Seimon et al., 2009). PYY is also released upon detection of bile salts via stimulation of the G-protein coupled bile acid receptor 1 (GPBAR1) (Ullmer et al., 2013).

PYY circulates in two forms, PYY_{1-36} and the truncated $PYY_{3-36.}$ Interestingly the longer form, $PYY_{1-36,}$ actively stimulates appetite (Kanatani et al., 2000). The two forms of PYY exert their opposing effects through differential interaction with YR that is expressed in specific brain areas (Batterham and Bloom, 2003, Batterham et al., 2002). The truncated PYY_{3-36} is the more prevalent form of the peptide (Kanatani et al., 2000).

1.4.3. Proglucagon-derived peptides

Glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glucagon, glicentin-related pancreatic polypeptide (GRPP) and oxyntomodulin are all derived from the proglucagon peptide *GCG* gene (Figure 1.9). These anorectic peptides are depicted separately in Table 1.2. Proglucagon is expressed in the pancreas, distal intestine and within the brainstem (Suzuki et al., 2010). In the intestine GLP-1, GLP-2 and oxyntomodulin are the predominant forms, and these are expressed in L-cells alongside PYY (Habib et al., 2013).

GLP-1, like PYY, is released after food intake and acts to induce satiety and slow gastric emptying (Naslund et al., 1998, Flint et al., 2001). GLP-1 interacts with its specific receptor the GLP-1R. GLP-1 is a potent incretin because it stimulates glucose-dependent insulin secretion and inhibits glucagon release (Schmidt et al., 1985, Cummings and Overduin, 2007, Nauck et al., 1993). GLP-1 is released on detection of bile salts (Parker et al., 2012, Ullmer et al., 2013) and nutrients including amino acids (Tolhurst et al., 2011, Greenfield et al., 2009), glucose (Reimann et al., 2008) and fatty acids (Poreba et al., 2012).

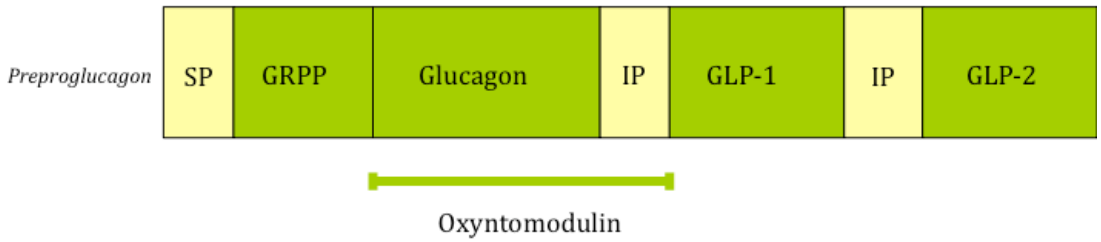


Figure 1.9. Preproglucagon-derived peptides.

Preproglucagon is a peptide derived from the *GCG* gene. It is cleaved into the following peptide products: Glicentin-related pancreatic polypeptide (GRPP), glucagon, oxyntomodulin, and glucagon like peptides -1 (GLP-1) and -2 (GLP-2). Signal peptides (SP) and intervening peptides (IP) locations are labelled. Oxyntomodulin exists as an extension of the glucagon sequence, prolonged by the IP sequence attached. Adapted from (Drucker, 2005).

In contrast, GLP-2 has no affect on satiety, but functions to stimulate intestinal epithelial cell growth. GLP-2 also facilitates uptake of nutrients by stimulating the up-regulation of nutrient specific transporters in enterocytes. For example, GLP-2 has been shown to increase fat uptake by directly up-regulating the expression of the fatty acid transporter CD36 (Hsieh et al., 2009). GLP-2 exerts its effects through interacting with its specific receptor GLP-2R.

Glucagon, a 29 amino acid peptide, is expressed by α-cells in the endocrine pancreas and is involved in the homeostatic control of blood glucose levels and energy storage (Suzuki et al., 2010, Cummings and Overduin, 2007). The glicentin-related pancreatic polypeptide (GRPP) exists as a cleavage product from glucagon. It is released in the pancreas and SI yet does not appear to convey any effects upon GI secretions or motility (Thim and Moody, 1982)

Lastly, oxyntomodulin is an anorectic peptide that slows gastric emptying and inhibits gastric acid secretion (Bataille et al., 1981, Flint et al., 2001). Oxyntomodulin can bind both the GLP-1R and the glucagon-R. Oxyntomodulin also possesses properties of an incretin effecter yet is not as potent as GLP-1 (Du et al., 2012).

1.4.4. Gastric Inhibitory Peptide (GIP)

Gastric inhibitory peptide (GIP), also known as glucose-dependent insulinotropic peptide, is a 42 amino acid mature peptide. Interestingly, GIP was the first gut hormone identified to have an incretin effect (Dupre et al., 1973).

EECs that contain GIP are classically termed K-cells. K-cells are expressed throughout the SI, with highest frequency in the duodenum (Diakogiannaki et al., 2012). K-cells release GIP postprandially upon stimulation by glucose, amino acids or fatty acids (Greenfield et al., 2009, Elliott et al., 1993, Karhunen et al., 2008, Beck et al., 1984, Tolhurst et al., 2011, Parker et al., 2009). GIP exerts its effects through interacting with its specific receptor, GIP-R. GIP also has a proliferative and anti-apoptotic role on pancreatic islets, contributing to maintenance of the endocrine pancreas (Kim et al., 2005, Friedrichsen et al., 2006, Yabe and Seino, 2011). GIP also has roles in energy control, helping to maintain glucose and fat metabolism, storage, and in regulating the insulin sensitivity of adipose tissue (Knapper et al., 1995, Zhou et al., 2005).

1.4.5. Neurotensin

Neurotensin is a 13 amino acid neuropeptide that is found widely distributed in the CNS and some parts of the GI tract. Within the CNS, neurotensin acts as a primary neurotransmitter and is a modulator of brain signalling pathways with particular roles in the regulation of dopamine pathways and analgesia (Mustain et al., 2011). Within the GI tract, EECs containing neurotensin are termed N-cells. N-cells are found throughout the length of the SI and release neurotensin upon stimulation by fatty acids or the presence of gastric acid in the duodenal lumen (Wallin et al., 1995, Ferris et al., 1985).

Like many of the GI peptides, neurotensin exerts an anorectic function and also serves an additional regulatory role upon GI processes. Neurotensin induces the release of bile and pancreatic juices into the duodenum whilst inhibiting gastric acid secretion (Wood et al., 1988, Gui and Carraway, 2001, Mustain et al., 2011).

Neurotensin exerts inhibitory effects on GI motility within the SI yet, interestingly, it has also been shown to stimulate GI motility in the colon in a region specific manner (Azriel et al., 2010). Neurotensin has also been shown to stimulate cell growth and prevent cell apoptosis in the intestine and pancreas (Mustain et al., 2011). Neurotensin exerts these effects through binding one of its three specific receptors; NTS1, NTS2 and NTS3/GP95 (Vincent et al., 1999).

1.4.6. Secretin

Secretin, a 27 amino acid peptide, was famously the first hormone to be discovered (Bayliss and Starling, 1902). EECs containing secretin are termed S-cells. S-cells are found throughout the SI in the crypts of Lieberkuhn, and their frequency decreases distally along the GI tract (Bryant and Bloom, 1979).

Secretin is released following detection of gastric acid (Glad et al., 1996) and subsequently acts to inhibit gastric acid secretion and thus is important for pH stabilisation within the intestinal environment. In addition, secretin also exerts a role to slow gastric emptying and induces the release of bile and pancreatic juice (Jin et al., 1994). Like neurotensin, secretin also acts as a neuropeptide within the CNS. Secretin exerts its effects through binding its specific receptor, the secretin GPCR (SCTR) (Siu et al., 2006).

1.4.7. Preproghrelin

The ghrelin gene *GHRL* encodes the preproghrelin peptide from which several key gut peptides are derived. These include acyl-ghrelin (ghrelin), des-acyl ghrelin (DAG) and obestatin, which are produced through posttranslational modifications of the preprohormone (Figure 1.10).

Ghrelin and its related hormones have highest expression in the stomach, particularly within the closed-type enteroendocrine X/A-cells, the majority of

which are concentrated in the oxyntic and pyloric glands, but these peptides are also found in open and closed type EECs of the intestine (Sakata et al., 2002).

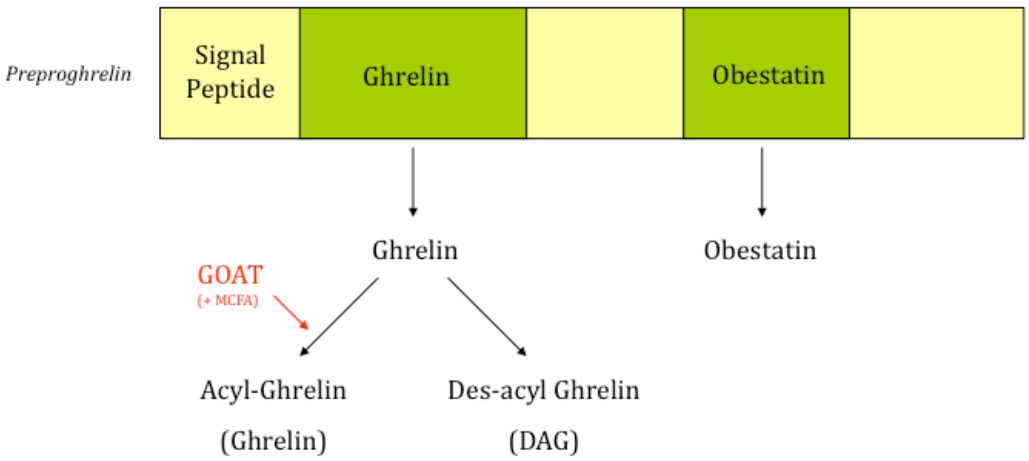


Figure 1.10. Preproghrelin derived products.

The preproghrelin sequence is encoded by the *GHRL* gene. Post-translational processing of the sequence generates the 28 amino acid peptide ghrelin and the 29 amino acid peptide obestatin. The ghrelin peptide sequence is acylated by the enzyme GOAT, which octanoylates ghrelin using a MCFA, which synthesises the production of 'active' ghrelin. Non-octanoylated ghrelin is known as des-acyl ghrelin (DAG). Adapted from (Tena-Sempere, 2008).

The most biologically active form of ghrelin, termed acyl-ghrelin, is a 28-amino acid peptide with an o-n-octanoyl- modification at serine 3 position. The enzyme 'ghrelin O-acyl transferase' (GOAT) catalyses the octanoylation of ghrelin (Figure 1.10). Octanoylation is essential for ghrelin binding to its receptor; the growth hormone secretagogue receptor-1 (GHSR-1) (Kojima et al., 1999). GOAT expression mirrors that of acyl-ghrelin (Kang et al., 2012, Kang et al.). It has been suggested that GOAT system acts as a nutrient sensor as it necessitates the presence of octanoic acid, a MCFA, obtained from the diet, to octanoylate the ghrelin peptide (Kirchner et al., 2009).

In comparison to the hormones described so far, ghrelin is an orexigenic hormone and has an opposite secretory pattern to the other GI hormones; that is high circulating levels during fasting and decreased levels after food intake

(Tschop et al., 2001, Cummings et al., 2001). In addition to its orexigenic role, ghrelin also has a role in the control of GI motility, insulin secretion and energy homeostasis via its action on glucose and lipid metabolism (Fujimiya et al., 2010, Fujimiya et al., 2012).

Des-acyl ghrelin (DAG) is the major form of circulating ghrelin, representing 90% of ghrelin in plasma (Chen et al., 2005). DAG is also 28 amino acids in length and is structurally identical to acyl-ghrelin, but lacks the o-n-octanoyl modification. For this reason it is considered the 'inactive' form of ghrelin because it cannot bind GHSR-1 (Fujimiya et al., 2010, Hosoda et al., 2000). DAG has been reported to possess many extra-endocrine roles including cardiotropic actions and even roles in adipogenesis (Thompson et al., 2004). However, more recently several papers have published data that suggest DAG to have the ability to antagonise many of the actions of acyl-ghrelin. It was shown that central and peripheral administration of DAG caused a decrease in food intake coupled with slowed gastric emptying. Mice over-expressing endogenous DAG lost body weight, ate less and had decreased fat mass compared to wild type (WT) (Asakawa et al., 2005). DAG has also been demonstrated to enhance release of GI peptides such as glucagon, somatostatin and pancreatic polypeptide, a property that opposes the actions of ghrelin (Fujimiya et al., 2010). These findings have since been reproduced in a number of experiments (Chen et al., 2009, Broglio et al., 2004, Delhanty et al., 2012, Kumar et al., 2010, Fujimiya et al., 2010) and suggest an additional facet to the function of ghrelin in the control of energy balance as DAG behaves in an inverse manner to ghrelin.

Finally, obestatin is a 23-amino acid peptide hormone cleaved from the C-terminal end of the preproghrelin sequence. Obestatin is expressed in the stomach (Zhang et al., 2005) and in SI (Zhang et al., 2008). Obestatin has been identified as the natural ligand for GPR39 and it has also been shown to exert effect through binding GLP-1R (Granata et al., 2008, Zhang et al., 2005). Data have been controversial whether obestatin has an anorectic function similar to that of DAG (Lacquaniti et al., 2011). Studies have demonstrated obestatin to reduce food intake in rats (Green et al., 2007, Zhang et al., 2005, Bresciani et al., 2006, Chartrel et al., 2007, Carlini et al., 2007, De Smet et al., 2007, Zizzari et al., 2007, Sibilia et al., 2006) however other labs have failed to reproduce these effects

(Gourcerol et al., 2007, Van Dijck et al., 2009, Kobelt et al., 2008, Mondal et al., 2005, Unniappan et al., 2008). In addition to its debatable role in satiety control, obestatin exerts roles in GI functioning. Obestatin causes delayed gastric emptying and increases delivery of pancreatic enzymes into the duodenum (Ariyasu et al., 2001, Lacquaniti et al., 2011, Kapica et al., 2007). These actions effectively enhance digestive processes.

Throughout this thesis, acyl-ghrelin will be referred to as 'ghrelin' and des-acyl ghrelin as 'DAG'.

1.5. Cholecystokinin and The Enteroendocrine I-cell

1.5.1. What are I-cells?

Cells that produce and release the hormone cholecystokinin (CCK) are called 'I-cells' according to the Wiesbaden classification system (Helander and Fendriks, 2012, Creutzfeldt, 1970). In this thesis the term 'I-cell' will be used to denote intestinal cells that express the hormone CCK. I-cells have typical 'open-type' EEC morphology: characteristically flask-shaped with a narrow apical membrane exposed to luminal contents and a broader basolateral membrane where secretory granules are concentrated (depicted previously in Figure 1.3). Studies have shown I-cells also possess processes that extend from their basolateral membrane and appear to interact with neighbouring enterocytes (Chandra et al., 2010). This morphological characteristic has led to the suggestion that I-cells may secrete hormones that act locally on neighbouring cells in a mechanism of paracrine signalling. This has been demonstrated in only one study where paracrine activity of CCK exerted a protective effect upon enterocytes to enhance the expulsion of bitter toxins from the intestine (Jeon et al., 2008).

The highest density of I-cells is found in the proximal duodenum and their number decreases moving distally along the SI. I-cells are not apparent in the

stomach and only a sparse detection of CCK protein has been recorded in the colon. CCK is released from I-cells in response to the presence of nutrient stimuli; namely the breakdown products of fat and protein; fatty acids, amino acids and peptides. FAs are known to stimulate CCK release in a chain length dependent manner. FA with a chain length of 10 (C>10) or more can stimulate CCK secretion, however C12 LCFA are significantly more potent than C10 MCFAs (McLaughlin et al., 1999, McLaughlin et al., 1998). This has been demonstrated *in vivo* in humans and *in vitro* in primary cell cultures and STC-1 cells. FA stimulation of I-cells is primarily mediated through receptors such as GPR40 and GPR120 (McLaughlin et al., 1999, McLaughlin et al., 1998).

Protein stimulates CCK release yet peptides and amino acids that are produced by proteolytic cleavage of ingested protein are significantly more potent (Meyer et al., 1976, Liddle, 1995). Interaction of aromatic amino acids with the CaSR expressed on plasma membrane of I-cells has been shown to elicit CCK release through raising intracellular Ca^{2+} concentrations (Wang et al., 2011, Liou et al., 2011c). Amino acids have been shown to also induce this effect through signalling via the taste receptor T1R1/R3 (Daly et al., 2013). Protein hydrosylates can also indirectly initiate CCK release through the uptake of oligopeptides by PepT1 (Liou et al., 2011b). This was covered in section 1.2.3.6.

It is not only nutrients that have the capacity to stimulate CCK release. Bitter tasting toxins have been shown to stimulate CCK secretion through signalling via the taste receptor type 2 (T2R). This is part of a protective-loop mechanism whereby CCK feeds forward to prevent absorption of toxic substances by enterocytes (Jeon et al., 2008).

Additionally the neurotetrapeptide bombesin can induce CCK release and for this reason has been used experimentally for four decades. This effect has primarily been shown in STC-1 cells (Chen et al., 2006, Wang et al., 2011) where bombesin stimulates CCK release through signalling through the G-Protein Gαq (Hira et al., 2009) and protein kinase C and calmodulin-dependent pathways (Takahashi et al., 2000). This has also been observed in mammalian *in vivo* studies where bombesin administration raises plasma CCK concentrations (Banks, 1980, Cuber et al., 1989, Konturek et al., 1976, Erspamer et al., 1974).

1.5.2. Cholecystokinin; Discovery and Functions

Cholecystokinin (CCK) was discovered in 1928 by Ivy and Oldberg who observed that infusion of intestinal extracts in dogs induced gallbladder contraction through the actions of a circulating peptide they named cholecystokinin (Ivy and Oldberg, 1928). In 1943 Harper and Raper independently found that intestinal extracts induced secretion of pancreatic enzyme into the duodenum and thus named the peptide 'pancreozymin' (Harper and Raper, 1943). Subsequent purification of the active peptides many years later showed pancreozymin and cholecystokinin to be the same peptide and the original name 'cholecystokinin' was adopted (Mutt and Jorpes, 1968).

CCK is released postprandially and circulating concentrations increase from approximately 1pM/l to 5-10pM/l upon ingestion of a standard fatty meal (Liddle et al., 1985). Accurately measuring circulating CCK concentrations accurately is notoriously difficult. The first reason for this is that CCK circulates at very low concentrations and in a variety of molecular forms, of which will be discussed shortly. A sensitive detection mechanism would therefore need to detect total CCK irrespective of molecular size and sequence. In addition to this, CCK shares C-terminal homology with gastrin, a peptide released from endocrine cells of the stomach (Wank, 1998). It is therefore important that assays can accurately distinguish between the two peptides. This is particularly challenging when considering the relatively low concentration of circulating CCK, which circulates at concentrations up to 50-fold lower than that of gastrin. Nevertheless, despite difficulties to accurately measure CCK concentrations, much is known about the physiological functions of the peptide.

CCK was the first hormone to be identified as an anorectic satiety-inducing hormone (Gibbs et al., 1973). CCK initiates a satiety response in the brain following food intake. This effect is largely relayed through binding its receptors on vagal afferent neurones, as CCK is laregely unable to cross the BBB. However, a portion of the BBB, located near the brainstem and hypothalamic regions, is leaky and therefore enables CCK to cross over in these regions.

therefore its satiety effect is dependent upon binding its receptors on vagal afferent neurones. The satiety effects of CCK are therefore entirely mediated by vagal afferent neurones and the vagus nerve. Signals from vagal afferents activate CCK receptors within brain areas causing a reduction in meal size and meal frequency. CCK also blocks the orexic activity of the hormone ghrelin in the brain (Kobelt et al., 2005, Gallmann et al., 2005).

Within the GI tract CCK is a major regulator in optimising digestive function. CCK acts to induce the secretion of pancreatic enzymes (Harper and Raper, 1943) and the release of bile from the gall bladder (Ivy and Oldberg, 1928). CCK also slows gastric emptying (Debas et al., 1975, Raybould and Tache, 1988, Forster et al., 1990), increases gastric acid secretion (Corazziari et al., 1979) and slows intestinal peristalsis (Moos et al., 1982, Meyer et al., 1989). These actions act to regulate the delivery of nutrients into the SI and control the release of enzymes and bile that is essential for hydrolysis of nutrients.

CCK also has an effect on the endocrine pancreas. Administration of CCK can lead to an increase in islet cell number under hyperglycaemic conditions, thus stimulating insulin secretion (Kuntz et al., 2004). This suggests a key role for CCK in the control of peripheral and central glucoregulatory pathways.

Lastly, CCK administration has also been shown to enhance FA absorption whereas CCK KO results in defective FA absorption (Lo et al., 2010, Zhou et al., 2012). This indicates a link for CCK to mediate nutrient absorption (Lo et al., 2010, Zhou et al., 2012).

The key functions of CCK are outlined in Figure 1.11.

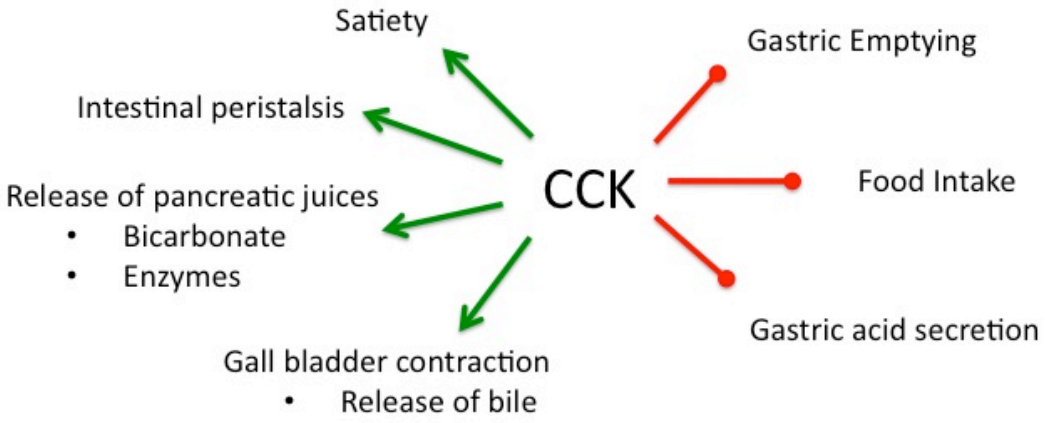


Figure 1.11. The biological actions of CCK.

Green arrows show stimulatory actions whilst red circular pointers indicate inhibitory actions.

1.5.3. CCK gene and structure

PreproCCK mRNA is ~750bp long. The expression pattern of CCK and its abundance in the body is dependent on a number of aspects including hormonal factors and nutrient presence that can directly up- or down-regulate CCK mRNA levels (Rehfeld et al., 2001). As is typical for many gut hormones, the preproCCK precursor peptide undergoes many post-translational modifications to produce a variety of molecular forms of biologically active CCK. CCK has a conserved C-terminal heptapeptide sequence (-Tyr77-Met-Gly-Trp-Met-Asp-Phe-Nh2–) in which the Tyr-77 residue is sulphated. Sulphation occurs at the trans-golgi apparatus, catalysed by sulfotransferases and is critical for the biological activity of the hormone as it determines binding efficacy to the CCK receptors, CCK-R_A and CCK-R_B. Non-sulphated CCK (CCK-NS) is 1000-fold less active than sulphated CCK at the CCK-R_A yet the two form bind CCK-R_B with equal potency (Rehfeld et al., 2001).

For this reason sulphated CCK is generally termed the 'active' form of CCK, as CCK-R_A is thought to convey the main effects of CCK especially within the GI tract. As such the activity of CCK-NS is considered as confined within the CNS or simply as an epithelial growth factor in the intestine. I-cells produce and release both sulphated and non-sulphated forms of CCK, alongside other splice variants (Bonetto et al., 1999).

The preproCCK sequence contains a 20 amino acid signal peptide at its N-terminus. Cleavage of this signal peptide results in a 95 amino acid CCK prohormone. Within the amino acid sequence [83-86] proteolytic cleavage occurs, catalysed by prohormone convertases and this is followed by further cleavage by carboxypeptidase E. Sequence [83-86] also possesses an amidation site that is amidated by peptidylglycine α-amidating monooxygenase (Rehfeld et al., 2008). Due to the position of this amidation site CCK-83 is the largest bioactive form of CCK and represents the amidated sequence 1-83 as proCCK. Further cleavage by prohormone convertases at monobasic sites produces the other major circulating forms of CCK, namely CCK-58, -33, -22 and -8 (Figure 1.12). These forms of CCK have been carboxyamidated and most contain the sulphated C-terminal heptapeptide amide chain (Rehfeld et al., 2001). Of these, CCK-33 is the most abundant form of CCK in the circulation, but CCK-8 is the most biologically potent (Rehfeld et al., 2001). Additional forms of CCK have also been isolated in intestine, brain and blood plasma, however not all exert a biological function (Chandra and Liddle, 2007). In I-cells, proCCK processing is achieved through prohormone convertase 1/3, which generates the active forms of CCK (Rehfeld et al., 2008).

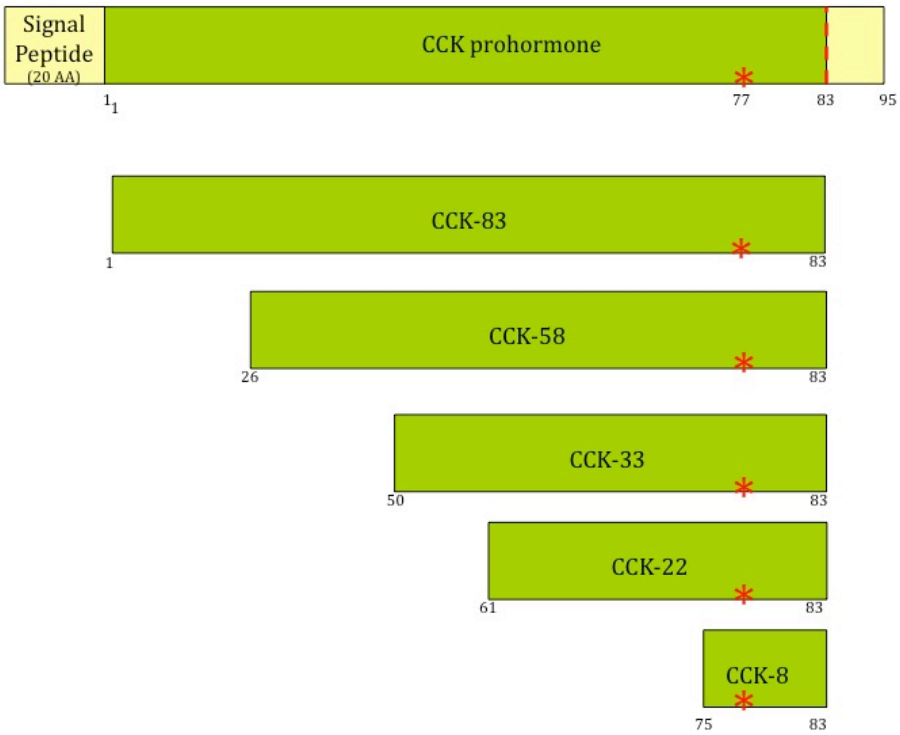


Figure 1.12. Processing of proCCK into bioactive forms of CCK.

The proCCK sequence undergoes many stages of posttranslational processing. The 20 amino acid (AA) signalling peptide is initially cleaved off to produce the 95 AA proCCK sequence. The CCK prohormone sequence is cleaved from AA position 83 to produce CCK-83 as the largest form of biologically active CCK. Further cleavage reactions generate the other forms of CCK; CCK-58, CCK-33, CCK-22 and CCK-8. Sulfotransferase enzymes sulphate the Tyrosine residue at position 77 (depicted by the red asterix). This residue is essential to convey its activity through binding its receptor. Adapted from (Beinfeld, 2003).

1.5.4. CCK receptors

Two CCK-specific GPCRs have been characterised. These are termed, CCK-R_A and CCK-R_B. Activation of these receptors by CCK activates the Gαq pathway causes intracellular Ca^{2+} concentrations to rise and also activates the enzyme Protein Kinase C. These receptor subtypes vary in tissue distribution, structure and affinity for different ligands (Moran et al., 1986).

The CCK-Rs have high and low affinity binding properties, for sulphated CCK; these have a *Kds* of 50-300pM and 30-200nM respectively for CCK-R_A (Jensen et

al., 1980, Jensen et al., 1989, Sandberg et al., 1988a) and 100-300pM and 2-5nM respectively for CCK-R_B (Huang et al., 1994, Dufresne et al., 2006). CCK-NS however, has a much lower binding affinity for CCK-R_A that is in the μM range. Alternatively CCK-NS binds CCK-R_B with equal affinity to that for sulphated CCK (Dufresne et al., 2006, Rehfeld et al., 2001).

Many of the specific roles of CCK-R_A and CCK-R_B have been uncovered through the use of CCK receptors antagonists. These act to block the receptor sites for the hormone. Some antagonists exist that non-preferentially block both CCK-R subtypes such as the drug proglumide. More widely used however are specific antagonist that preferentially inhibits the activity of either CCK-R_A or CCK-R_B such as loxiglumide (R_A) or YM022 (R_B) (Jensen et al., 1986, Jeon et al., 2008).
The use of such CCK-R antagonists have enabled many of the functions of CCK to be established *in vivo* (Schmidt et al., 1991) as well as in cell models (Jensen et al., 1986, Jeon et al., 2008).
Of these, loxiglumide is a well-established specific inhibitor of CCK-R_A (Konturek et al., 1995, Beglinger et al., 1992). Loxiglumide is approximately 3000 times more potent than proglumide (Otsuki et al., 1989) and is 64-fold selective for CCK-R_A over CCK-R_B with an IC_{50} of 3μM (Taniguchi et al., 1996). On the other hand, YM022 has been used as a potent and selective CCK-R_B antagonist, with 1000-fold higher selectivity to CCK-R_B than CCK-R_A (Yuki et al., 1997).

CCK-R_A is expressed in the pancreas, gall bladder, smooth muscle of the GI tract, pylorus in the stomach, and within specific areas of the brainstem. CCK-R_A is also highly expressed on enteric and vagal neurones, in particular capsaicin-sensitive C-fibres of vagal afferents that innervate both the stomach, pancreas and the lamina propria of the SI (Dockray, 1987, Moran, 2000). The tissue expression of CCK-R_A mimics the main sites of CCK activity, namely its control on satiety and GI functioning (Moran et al., 1997). Interestingly, signals of gastric distension which are transmitted by capsaicin-insensitive afferents, are intensified by CCK binding to local CCK-R_A (van de Wall et al., 2005). As a result of this action, mice lacking the CCK-R_A eat much larger meals than wild type because the potentiating effect of CCK on the gastric distension signal is absent. Receptor agonists and

antagonists such as devazepide and loxiglumide have also shown that CCK-R_A conveys the satiety effects of endogenous peripheral CCK (Chang and Lotti, 1986, Varga et al., 1998, Otsuki et al., 1989). Stimulation of CCK-R_B does not produce these effects, thus providing a possible therapeutic target of CCK-R_A in diseased states (Asin et al., 1992).

CCK-R_B is more widely expressed in the CNS, specifically within the homeostatic centres of the hypothalamus, brainstem and cerebral cortex. CCK-R_B is also expressed on vagal afferents (Dockray, 1987, Moran, 2000). CCK-R_A has a much higher affinity to sulphated CCK therefore CCK-NS is considered to preferentially bind CCK-R_B which binds CCK and CCK-NS with equal affinity. Additionally, due to the shared C-terminal sequence homology of CCK and gastrin, gastrin can bind and activate CCK-R_B with the same affinity as CCK (Wank et al., 1992, Rehfeld et al., 2001, Miller and Gao, 2008). Gastrin is released from endocrine cells in the stomach in response to food and exerts its role in regulating the release of acid in the stomach through binding CCK-R_B expressed on local parietal cells (Wank, 1995, Ozcelebi et al., 1996, Wank, 1998). The importance of CCK-R_B in the stomach has been depicted in a CCK-R_B KO model that demonstrated deficient ability to generate parietal and enterochromaffin cells in the gastric mucosa coupled with disrupted gastric acid production (Koh et al., 1997, Nagata et al., 1996, Wank, 1995, Ozcelebi et al., 1996, Wank, 1998). Human studies have also shown that activation of CCK-R_B expressed on vagal afferents modulates pancreatic secretion (Ozcelebi et al., 1996, Wank, 1998).

1.6. I-Cell Research

It is widely known that EECs play a key role in the body's ability to handle ingested nutrients and upon other actions such as regulating satiety signals and influencing food intake and energy availability (Dockray, 2004, Cummings and Overduin, 2007, Rehfeld, 1998). Although EECs in the GI tract constitute the largest endocrine gland in the body they represent only ~1% of the cells lining the epithelia (Moran et al., 2008). A major hindrance in EEC research has been the difficulty of isolating these cells with good specificity and cell viability. The inability to identify or isolate EECs or even to accurately measure CCK secretion due to lack of an accurate sensitive radioimmunoassay technique has previously hindered research into understanding these cells.

Towards the end of the 20th century several techniques were employed, with differing degrees of success, to isolate I-cells and to study them in culture. A major hurdle encountered by workers in the field was ensuring that cell preparations were sufficiently enriched in EECs. The most successful technique used a counter flow elutriation technique using preparations from canine jejunum. This enabled enrichment of I-cells in a mixed culture and allowed a selection of functional experiments to be performed (Liddle et al., 1992, Barber et al., 1986, Koop and Buchan, 1992). These enriched cultures could be maintained up to 40 hours and enabled at selection of secretory studies to be performed. This advancement however still had its limitations, as it remained difficult to determine a direct function of I-cells. Ultimately, the difficulties faced in the isolation of I-cells led to the development and use of surrogate cell-line models.

The most common cell line model used in I-cell research was the STC-1 cell model. STC-1 cells are a murine cell-line developed from an intestinal endocrine tumour from the upper SI of a double transgenic mouse (Rindi et al., 1990). STC-1 cells were found to release CCK upon exposure to LCFAs (McLaughlin et al., 1998, Sidhu et al., 2000) and experiments ensued that used the cell-line as a model for I-cells (Rindi et al., 1990, Mangel et al., 1995). Additional stimulants of CCK release from STC-1 cells included bombesin and amino acids (Snow et al., 1994,

Chang et al., 1998, Mangel et al., 1995, Chang et al., 1994). However, STC-1 cells have also been found to contain many GI hormones as well as CCK (McLaughlin et al., 1998) including multiple products of the proglucagon gene, secretin (Rindi et al., 1990), GIP and islet amyloid polypeptide, (Habib et al., 2012), and PYY (Geraedts et al., 2009, Kieffer et al., 1995). This hindered the idea that STC-1 cells could be used as a robust model for I-cells as specific EEC subtypes were typically considered to contain only one hormone type. Although STC-1 cells remained the closest comparison.

Another closely related EEC model used was the GLUTag cell line. The GLUTag cell-line was isolated from a glucagon-producing EEC tumour (Lee et al., 1992) and robustly expresses proglucagon gene products and secretes glucagon-like peptides upon stimulation by LCFAs, amino acids and glucose (Brubaker et al., 1998, Reimann and Gribble, 2002, Oya et al., 2012). GLUTag cells also possess the ability to secrete CCK (Sidhu et al., 2000) however due to the lower expression of CCK in these cells, did not remain an alternative model for I-cells but more as a general model of EECs.

Over the years cell-line models, particularly STC-1 cells have been a useful tool with which to conduct I-cell research. They have helped to provide insight into the pathways involved in stimulating the release of hormones from EECs. Their true value, however, has considered limited as they contained more than one hormone, a property that was believed atypical to I-cells.

In more recent years, the use of transgenic animals and fluorescent markers to identify and isolate cells has led to many breakthroughs in EEC research. Reimann and colleagues have successfully engineered mice expressing pro-glucagon labelled with the yellow fluorescent protein 'Venus'; enabling fluorescing L-cells to be easily identified and effectively isolated. Using this mouse model, Reimann et al. isolated L-cells and went on to elegantly characterise these cells using electrophysiology, hormone expression analysis and fluorescent calcium imaging techniques (Reimann et al., 2008). This has led to substantial discoveries in the field of L-cell biology. Of particular excitement

was the ability to use isolated L-cells to record electrical activity upon administration of glucose and other compounds. Isolated L-cells were also shown to contain CCK, PYY and gastrin inhibitory peptide (GIP) mRNA. Similar experiments have since been repeated for various enteroendocrine subsets including the characterisation of GIP-producing K-cells (Parker et al., 2009, Habib et al., 2012).

A transgenic mouse strain has been developed with eGFP driven by the CCK promoter providing the means to characterise CCK-containing I-cells. The availability of this mouse model has enabled the characterisation of I-cells and has furthered the field of I-cell research no end. Sykaras and colleagues have provided methodology into successfully sorting fluorescing cells using a fluorescence activated cell sorting (FACS) technique to enable extensive molecular analysis of I-cells (Sykaras et al., 2012). Using this mouse model, analysis of molecular transcripts have also been successfully performed in other laboratories (Samuel et al., 2008, Egerod et al., 2012) and furthermore, the ability to isolate primary I-cells has also enabled secretion studies to be conducted (Liou et al., 2011c, Liou et al., 2011b, Liou et al., 2011a). Through the use of transgenic mouse models, these novel experiments have provided the foundation from which most of the latest knowledge about I-cell activity has been based. Furthermore protein analysis using tissue and cell samples isolated from these transgenic models have enabled characterisation into the hormonal properties of these cells (Chandra et al., 2010, Sei et al., 2011). This has in fact demonstrated that the STC-1 cell line was a more accurate model for I-cells than previously thought.

The ability to isolate and characterise native I-cells has opened a gateway to enable the investigation of hormonal expression, chemosensing properties, and gene expression profile of these fascinating cells.

1.7. Thesis Aims and Hypotheses

Chapter Title: Characterisation of a Multi-Hormonal Complement Expressed in CCK-cells of Mouse Duodenum

To facilitate the use of an eGFP-CCK transgenic mouse model to target the null hypotheses; CCK-cells in mouse duodenum express only CCK

The specific aims of this chapter were set to establish that eGFP cells of the eGFP-CCK mouse model represented CCK-cells and to determine and quantify the expression of key GI peptides within eGFP-cells. Finally cellular co-expression of GI peptides was analysed.

The specific aims were to:

- Determine the validity of the eGFP-CCK mouse model as a mode to identify and isolate CCK-cells.
- Use immuno-staining techniques to determine the expression of key GI peptides within duodenal eGFP-cells
- Use immuno-staining techniques to determine the co-expression of GI peptides within duodenal EECs.

The null hypotheses were as follows:

- Duodenal eGFP-cells in eGFP-CCK transgenic mice do not contain CCK
- Duodenal eGFP-cells do not express a repertoire of anorectic GI peptides
- Duodenal eGFP-cells do not contain the orexigenic peptide ghrelin
- Duodenal eGFP-cells do not express multiple hormones throughout their life span

Chapter Title: Modulation of Enterocyte Fatty Acid Uptake by Gut Hormones

To investigate the ability of GI peptides to modulate FA uptake in intestinal cells to target the null hypothesis; CCK does not have an effect upon FA uptake by enterocytes.

The specific aims of this chapter were set to establish a reliable methodology with which to perform FA uptake studies and application of this methodology to investigate the effects of CCK upon FA uptake in isolated intestinal cells.

The specific aims were to:

- Develop a robust method to measure enterocyte FA uptake
- Determine whether CCK modulates FA uptake by enterocytes
- Determine the basic mechanism of CCK-induced enterocyte FA uptake
- Determine whether CCK expressing EECs influence FA uptake in enterocytes.

The null hypotheses were as follows:

- Incubation with CCK does not enhance FA uptake in enterocytes
- Carrier-mediated transport is not implicated in CCK-induced enterocyte FA transport
- Ligand-stimulated release of CCK from CCK-cells does not induce FA uptake.

Chapter Two

Materials & Methods

2.1. Animals

Mice were either bred in-house by the Biological Services Unit, Faculty of Life Sciences, The University of Manchester (eGFP-CCK and CCK^{LacZ}) or were purchased directly from Harlan Laboratories (HillCrest, UK). All mice were kept on a 12 h light: dark cycle with *ad libitum* access to standard chow (Special Diets Services, Rodent Breeder & Grower, BK001) and water. Animal care and experiments were carried out in accordance with Home Office guidelines. Experiments were carried out in accordance with the UK Animals Scientific Procedures Act (1985).

2.1.1. eGFP-CCK mice

Transgenic eGFP-CCK mice (strain: Tg(eGFP)BJ203GSAT) were purchased from the Mutant Mouse Regional Resource Centre (USA) (MMRRC). This strain of transgenic mice express enhanced green fluorescent protein (eGFP) under the control of the CCK promoter, thus enabling the identification of CCK-containing cells in the mouse intestine. The Tg(eGFP)BJ203GSAT strain was originally created as part of the Gene Expression Nervous System Atlas (GENSAT) project at Rockefeller University, USA (Gong et al., 2003). The presence of the transgene was confirmed by genotyping, according to the instructions provided by MMRRC (http://www.mmrrc.org/catalog/sds.php?mmrrc_id = 249).

2.1.2. CCK Knockout (KO) mice

Tissue from CCK knockout (KO) mice was obtained from a CCK^{LacZ} transgenic mouse strain. CCK^{LacZ} mice have the CCK locus replaced with a knock-in LacZ cassette, rendering homozygotes of the transgene as CCK KO animals (Worthington et al., 2013). Mice were a generous gift from Professor John McLaughlin (University of Manchester).

2.1.3. CD1 and C57 mice controls

Crl:CD1(ICR) Swiss mice (strain code 022) and C57BL/6N (strain code 027) were used as wild-type (WT) control for eGFP-CCK and CCK^{LacZ} transgenic mice strains respectively.

Where applicable transgene was confirmed by genotyping. Adult mice (8-12 weeks old) were used for experiments.

2.2 Immunostaining of Paraffin embedded tissue sections

A series of immunostaining experiments were performed firstly to validate the eGFP-CCK mouse model and secondly to determine the hormonal content of CCK cells in the mouse small intestine.

2.2.1. Tissue Preparation

Tissues were prepared for immunostaining from 3 eGFP-CCK transgenic mice, 3 CCK^{LacZ} mice, 3 Crl:CD1(ICR) Swiss mice and 3 C57BL/6N mice. In the first instance the latter were used as a control. Mice were asphyxiated with CO_2 and killed by cervical dislocation. The small and large intestine was removed and flushed with ice–cold phosphate buffered saline (PBS). The small intestine was divided into three portions; the duodenum, jejunum and the ileum. The duodenum was defined as the first 10cm of the SI commencing from the pyloric sphincter of the stomach, the jejunum represented the following 10cm and the ileum represented the final section (~10cm) of the SI that ends at the caecum. 1cm sections were taken from mid-duodenum, jejunum and ileum. Tissues were then fixed and paraffin embedded.

2.2.2. Tissue Fixation and Paraffin Embedding

Tissues were placed in tissue cassettes and fixed for 5 hours in 4% paraformaldehyde (P6148, Sigma, UK)-PBS (PFA-PBS). This was followed by a dehydration cycle of ethanol gradient: 70%, 96%, 99% for 2 hours each and in xylene for 10 hours. Sections were then paraffin embedded (ThermoFisher, Shandon, Runcorn, W1) at the University of Manchester Histology suite. Transverse sections (4.5µm) were cut using a Leica RM2255 microtome (Leica, Germany) and mounted onto SuperfrostPlus slides.

2.2.3. Immuno-staining using paraffin sections

Sections were de-paraffinised by placing in xylene overnight and then rehydrated through applying an ethanol gradient followed by water (99% ethanol 30mins, 96% ethanol 20mins, 70% ethanol 10mins and dH_2O for 2mins). Antigen retrieval was performed using TEG (Tris 10mM, EGTA 1mM, dH_2O, pH9) buffer in a standardised microwave technique as follows; heated in the microwave at full power for 3mins until boiling and then onto a low power to simmer for 10mins. Slides were then left to cool to room temperature (RT).

Antigen sites were blocked for 30mins by incubation in 50mM NH_4Cl-PBS at RT and tissue permeabilised in 1% BSA, 0.2% gelatine, 0.05% saponin-PBS. Sections were then incubated overnight at 4^oC with mixtures of the appropriate antisera used at optimised dilutions in antiserum buffer; 0.1% BSA, 0.3% Triton-X in PBS. Double staining involved simultaneously diluting two primary antisera in the antiserum buffer for overnight incubation. The primary antisera used are listed in Table 2.1.

Antiserum	*Source*	*Species*	*Epitope*	*Dilution*
CCK (L421)	Professor Dockray (University of Liverpool) (Sykaras et al., 2012)	Rabbit	Raised to sequence: Genbank Protein Accession No. AAH28487	1.500
Ghrelin (GA1)	Professor Yuxiang Sun (Baylor college of Medicine) (Ma et al., 2011)	Rabbit	GSS*FLSPEHQKAQQRKESKK PPAKLQPR (*Ser-O-C(=O)-(CH2)6-CH3)	1.250
Ghrelin 1882 (GA2)	Dr Tomasetto (University of Strasbourg) (Xu et al., 2005)	Rabbit	CRKESKKPPAKLQPR	1.250
GIP	Professor Wice (Washington University) (Wang et al., 2004)	Guinea-Pig	Raised to sequence: Genbank Protein Accession No. AAA53192	1.100
Proglucagon FL-180	Santa Cruz Laboratories	Rabbit	Raised to sequence: Genbank Protein Accession No. P55095	1.100
PYY 16066	Progen BioTechnik	Guinea-Pig	Raised against synthetic porcine peptide YY. Genbank Sequence No. P68005	1.100
GFP SC-9996	Santa Cruz Laboratories	Mouse	Raised to sequence: Genbank Accession No. AAA27722	1.1000
GFP AB6673	AbCam	Goat	Raised to sequence: Genbank Accession No. AAA27722	1.1000
Ki-67 M3064	Spring Bioscience	Rabbit	Raised to C terminus of sequence: Genbank Accession No. CAD99007	1.100

Table 2.1. Table of primary antisera.

The full list of primary antisera. Listed are antisera name, source, species it was raised in, the targeted epitope or, in the case of proprietary sequences, the targeted gene product, and the optimised dilution employed.

Slides were removed from incubation at 4°C and allowed to warm to RT. Sections were washed three times in buffer (0.1%BSA, 0.2%gelatine, 0.05%saponin-PBS) and secondary antisera applied at a dilution of 1.1000 in antiserum buffer and then sections were incubated at RT for 90mins. The secondary antisera used are listed in Table 2.2.

Following exposure to the secondary antisera, sections were washed 3 times in PBS, incubated for 15mins with the nucleic acid stain Hoechst 33342 (Invitrogen, UK) at a final concentration of 0.5ng/µl, washed in dH_20 and then coverslips were mounted using glycergel (DAKO, UK).

Antiserum	*Source*	*Species Raised in*	*Dilution*
Anti-Rabbit IgG Alexafluor594 (CatNo. A11012)	Molecular Probes, Invitrogen, UK	Goat	1.1000
Anti-Rabbit IgG Alexafluor555 (CatNo. A-31572)	Molecular Probes, Invitrogen, UK	Donkey	1.1000
Anti-Rabbit IgG Alexafluor488 (CatNo. A-21206)	Molecular Probes, Invitrogen UK	Donkey	1.1000
IgG Anti-Goat Alexafluor488 (CatNo. A-11055)	Molecular Probes, Invitrogen UK	Donkey	1.1000
Anti-Guinea-Pig IgG Alexafluor594 (CatNo. A-11076)	Molecular Probes, Invitrogen UK	Goat	1.1000
Anti-Mouse IgG Alexafluor488 (CatNo. A-11029)	Molecular Probes, Invitrogen UK	Goat	1.1000

Table 2.2. List of secondary antisera.

Antisera are listed by their full names and catalogue number, where it was sourced, the species it was raised in and the dilution employed.

2.2.4. Double staining using two antisera raised in the same species

For double staining experiments using primary antisera that were raised in the same species, a protocol that utilised fab fragments to block antigenic sites was used. The immunostaining protocol was followed as described in section 2.2.3. up to overnight incubation at 4°C with the first primary antiserum diluted in antiserum buffer. Following incubation, slides were removed from incubation at 4°C and allowed to warm to RT. Sections were washed three times in buffer (0.1%BSA, 0.2%gelatine, 0.05%saponin-PBS) and the first secondary antiserum, anti-rabbit IgG Alexafluor594 (Table 2.2) diluted in antiserum buffer, was applied to sections and incubated for 90mins. Thereafter, the secondary antisera was removed and sections were washed three times in PBS and incubated with 10% AffiniPure Fab Fragment Goat Anti-Rabbit IgG in PBS (Jackson ImmunoResearch) for 30mins to block any free remaining IgG antigenic sites from the first primary antiserum reaction. Sections were then washed in wash buffer (0.1%BSA, 0.2% gelatine, 0.05% saponin in PBS) and the second primary antiserum in antiserum buffer was applied and incubated overnight at 4°C.

Slides were removed from incubation at 4°C and allowed to warm to RT. Sections were washed three times in wash buffer (0.1%BSA, 0.2%gelatine, 0.05%saponin in PBS) and second secondary antiserum, anti-rabbit IgG Alexafluor488, applied at a dilution of 1.1000 in antiserum buffer. Sections were incubated with secondary antiserum at RT for 90mins. Following exposure to the secondary antisera, sections were washed 3 times in PBS, washed in dH_20 and then coverslips mounted using glycergel (DAKO, UK).

Due to the added complexity of this protocol, additional control experiments were included to test the protocol and ensure complete blockade of IgG sites of the first primary antisera, and to validate the specificity of antisera.
When using fab fragments, the most important control experiment, is the omission of the second primary antisera from the experimental protocol. This confirms successful blocking of IgG sites by fab fragments. Insufficient blocking of IgG antigenic sites is a problem, as it would enable second secondary antisera to

bind with the first primary antisera and produce a false positive signal. An additional control where both primary antisera were omitted is included to validate the specificity of the secondary antisera involved. In this instance there should be no positive staining of cells and tissue auto-fluorescence can be determined as background signal.

2.3. Microscopy

Immunostained sections were visualised under fluorescent light for analysis.

2.3.1. Imaging of dual-labelled sections

Slides were visualised using an Olympus BX51 upright microscope with a 20X, 40X or 60X objective. Images were captured using a coolsnap ES camera (Photometrix) through MetaVue Software (Molecular Devices) and processed using ImageJ software. For each experiment, the average background was measured and a threshold background intensity value was set. Cells were considered positively stained when intensity was above the set threshold of background.
The total tissue area was captured by taking sequential fields-of-view, until whole tissue coverage was achieved.

2.3.2. High magnification microscopic imaging of sections that were double-stained for two hormones.

Images were collected on a Leica TCS SP5 AOBS inverted confocal microscope using a 63X 1.40 Oil Plan Fluotar objective and 3X confocal zoom. The microscale settings were as follows; pinhole 1 airy unit, scan speed 400Hz unidirectional. Images were collected using the following detection mirror settings; Alexafluor488 (498.8-569.7) and Alexafluor594 (603.2-679.6) laser lines. The

images were collected sequentially to eliminate bleed-through between channels. When acquiring 3D optical stacks the confocal software was used to determine the optimal number of z-stack slices. Optical sections were taken at 0.18μm spacing and 512 x 512 format for each z-stack. Only the maximal intensity projections for 3D stacks are shown in the results. Images were analysed using the Leica Application Suite Advanced Fluorescence (LAS-AF) software package (Leica Microsystems)

2.3.2.1. Co-localisation analysis of pixel fluorescence

To determine if co-localisation of two fluorophores was present, the LAS-AF software package was used.

Background Subtraction

Pixel fluorescence intensity was normalised by subtracting the background fluorescence from every image of the z-stack. This was achieved by selecting a region of interest (ROI) that had no positive labelling by either antiserum. For each channel the intensity of pixel fluorescence was viewed using an 'intensity histogram'. The highest intensity value for each channel within the ROI was subtracted from every image of the z-stack as background. This is depicted in Figure 2.1.

Determining Pixel Co-localisation

Once the background fluorescence had been subtracted the image was analysed using the co-localisation package in the LAS-AF software. The red and green intensity of every pixel was plotted on a cytofluorogram scatter graph. The x-axis of the cytofluorogram represents intensity of the green channel and the y-axis represents intensity of the red channel (Figure 2.1). In this way pixels with equal intensity for green and red are distributed in the centre of the scatter graph whereas those with signal for only one channel lie close to an axis.

Threshold values of 50% were set for each channel; this is the standard threshold value employed by co-localisation packages. It was applicable to use this default setting as pixel colour intensities were evenly distributed in every image, with a Pearson's Correlation value near to 1. Setting a threshold enabled exclusion of pixels that did not demonstrate significant overlap between the two channels. Pixels that had a minimum of 50% value for green and red intensity were considered as co-localised and this was represented as a percentage value. Percentage co-localisation was calculated for individual z-stack images and averaged. Snapshot images of maximal 3D projection of the total z-stack image were acquired to demonstrate pixel distribution through the cell. Images that contained only one stained cell were cropped to centralise the cell for display purposes.

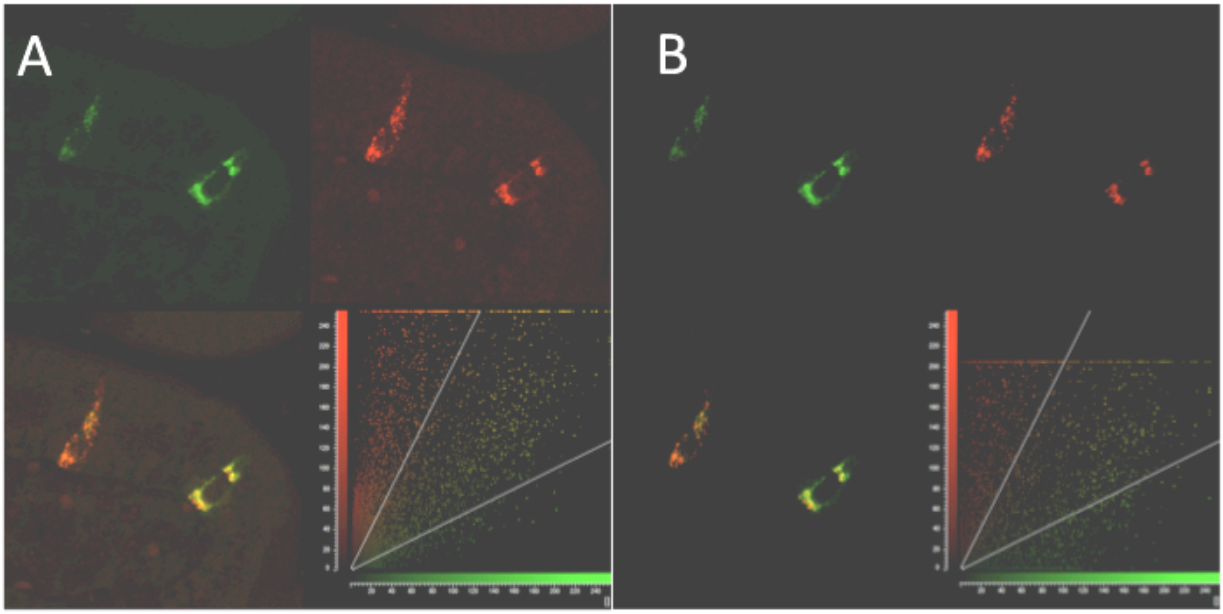


Figure 2.1. Cytofluorogram representing pixel signal intensity from the red and green channel; before (A) and after (B) subtraction of background fluorescence.

Micrograph images were normalised by subtracting background fluorescence. This was achieved by calculating the highest intensity signal from a background region of interest, which was then subtracted from every image in the z-stack. The remaining pixels represent positive signal of antisera labelling.
Green channel and red channel are shown separately in top panels of both images.
A) Before subtraction of background fluorescence. Autofluorescence is apparent in the background. B) After subtraction of background fluorescence. Fluorescent signal is exclusive to labelled cells. The fluorescence intensity for green and red channels are represented for every pixel in the image on the cytofluorograms. Pixels with an intensity of more than 50% for both channels were considered to be co-localised.

2.4. Control experiments aimed at assessing antisera specificity

It was necessary to determine whether a given antiserum was fit for purpose. The most robust control for antisera validation is using tissue from a KO animal, which lacks the specific antigen.
This was performed for anti-CCK antisera L421 due to the availability of CCK KO mouse model on site. This was also performed for the anti-ghrelin antisera, this was necessitated to support the unforeseen results obtained using these antisera.

2.4.1. Verification of anti-CCK antiserum using duodenum from CCK KO mice

To test the specificity of the anti-CCK antiserum, immunostaining was performed on tissue from CCK KO mice and compared to that of WT mice
Tissue sections prepared from CCK^{LacZ} duodenum were stained using anti-CCK antisera. The immunostaining protocol was followed as in Section 2.2.3. with application of just one primary antiserum, anti-proCCK. This protocol was repeated for anti-ghrelin (GA1), anti-proglucagon and anti-PYY antisera to distinguish whether KO of CCK affected the expression of other GI peptides.

2.4.2. Verification of ghrelin antisera

The specificity of anti-ghrelin antisera GA1 and GA2 were confirmed using stomach tissue from ghrelin KO and GOAT KO mice.

2.4.2.1. Tissue obtained from ghrelin KO and GOAT KO mice

Cryoblocks were prepared from control material; stomachs of ghrelin KO (Ma et al., Sun et al., 2003) or *Mboat4*/GOAT KO mice (Kirchner et al., 2009). *Mboat*4 encodes the enzyme Ghrelin O-acyl transferase (GOAT) that is necessary to octanoate and thus activates ghrelin; therefore disruption of the *Mboat4* gene

renders the mice GOAT deficient and thus termed GOAT KO. Ghrelin KO mice were donated by Dr Yuxiang Sun, Baylor College of Medicine, Houston, Texas and GOAT KO mice donated by Professor Tschöp, University of Cincinnati.

2.4.2.2. Tissue fixation and cryoblock preparation

Tissue sections from ghrelin KO and GOAT KO mice were cleaned by flushing with PBS and immediately fixed in 4% PFA-PBS for 60mins. Tissues were then cryo-protected overnight in 30% sucrose in PBS at 4^{o}C. Fixed-tissues were mounted in optimal cutting temperature (OCT) embedding media (Raymond Lamb, ThermoScientific) and snap-frozen in liquid nitrogen. Transverse 6µm cryosections were cut using a Leica CM3050S cryostat (Leica, Germany) and thaw-mounted onto SuperfrostPlus slides (VWR International). Slides were stored at -20^{o}C.

2.4.2.3. Immunostaining using cryosections

Directly before use, cryosections were thawed at 60^{o}C for 30mins, placed in 4% PFA-PBS for 15mins and washed in dH_20 for 2mins. Antigen sites were blocked for 30mins in 50mM NH_4Cl-PBS, and permeabilised in buffer (1% BSA, 0.2% gelatine, 0.05% saponin-PBS). Sections were incubated overnight with mixtures of the appropriate antisera at their optimised dilutions in antiserum buffer (0.1% BSA, 0.3% Triton-X in PBS). The protocol was then followed as for paraffin sections stated in section 2.2.3; following application of primary antiserum Sections were imaged as described in section 2.3.1.

2.5. Single cell isolation for FA uptake experiments

The method used to dissociate small intestinal cells was adapted from those described by Parker and colleagues (Parker et al., 2009, Sykaras et al., 2012) and Sykaras and colleagues (Sykaras et al., 2012). The basic method depends upon on the capacity of EDTA to dissociate cells by collapsing tight junctions connecting epithelial cells. Two adult eGFP-CCK mice were asphyxiated by CO_2 followed by cervical dislocation. The small intestine, from the pyloric sphincter to the caecum, was removed and flushed with ice-cold PBS to remove intestinal contents. Fat was stripped from the tissue using tweezers and whole tissue was rinsed with PBS several times before dissecting the intestine longitudinally to expose the epithelium. Dissected tissue was then cut into ~2mm pieces that were washed with PBS until the PBS ran clean. Single cells were dissociated using a chemical and mechanical method. Briefly, tissue fragments were placed into a 50ml falcon tube containing Ca^{2+} and Mg^{2+} free Hanks Buffered Salt Solution (HBSS) supplemented with 5% foetal bovine serum (FBS) and 1mM 2,29, 20, 20'-(Ethane-1, 2-diyldinitrilo) tetraacetic acid (EDTA). Epithelium was dissociation into single-cells by shaking at 37°C for 30mins as follows: For the first 15mins samples were shaken at 175g in an orbital shaker thereafter samples were shaken at 100g in an orbital shaker for the remainder. During this treatment samples were removed from the incubator and shaken by hand every 5mins. The resultant cell suspension was collected in a 50ml falcon tube and pelleted by centrifugation at 150RCF at RT.

The cell pellet was washed in 10ml PBS to remove any residual EDTA and centrifuged again. The resultant pellet was resuspended in culture medium (pH 7.4) comprising of HBSS with 1.2mM $CaCl_2$, 10mM HEPES, 5mM glucose, 10% FBS. 500µl aliquots were transferred to 1.5ml eppendorf tubes and incubated for 60mins at 37°C supplied with 5% CO_2. For experiments that involved treatment of intestinal cells, the relevant treatment was added after cells had incubated for 45mins after which they were incubated for the 15mins.

2.6. Bodipy-Fatty Acid Uptake

The methods for Bodipy-FA uptake were adapted from those previously described by Yang and colleagues and by Gimeno and colleagues (Yang et al., 2007, Gimeno et al., 2003, Nassir et al., 2007).

Continuing from incubation of cells in section 2.5, tubes were removed from incubator, centrifuged at 800RCF for 5mins, and cell pellets washed in Dulbecco's complete PBS containing Ca^{2+} (0.9mM) and Mg^{2+} (0.5mM) (DPBS) to remove any presence of serum. Cells were pelleted again and resuspended in 500µl DPBS with 10µM fatty acid free BSA (Sigma, UK, Cat no. A6003) and 5µM 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY(R) 500/510 C1, C12) Bodipy fluorescent fatty acids (Bodipy-FA) (Invitrogen, UK)(Cat No. D-3823), at a BSA: Bodipy-FA ratio of 2.1. Prior to its addition to cells, the Bodipy-FA solution was sonicated for 15mins to evenly disperse fatty acids into solution.

Uptake experiments were carried out in triplicates. Reactions were incubated with Bodipy-FA for 2mins in the dark, to protect fluorescence integrity of the Bodipy, and then immediately centrifuged at 800RCF for 5mins. Pellets were washed twice in 500µl ice-cold 'stop buffer' consisting of DPBS, 1% fatty acid free BSA and 200µM phloretin which was included to remove surface-associated Bodipy-FA and prevent further FA flux. Cell suspensions were centrifuged at 800RCF for 5mins between washes. Resultant cell pellets were resuspended in 500µl stop buffer and kept on ice until fluorescence was measured. Phloretin (CatNo.P7912, Sigma, UK) was solubilised in dimethyl sulphoxide (DMSO) (Sigma, UK) before use. Cellular Bodipy fluorescence was measured by flow cytometry with fluorescence-activated cell sorting (FACS).

2.6.1 FACS analysis of cells exposed to Bodipy-FA

Cells were analysed on a Beckman Coulter CyAn ADP cell analyser using Summit software (version 4.3) and cellular fluorescence measured. A 488nm laser was used for excitation and a fluorescent signal was detected as a 530/30nm bandpass.

Events were represented first in a plot of pulse width vs. forward scatter from which single cells could be selected. Selected events were then represented on a plot with side scatter vs. forward scatter values, which gated events by size and were used to eliminate larger 'debris'. Gating is depicted in Figure 2.2. Fluorescence was measured for 10000 events from each sample, represented upon a fluorescence histogram (Figure 2.3) and a mean value was obtained.

To enable gating parameters to be set, cells were also analysed in the absence of Bodipy-FA (control samples).

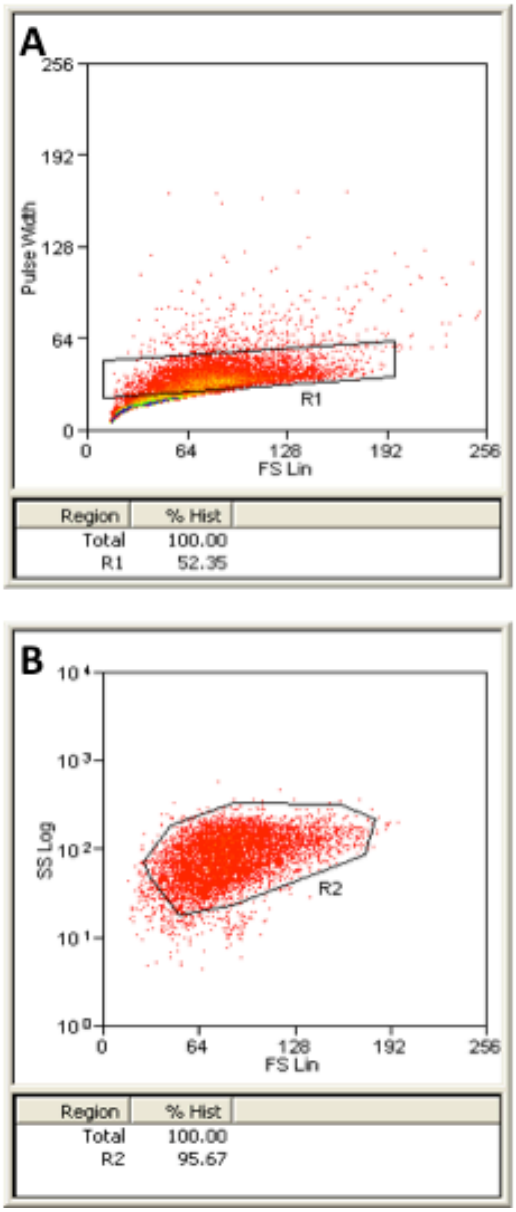


Figure 2.2. FACS data sheet to reflect the gating process to select cells for analysis following incubation with Bodipy-FA.

Cell samples were gated according to their properties of size and viability. Red dots represent analysed 'events'.
A) Forward scatter (x-axis) vs. pulse width (y-axis). Forward scatter reflects the cell-surface size. R1 gating excludes events that do not display properties typical to cells or are very big and may represent debris or cell clumps.
B) Forward scatter (x-axis) vs. side scatter (y-axis). This measures cell events according to size and in proportion to cell granularity. R2 gating again enables gating and takes forward events that represent the properties of single, viable cells.

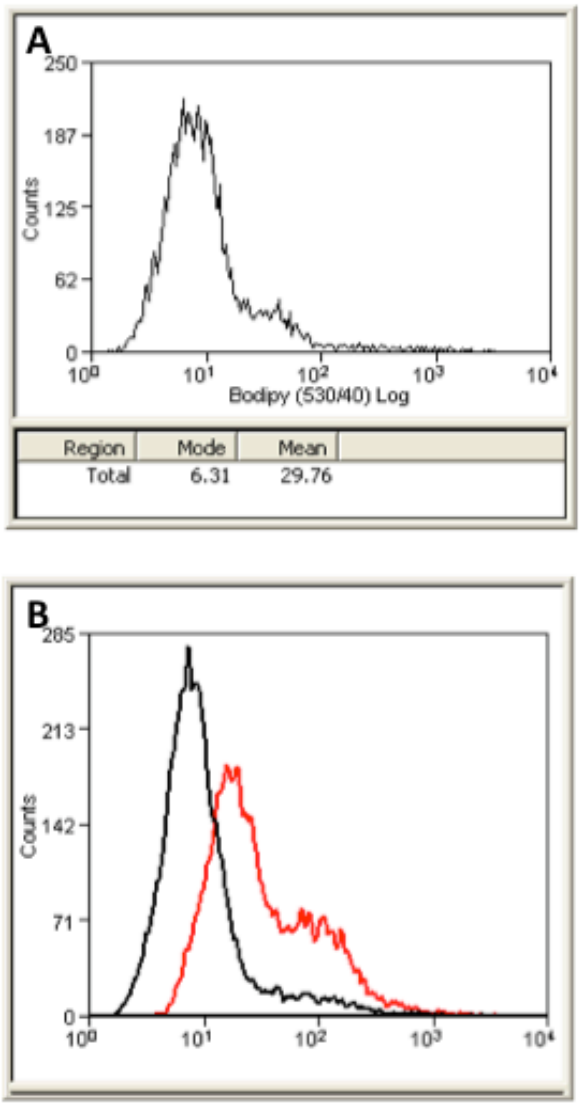


Figure 2.3. After incubation with Bodipy-FA, cell samples were analysed according to cell fluorescence.

Within each cell sample, 10000 events were analysed according to fluorescence. These values are represented on a histogram. Histogram: x-axis represents the logarithmic scale for Bodipy (530/40) fluorescence value and y-axis represents cell count. Computer analysis of these data acquires a mean fluorescent value for the sample that is represented as a relative arbitrary unit (RAU).
A) Histogram data sheet example. Represents fluorescent value against cell count. B) Histogram overlay for Bodipy-FA uptake data from non-treated cells (black line) and cells pre-treated with CCK (10pM) (red line data). Treatment with CCK evokes an obvious rightward shift in cell fluorescence.

2.7. Optimisation of Bodipy-FA uptake methodology

The experimental parameters and the concentration of Bodipy-FA to be used were first optimised. It was important to determine whether the dynamics of Bodipy-FA uptake were reflective of typical FA uptake and thus supports the use of the Bodipy-C12 FA analogue as a means to measure cellular FA uptake. It was also deemed important to construct a protocol with the capacity to differentiate changes in FA uptake within the physiological range and that the protocol should relatively simple, thus limiting potential user errors, and quick, thus minimising any temporal effects due to sample degradation.

2.7.1. Determining an effective FA uptake incubation time

A time course of Bodipy uptake was performed to determine the optimal incubation time. Single cells were isolated as described in section 2.5. Briefly, cell pellets were washed with DBPS and re-pelleted by centrifugation at 800RCF for 5mins. Cell pellets were suspended in 500µl DPBS with 5µM Bodipy-FA. Cells were incubated in 500µl Bodipy-FA solution (5µM) for 0.5mins, 2mins, 5mins, 10mins or 30mins. Samples were centrifuged and washed stop buffer as described in Section 2.6. An optimal incubation period of 2mins was decided.

2.7.2. Bodipy-FA uptake concentration curve

To determine the optimal concentration of Bodipy-FA to use, cells prepared as described in section 2.5. were pelleted by centrifugation at 800RCF for 5mins and washed in DPBS. Cell samples were centrifuged again and pellets re-suspended in DPBS with concentrations of Bodipy-FA ranging between 2.5-50µM, with a BSA: Bodipy Molar ratio of 2:1. Cells were incubated for 2mins before washing in stop solution. An optimal concentration of 5µM Bodipy-FA was decided.

2.7.3. Determining the effect of concentrations of stimulators of FA uptake

Oleoylethanolamide (OEA) stimulates FA uptake via up-regulation of the membrane protein CD36 (Yang et al., 2007). Bodipy-FA uptake into intestinal cells was measured after cell incubation with OEA (Phoenix Pharmaceuticals, USA) at concentrations ranging between 10nM – 50μM.
The GI peptide glucagon-like peptide-2 (GLP-2)(Hsieh et al., 2009) has also been shown to stimulate FA uptake. Cells were incubated in media supplemented with GLP-2 (Tocris, UK) to a final concentration in the range of 10pM – 100nM.

Cells were prepared as described in section 2.5. Defined concentrations of OEA or GLP-2 were added to resultant cell suspensions and incubated for 15mins 37^{o}C. The Bodipy-FA uptake protocol was then followed as for section 2.6.
Optimal concentrations of OEA (100nM) and GLP-2 (10pM) were then decided.

2.7.4. Determining the effect of sulphated CCK (CCK) on FA uptake

To test whether CCK stimulates FA uptake into intestinal cells, cells were incubated with sulphated CCK octapeptide (CatNo.1150 Tocris, UK) at concentrations ranging between 1pM – 10nM.
Cells were prepared as described in section 2.5. Concentrations of CCK were added to cell suspensions and incubated for 15mins 37^{o}C. The Bodipy-FA uptake protocol was followed as for section 2.6.
An optimal working CCK concentration of 10pM was decided.

2.7.5. Determining the effect of non-sulphated CCK (CCK-NS) on FA uptake

To determine the effect of non-sulphated CCK on Bodipy-FA uptake into intestinal cells, the non-sulphated form of CCK (CCK-NS) (CatNo.1166 (Tocris, UK) was added to incubating cells to a final concentration that ranged between 1pM - 10nM.

Cells were prepared as described in section 2.5. Concentrations of CCK-NS were added to cell suspensions, which were incubated for 15mins 37^{o}C. The Bodipy-FA uptake protocol was followed as for section 2.6. No significant increase in Bodipy-FA uptake was observed.
A positive control using a known stimulator of Bodipy-FA uptake was included in every experiment to ensure cells were viable and capable of FA uptake response.

2.7.6 Confirmation that CCK incubation increases Bodipy-FA uptake in different mouse models.

To validate the choice to use eGFP-CCK mice as the mouse model for data presented on Bodipy-FA uptake, intestinal cells from eGFP-CCK, CCK^{LacZ} and C57BL/6N (C57) WT mice were independently tested and compared. Small intestinal cells from each mouse strain were isolated as described in section 2.5. CCK 10pM was added to incubating cells and left at 37^{o}C for 15mins. The protocol was then followed as in section 2.6.
Each data set were normalised to the mean of lowest control values to enable comparisons. Comparable increases in uptake were recorded for cells from each mouse strain.

2.8. Outlining the Mechanistic Pathway

2.8.1. Determining the involvement of CCK-R_A in CCK-stimulation of cellular FA uptake

To test whether CCK binding to the CCK-R_A mediated the CCK-stimulated FA uptake, cells were treated with the selective CCK-R_A inhibitor, Loxiglumide.
Intestinal cells were prepared as described in section 2.5. Cells were incubated in standard media for 45mins. Incubating cells were then supplemented with Loxiglumide (CatNo. SML0130, Sigma Aldrich, UK) to a final concentration of 100μM. Alternatively cells were incubated with Loxiglumide (100μM) alongside CCK (10pM), or with CCK (10pM) alone. Treated cells were incubated at 37°C for 15mins. The Bodipy-FA uptake protocol was followed as for section 2.6.

2.8.2. Determining the effect of phloretin upon Bodipy-FA uptake

Phloretin is a potent and non-specific inhibitor of many membrane transport proteins therefore inhibits many facilitated transport processes within a cell (Fenton et al., 2004, Potter et al., 2006). Phloretin applied at 200μM has been shown to significantly reduce FA uptake in muscle cells (Luiken et al., 1999, Luiken et al., 2001).
Isolated intestinal cells were prepared as described in section 2.5. Phloretin (200μM), Phloretin (200μM) alongside optimised concentrations of stimulator, or stimulator alone, were added to cell suspensions and incubated for 15mins at 37°C. The stimulators of FA-uptake tested were OEA (100nM), GLP-2 (10pM) and CCK (10pM).
The Bodipy-FA uptake protocol was followed as for section 2.6.

2.8.3. Determining the contribution of the FA transport protein CD36 upon Bodipy-FA uptake

CD36 is indicated as the major fatty acid transporter in enterocytes (Lynes et al., 2011, Nassir et al., 2007). To test whether CD36 mediated the peptide-induced increase in Bodipy-FA uptake, the CD36-specific inhibitor sulfo-N succinimidyl oleate (SSO) was employed (Coort et al., 2002). SSO was kindly donated by Professor Glatz at Maastricht University, The Netherlands. A stock solution of SSO was prepared by dissolving SSO in DMSO and applied to cells to a final concentration of 1μM.

Isolated intestinal cells were prepared as described in section 2.5. SSO (1μM), SSO (1μM) and stimulator, or stimulator alone were added to cell suspensions and incubated for 15mins 37^{o}C. The stimulators of FA-uptake tested were OEA (100nM), GLP-2 (10pM) and CCK (10pM). The Bodipy-FA uptake protocol was followed as described in section 2.6.

2.9. Determining the involvement of I-cells upon CCK stimulation of cellular FA uptake

The experiments described below were aimed at establishing whether CCK released by EECs stimulated FA uptake, thus suggesting a paracrine action of 'I-cells' to increase local FA uptake. To do this, treatments were selected that had previously been reported by others to stimulate I-cell release of CCK. These agents were bombesin, or amino acids. These experiments also made use of CCK^{LacZ} mice and FACS sorting to prepare populations of cells depleted in I-cells.

2.9.1. Stimulating I-cells activation using Bombesin

Bombesin has been used experimentally for decades as a model of inducing the release of CCK (Banks, 1980, Chen et al., 2006, Wang et al., 2011, Cuber et al., 1989). The effects of bombesin on CCK release have been recorded in both STC-1

cells and *in vivo* (Snow et al., 1994, Cuber et al., 1989). Bombesin was utilised in this experiment to determine its effects on Bodipy-FA uptake.

Intestinal cells were isolated as described in section 2.5. Bombesin acetate salt hydrate (CatNo.B4272, Sigma, UK) was added to incubating cell suspensions to a final concentration ranging between 1pM – 1μM. Cells were left to incubate for a further 15mins and the Bodipy-FA uptake protocol was followed as described in section 2.6. An optimal working concentration of bombesin (1nM) was decided.

2.9.2. Stimulating I-cells activity using amino acids

Aromatic L-amino acids have been shown to cause CCK release through acting through the CaSR (Wang et al., 2011, Liou et al., 2011c). L-Phenylalanine (10mM), L-Tryptophan (10mM), L-Histidine (10mM), L-Alanine (10mM) and the antibiotic neomycin (100μM), a known agonist of the CaSR, were dissolved in culture medium and added to isolated intestinal cells (section 2.5). Cells were incubated with amino acid solutions for 60mins at 37^{o}C. The Bodipy-FA uptake protocol was then followed as in section 2.6.

2.10. Determining the effect of endogenous CCK expression on FA uptake experiments using a CCK KO model

A number of the above methods were performed on CCK^{LacZ} a mouse model in which CCK is not expressed. This provided a model to compare the data acquired using the eGFP-CCK mouse model to determine the effects of endogenous CCK upon cellular FA uptake. Experiments were performed as previously described.

2.10.1. Determining the effect of CCK on FA uptake in cells from CCK KO mice

Intestinal cells were prepared from CCK^{LacZ} mice as described in section 2.5. Cells were incubated in CCK for 15mins at 37°C, at final concentrations ranging from 1pM-10nM CCK as described in section 2.7.4.

2.10.2. Determining the effect of other stimulators of FA uptake in cells from CCK KO mice

Intestinal cells were prepared from CCK^{LacZ} mice as described in section 2.5. Cells were incubated for 15mins at 37°C, in optimised concentrations of OEA (100nM), GLP-2 (10pM) and bombesin (1nM). The Bodipy-FA uptake protocol was followed as for section 2.6.

2.10.3. Determining the effect of amino acid incubation upon FA uptake in cells from CCK KO mice

Intestinal cells were prepared from CCK^{LacZ} mice as described in section 2.5. Cells were incubated for 60mins at 37°C, in culture media containing L-Phenylalanine (10mM), L-Tryptophan (10mM), L-Histidine (10mM), L-Alanine (10mM) and the antibiotic neomycin (100μM) as described in section 2.9.2. The Bodipy-FA uptake protocol was followed as for section 2.6.

2.11. Using a eGFP–ve cell population as a model of excluding native I-cells

Using the methods from (Sykaras et al., 2012), small intestinal cells of eGFP-CCK mice were subjected to FACS sorting. Green fluorescing cells were sorted out of the cell population to be used as a model of intestinal epithelial cells minus I-cells. Briefly, cells were isolated from eGFP-CCK mice as described in section 2.5. After the EDTA dissociation step, cells were pelleted by centrifugation for 5mins at

200RCF and re-suspended in PBS containing 3% FBS. Cells were passed through a 40μm cell strainer and subjected to FACS cell sorting on a BD FACS Aria (DIVA version 5 software) cell sorter (BD Biosciences). Cells were gated using a forward scatter to side scatter plot to exclude debris and examine cells for viability (Figure 2.4). Cell events were then analysed for eGFP fluorescence using a 488nm laser for excitation and measuring signal at 530/30nm bandpass. A control sample (intestinal cells isolated from WT/CD1 mouse) was used to establish the background fluorescence and enable gating for eGFP-positive cells (Figure 2.4). 1.5million eGFP negative cells were sorted into HBSS buffer. An additional cell sample was subjected to FACS yet sorted into 1.5million total cell population sample as a positive control. Cells were spun down and placed into culture media buffer ready for incubation.

For the purposes of this experiment, cells were incubated in CCK 10pM for 15mins to verify cells were responsive and with L-phenylalanine 10mM for 60mins. L-Phenylalanine was used in this instance to determine whether the responses seen in previous experiments were attributable to I-cells. The Bodipy-FA uptake protocol was continued as described in Section 2.6.

2.12. Statistical Analysis

On graphs, data are presented as mean values along with values for standard error of the mean (S.E.M). Statistical analysis was carried out using GraphPad Prism for Mac, Version 6. When optimising parameters of Bodipy-FA uptake protocol, data were analysed using a non-linear fit of linear regression alongside analysis using the Michaelis-Menten equation. Where sample mean values are compared to mean control values an Analysis of Variance (ANOVA) test followed up with the post-hoc Dunnets test was performed. When comparing pre-selected pairs of data, the ANOVA test was performed, followed by the post-hoc Bonferroni test. When comparing only two sets of mean values a paired t-test was used. In all cases a p value ≤ 0.05 was considered significant. Significance symbols are represented on the graphs.

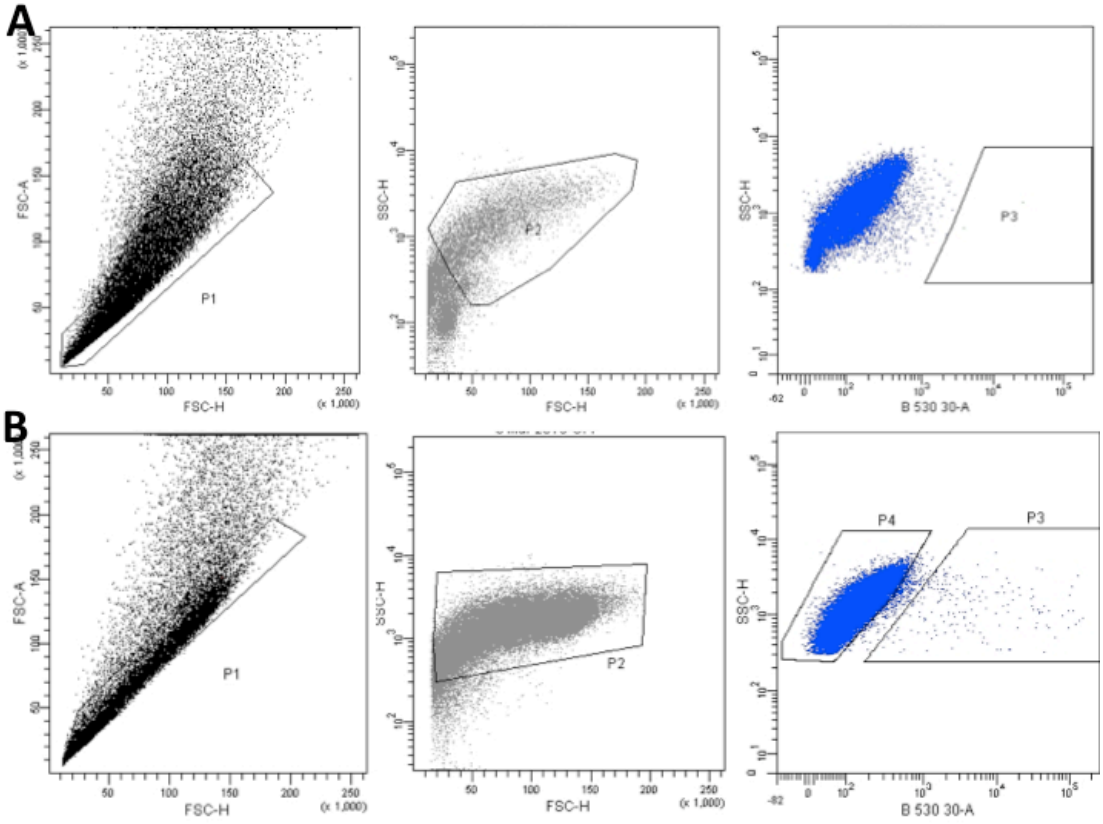


Figure 2. 4. FACS sorting of eGFP-ve cells from total cell population.

Dissociated small intestinal cells from A) CD1 WT mice and B) eGFP-CCK mice were FACS sorted using a BD FACS Aria cell sorter.
Cells were gated using a forward scatter to side scatter plot to exclude debris and examine cells for viability (P1) and (P2) populations. Right hand panels; Cells were analysed for eGFP fluorescence. Events are represented according to side scatter (y-axis) against eGFP fluorescence (x-axis). P3 population represents cells with green fluorescence. Green fluorescing cells are absent from control sample (Row A). P4 represents non-eGFP cells.
P4 gated cells from eGFP-CCK mice (Row B) were isolated and used as eGFP negative cell population. P2 gated cells from eGFP-CCK mice were sorted as a representation of total cell population (positive control). 1.5 million cells from eGFP-CCK mice were sorted from P2 and P4 regions and used experiment to compare the response of eGFP negative cells vs. total cell population.

Chapter Three

Characterisation of a Multi-Hormonal Complement Expressed in CCK-cells of Mouse Duodenum

3.0. General Overview

Several studies have recently reported the expression of multiple gut hormones in the distal SI and colon (Egerod et al., 2012, Habib et al., 2012). Whether this holds true for the proximal SI, in particular within CCK-expressing cells (I-cells) remains to be elucidated.

The aims of this chapter were set to test the null hypothesis that CCK-cells in the duodenum express only CCK.

Initially, duodenal sections were immunostained with an eGFP antiserum to confirm eGFP expression and then counterstained sections with a CCK antiserum to validate the fidelity of the reporter gene. Duodenal sections were dual-immunostained for eGFP and key GI peptides, and the frequency and location of dual-labelled cells was determined. Finally, duodenal sections were dual-stained against a complement of GI peptides to establish peptide co-localisation within EECs.

The specific aims were to:

- Determine the validity of the eGFP-CCK mouse model as a mode to identify and isolate CCK-cells
- Use immuno-staining techniques to determine the expression of key GI peptides within duodenal eGFP-cells
- Use immuno-staining techniques to determine the co-expression of GI peptides within duodenal EECs

The null hypotheses were set as:

- Duodenal eGFP-cells in eGFP-CCK transgenic mice do not contain CCK
- Duodenal eGFP-cells do not express a repertoire of anorectic GI peptides
- Duodenal eGFP-cells do not contain the orexigenic peptide ghrelin
- Duodenal eGFP-cells do not express multiple hormones throughout their life span

3.1 Results

Validation of the eGFP-CCK mouse model.

In the present study, we utilised a eGFP-CCK transgenic mouse strain to facilitate identification of intestinal CCK-containing 'I-cells' to enable characterisation of these cells. The first aim was to validate this mouse model as a model for studying duodenal CCK-expressing cells - I-cells.

To do this, tissue from eGFP-CCK mice was paraffin embedded and immunohistochemistry techniques were performed. It was noted that during processing of paraffin embedded tissue, the microwave treatment step for antigen retrieval obliterated the fluorescence signal of endogenous eGFP possibly by heat-induce denaturation. To overcome this problem, staining with an anti-GFP antibody was employed to visualise cells containing the eGFP epitope.

3.1.1 Mapping the presence and morphology of eGFP cells

Green fluorescing cells were seen throughout the length of the small intestine (Figure 3.1 A-C). Green fluorescence filled the cytoplasm of cells and was confined to the epithelium. Green fluorescing cells typically had a flask-shaped morphology and were scattered with an even distribution pattern throughout the intestinal epithelium. Green fluorescing cells were present in the villi and within the crypt regions. Qualitatively there appeared to be a higher abundance of eGFP-cells within the duodenum, decreasing in number in the jejunum and ileum. A small number of eGFP-positive cells were seen in the colon (Figure 3.1-D).

3.1.2. eGFP positive cells represent both proliferating and non-proliferating cells

To determine the distribution and proliferative activity of eGFP-cells within the duodenal epithelium, paraffin embedded tissue sections from eGFP-CCK mice were dual-stained with anti-GFP and anti-Ki-67 antisera. Ki-67, a marker of cell proliferation is expressed in the cell nucleus during mitosis. Therefore, Ki-67 antiserum labels highly proliferative cells, a feature of stem cells that are located in the intestinal crypts. Anti-Ki-67 staining was highly concentrated in cells of the crypts. A small number of Ki-67 positive cells were seen to extend to the villi, yet these had a sparse distribution. eGFP-positive cells were evenly distributed throughout the crypts and villi of duodenal sections. Anti-eGFP and anti-Ki-67 labelling was co-localised in some instances, particularly in cells in crypt regions (Figure 3.2).

3.1.3. eGFP cells represent CCK containing I-cells.

Duodenal tissue sections from 3 transgenic mice were dual-stained with anti-eGFP and a well-characterised anti-proCCK antiserum, L421. eGFP-positive cells in the duodenum were observed to stain positively with anti-proCCK (Figure 3.3). Anti-proCCK staining was specifically localised to eGFP-cells, of which 89% (±1.25 n=19 slides prepared from 3 mice) were labelled positively for proCCK (n=3000 cells). ProCCK staining was strongest towards the basolateral membrane of eGFP-cells where the secretory granules are known to be located. Figure 3.3 shows merge images for positively stained cells in the duodenum with A) 20X objective B) 40X objective and (C) a 100X objective. Control staining was performed using paraffin embedded duodenum of a CCK knockout model, CCK^{LacZ} (Figure 3.4 A). Secondary antibody only control image is also included (Figure 3.4 B). These images display no signal from antisera labelling, which corresponds to the red channel in both images. Together these data confirmed that eGFP was a reliable reporter for CCK in our mouse model and thus eGFP-positive cells faithfully represented CCK I-cells.

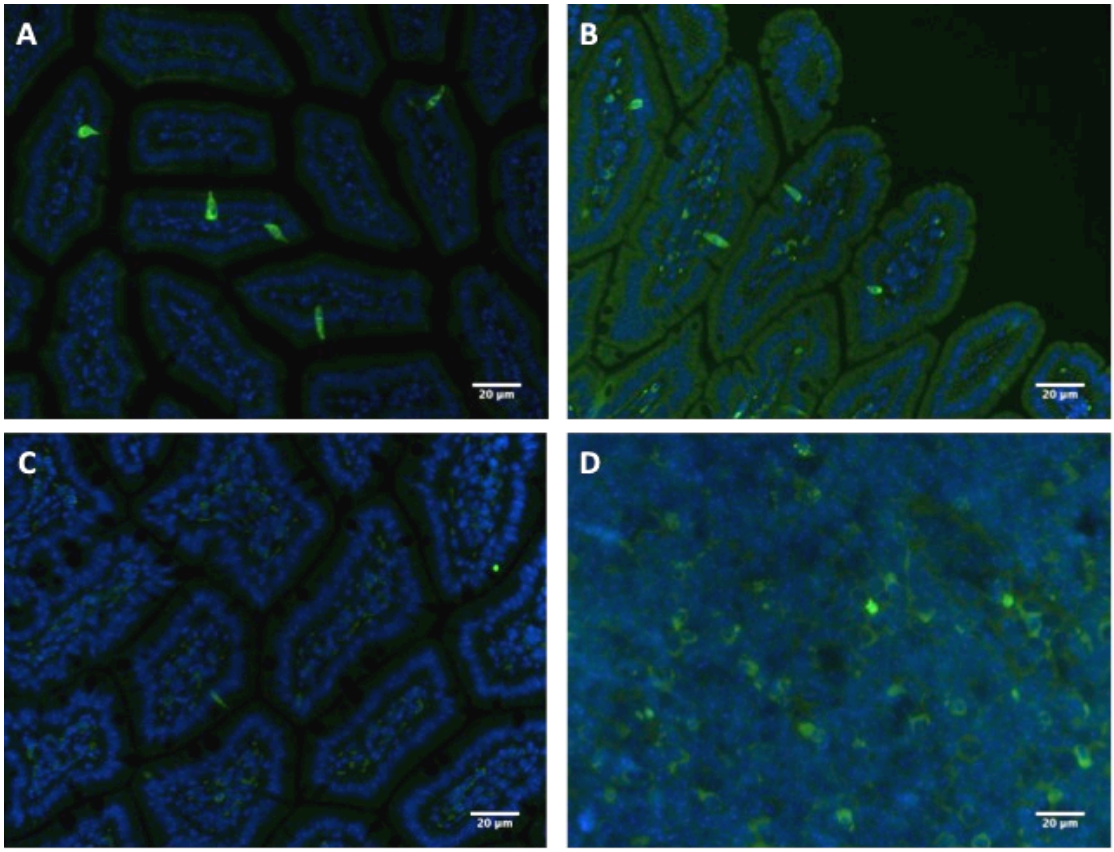


Figure 3. 1. Anti-eGFP immunostaining of paraffin embedded tissue sections from eGFP-CCK mice.
Sections (4µm) were immunostained with an eGFP antiserum to confirm expression of eGFP in intestinal epithelium. A) Duodenum, B) Jejunum and C) Ileum were positively stained for eGFP and displayed typical enteroendocrine characteristics. D) eGFP-positive cells were observed in colon. Blue fluorescence represents nuclei staining with Hoechsst 33452, and green fluorescence represents labelling with eGFP-antiserum. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

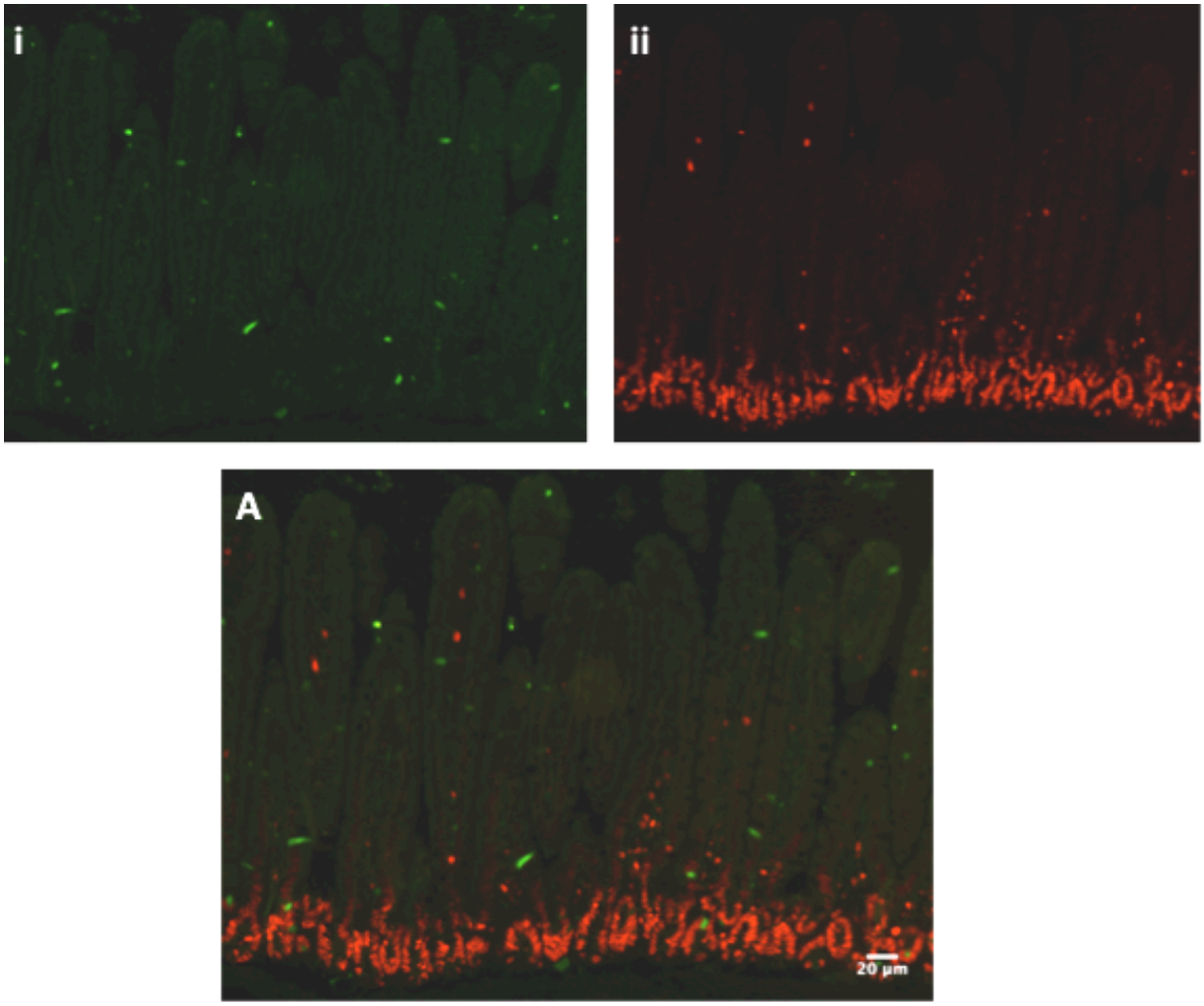


Figure 3. 2. Anti-Ki-67 and anti-eGFP immunostaining of paraffin embedded mid-duodenal sections from eGFP-CCK mice.

Sections (4µm) were dual-immunostained using anti-Ki-67 and anti-eGFP antisera. i) Green fluorescence represents anti-GFP and ii) Red fluorescence represents the anti-Ki-67 immuno-label. A) Merged image of red and green channels.
Ki-67 positive cells are concentrated in the crypt areas with a small number of positive cells along the villi whereas eGFP-labelled cells were equally distributed between the crypt and villi regions. Some staining was co-localised to the same cell. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

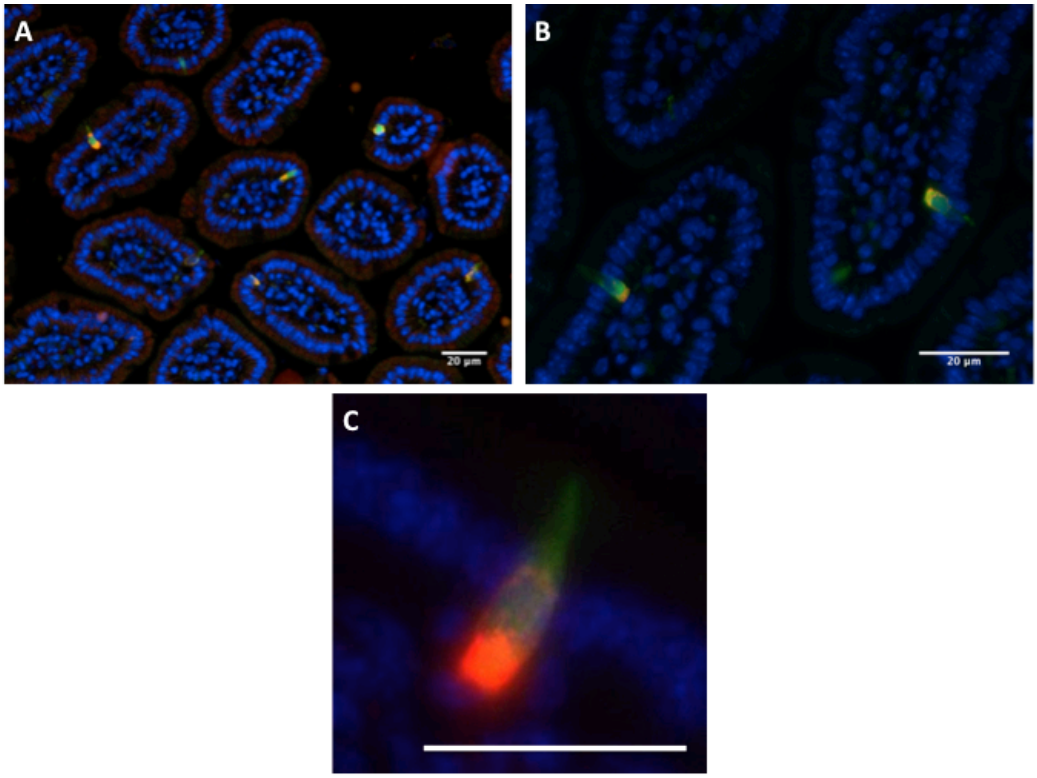


Figure 3.3. Anti-proCCK immunostaining of eGFP-positive cells of paraffin embedded duodenum from eGFP-CCK mice.

Sections (4µm) were immunostained with anti-proCCK and anti-GFP antisera. 89% (±1.25 n=19) eGFP positive cells (n=3000 cells) were immunostained for CCK.
Green fluorescence represents anti-GFP label and red fluorescence represents labelling with anti-proCCK antiserum. Blue fluorescence represents nuclei staining with Hoechsst 33452. A) 20X objective B) 40X objective C) 100X objective images of cells positively labelled for eGFP and CCK. Images clearly show CCK staining to be concentrated at the basolateral pole of eGFP-positive cells. CCK staining was restricted to eGFP-cells. Images were taken on a snapshot widefield microscope. Scale bar represents 20µm.

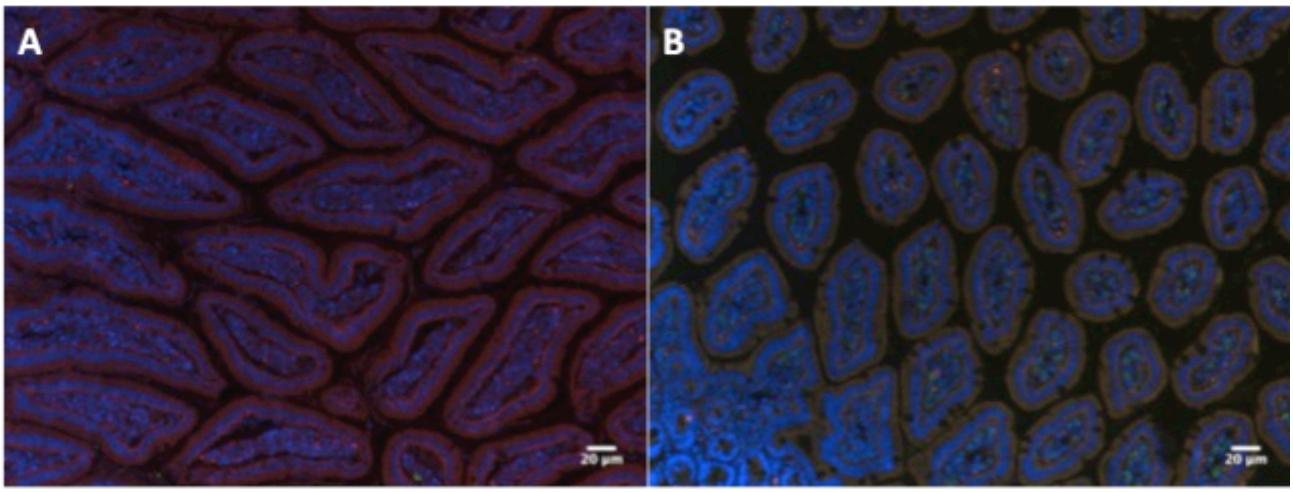


Figure 3.4. Control experiments for Anti-proCCK L421 antiserum

A) Application of anti-proCCK to mid-duodenal section from CCK^{Lacz} mice.
B) Omission of anti-GFP and anti-proCCK antiserum as a control reaction to test specificity of secondary antisera.
Blue fluorescence represents nuclei staining with Hoechsst 33452. Red represents anti-proCCK labelling with Alexafluor594 and green represents anti-GFP labelling with Alexfluor488. There is an absence of green and red fluorescence labelling in both pictures. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

3.2. eGFP-positive cells contain CCK, but do they contain other gut peptides?

Dual-staining of mid-duodenal sections for eGFP and other key GI peptides.

Double staining experiments utilised an anti-eGFP antiserum alongside antisera specific for key GI peptides. Dual labelling was preformed to gain insight whether eGFP-cells from eGFP-CCK mice contained only CCK and if not to ascertain which hormones are expressed within eGFP-cells. In all images green represents staining by anti-eGFP and red represents labelling by antisera targeted against the relevant hormone. For secondary only controls see Appendix (section 6.0).

Dual-staining of mid-duodenal sections for key GI peptides alongside an anti-Ki-67 antiserum as a robust marker of proliferating cells

Recent data in the literature have indicated eGFP-cells from eGFP-CCK mice which displayed properties of hormone co-expression were exclusively located in the crypts (Sei et al., 2011). It was further suggested that these cells have stem cell-like properties, and do not represent mature and functional CCK-cells that are located within the villi. To investigate this claim, co-staining was performed on duodenal sections using an anti-Ki-67 serum, which is a marker of cell proliferation, alongside antisera targeting key GI peptides. Anti-Ki-67 labelling is represented by green fluorescence, and anti-peptide labelling represented by red fluorescence in every image. For secondary only controls see Appendix (section 6.0).

Experiments were performed using duodenal tissue prepared from 3 eGFP-CCK mice.

3.2.1. Dual staining experiments using an anti-PYY antiserum

3.2.1.1. Co-expression of eGFP and PYY

Duodenal sections were stained with anti-GFP and anti-PYY antisera. Antisera labelling was seen to co-localise in cells found along the villi and in the crypt areas where stem cells and epithelial differentiation originates. Figure 3.5 is a representative image showing two cells within the villi that have been double-stained (Figure 3.5-A). 45% (±3 n=9 slides) of eGFP-positive cells (n=400 cells) were labelled with anti-PYY. However, 32% (±3 n=9 slides) of total PYY-positive cells (n=260 cells) did not contain eGFP. Figure 3.5-B shows a high magnification image (60X objective) of a dual-labelled cell. Anti-PYY staining was strongest towards the basolateral membrane of cells where secretory granules are concentrated.

3.2.1.2. Co-expression of Ki-67 and PYY

Anti-Ki-67 and anti-PYY co-stained sections are represented in Figure 3.6. Anti-Ki-67 labelling is conserved within the cell nucleus, of which highest expression is located to the intestinal crypts. Some Ki-67 positive cells are seen further along the villi yet at a much lower frequency. As before, PYY-positive cells are located within the crypts and the villi. A small degree of co-localisation of Ki-67 and PYY was observed and this occurred predominantly in cells in the crypts regions. These data indicate cells containing PYY are not confined to proliferating cells.

3.2.2. Dual staining experiments using an anti-GIP antiserum

3.2.2.1. Co-expression of eGFP and GIP

The pattern of anti-eGFP and anti-GIP staining was similar to that seen for PYY. Co-stained cells were observed in both the villi and the crypt regions. Figure 3.7 is a representative image showing dual-labelled cells within the villi epithelia

(Figure 3.7-A) and a higher magnification (60X objective) image of a dual-labelled cell (Figure 3.7-B). Of the total eGFP positive cells (n=600), 37.1% (±1.9 n=10 slides) were co-stained for GIP. However, 18.2% (±3.2 n=10 slides) of cells labelled with GIP (n=215 cells) were not eGFP-positive. As seen for PYY, anti-GIP staining was strongest towards the basolateral pole of the cell.

3.2.2.2. Co-expression of Ki-67 and GIP

Anti-Ki-67 staining alongside anti-GIP is represented in Figure 3.8. These images show nuclear staining by anti-Ki-67 that is concentrated to the highly proliferative cells of the intestinal crypts. Only a very low number of Ki-67 labelled cells are found in the villi regions whereas GIP-positive cells are evident in the crypts and the villi. Co-localisation of Ki-67 with GIP is shown to be exclusive to crypt regions. These data indicate that GIP-containing cells in the villi are not representative of proliferating cells.

3.2.3. Dual staining experiments using an anti-proglucagon antiserum

3.2.3.1. Co-expression of eGFP and Proglucagon

A small number of eGFP positive cells in the duodenum were seen to label positively with proglucagon antisera. Anti-proglucagon staining was specifically localised to eGFP-cells, of which 14% (±2. n=10) eGFP-cells (n=624 cells) labelled for proglucagon. Figure 3.9 is a representative image showing a cell within the villi that has been dual-stained (Figure 3.9-A) and a higher magnification image of a dual-labelled cell (Figure 3.9-B). Again, staining was strongest towards the basolateral membrane of eGFP-cells.

3.2.3.2. Co-expression of Ki-67 and Proglucagon

Data were lacking for Ki-67 and proglucagon due to the low number of proglucagon cells in this region and difficulties in performing this experiment using two antisera from the same species.

3.2.4. Dual staining experiments using ghrelin antisera

In summary, eGFP positive cells contain several key gut hormones, but do they contain the orexigenic hormone ghrelin? The experiments that follow aimed to answer this question.

3.2.4.1. Verification of anti-ghrelin antisera using stomach tissue from ghrelin KO and GOAT KO mice.

Anti-ghrelin antisera were verified to confirm specificity for their epitope. Tissue from genetic knockout mice were used as trustworthy controls for GA1 and GA2 ghrelin antisera (Figure 3.10). GA1 and GA2 were applied to cryosections of stomach prepared from either ghrelin knockout or GOAT knockout animals along with stomach tissue from eGFP-CCK mice as wild-type tissue. GOAT is the enzyme that catalyses ghrelin octanoylation to produce the 'active' form of ghrelin.
GA1 and GA2 showed the same staining patterns: The anti-ghrelin antisera GA1 and GA2 successfully labelled many positive cells in stomach from WT mice. The labelled cells were round shaped, typical of 'closed-type' ghrelin (X/A) cells in the stomach. For ghrelin KO tissue there was an absence of ghrelin staining. Staining of GOAT KO tissue was similar to the staining recorded using tissue from wild type animals. Together these data demonstrate that GA1 and GA2 are specific for ghrelin, but do not differentiate between octanoylated and non-octanoylated ghrelin. Both antisera showed minimal non-specific background staining.

3.2.4.2. Co-expression of eGFP and ghrelin (GA1)

Duodenal sections were stained using a GFP-targeted antisera and anti-ghrelin (GA1) serum. Staining was seen to co-localise for anti-GFP and anti-ghrelin in open-type cells located along the villi (Figure 3.11-A) and in crypts. Ghrelin staining was also seen in 'closed-type' enteroendocrine cells (Figure 3.11-A-Inset). The closed-type ghrelin-positive cells were circular in shape and did not extend to the intestinal lumen like the classical open-type EECs. Figure 3.11-B shows a high magnification image of an open-type cell that has been dual-labelled by the two antisera. Ghrelin staining (red fluorescence) was concentrated at the basolateral membrane and around the cell nucleus. Additional information of cell localisation is provided in Figure 3.12 that shows dual-labelled cells were found located in both the crypt and along the villus. Staining with the ghrelin antisera GA1 localised to 50.1% (±1.7 n=25) of eGFP-positive cells (n=3353 cells).

3.2.4.3. Confirming co-expression of eGFP and ghrelin (GA2)

Because the results obtained using GA1 antiserum were so surprising and unexpected it was deemed necessary to confirm these data using a different ghrelin-targeted antiserum. To this end, duodenal sections were dual-stained with anti-eGFP and antisera GA2. GA2 was an independently sourced ghrelin antiserum raised to a different epitope sequence and in a different laboratory to GA1. As before, labelling for eGFP and ghrelin was co-localised. Figure 3.13 shows representative images of cells dual-labelled for eGFP and GA2. Therefore, two independently raised anti-ghrelin antisera were observed to stain eGFP positive open-type cells.

3.2.4.4. Co-expression of Ki-67 and ghrelin (GA1).

Anti-Ki-67 and anti-ghrelin co-staining is represented in Figure 3.14. These images display typical nuclear staining of Ki-67 concentrated within the crypt regions. A low number of cells in the villi were also stained for Ki-67. Ghrelin-

positive cells were present in the crypts and villi. A number of dual-labelled cells are located in the crypt areas and one dual-labelled cell is present in the villi.

As the anti-Ki-67 and anti-ghrelin antisera were raised in the same species rigorous controls experiments were performed to control for the respective secondary antiserum binding. Fab fragments were used to block unoccupied IgG sites following application of the first primary and first secondary antibody. The fab control is shown in Figure 3.14-B where background auto-fluorescence of the tissue sample is apparent with an absence of a positive signal. This sufficiently supports the reliability of these data. In this instance, application of anti-Ki-67 antiserum has been omitted from the experiment. The absence of green fluorescence proves IgG blocking by fab fragments was successful. These data indicate that ghrelin-cells in the duodenal villi are not restricted to proliferating cells.

3.2.4.5. Co-expression of eGFP and Obestatin

Presence of the ghrelin gene within eGFP-positive cells was further probed by immunostaining with an anti-obestatin antiserum. Obestatin is an additional peptide product of the preproghrelin gene. Dual-staining was performed using an anti-obestatin antiserum alongside anti-GFP. Dual-labelling was observed, but the frequency was much lower than with either GA1 or GA2 (Figure 3.15). Positive staining was concentrated at the basolateral pole of a number of eGFP cells. There was high non-specific background staining which made it difficult to interpret results. Unfortunately it was difficult to achieve many successful experiments with this antiserum so quantitative data are unavailable.

3.2.5. eGFP-cells in duodenum of transgenic eGFP-CCK mice express several key GI peptides

Duodenal sections showed a high degree of co-localisation of eGFP and staining for each respective GI peptide. The percentage of eGFP cells which were co-

stained with proCCK (89% ±1.3 n=19), PYY (45% ±3 n=9), GIP (37% ±1.9 n=10), proglucagon (14% ±2. n=10) or ghrelin (50% ±1.7 n=25) are represented along with the S.E.M on Graph 3.1. Standard error bars are small and therefore support the reliability of the data. From these numbers it is evident there is a degree of co-localisation within single eGFP-cells. This would imply that EECs have the potential to express peptides derived from more than one peptide precursor.
The expression of multiple GI peptides within duodenal eGFP-cells of eGFP-CCK mice rejects the null hypothesis that CCK is exclusively expressed within these cells.

3.2.6. Immunostaining of sections from CCK^{LacZ} mice using antisera against a selection of GI peptides.

To determine whether the duodenal hormone expression observed in CCK cells was reliant on the expression of CCK, additional experiments were carried out using CCK^{LacZ} mice. Paraffin embedded tissue samples from CCK^{LacZ} mice were used as a model of CCK KO mice. Immunostaining experiments were carried out to see if knockout of the CCK gene influenced the expression of other peptides within the duodenum. Mid-duodenal sections were stained with anti-proCCK antisera and saw no cells to stain positively for CCK (Figure 3.16-A). This validated the specificity of the anti-proCCK antiserum. Sections were further stained for anti-PYY (Figure 3.16-B), anti-ghrelin (GA1) (Figure 3.16-C) and anti-glucagon (Figure 3.16-D) all of which positively stained epithelial cells. Stained cells show characteristics typical to enteroendocrine cells, displaying a flask-shaped morphology and sparse distribution through the epithelial layer of villi. This shows that deletion of endogenous CCK had no effect on the generation of other EECs.

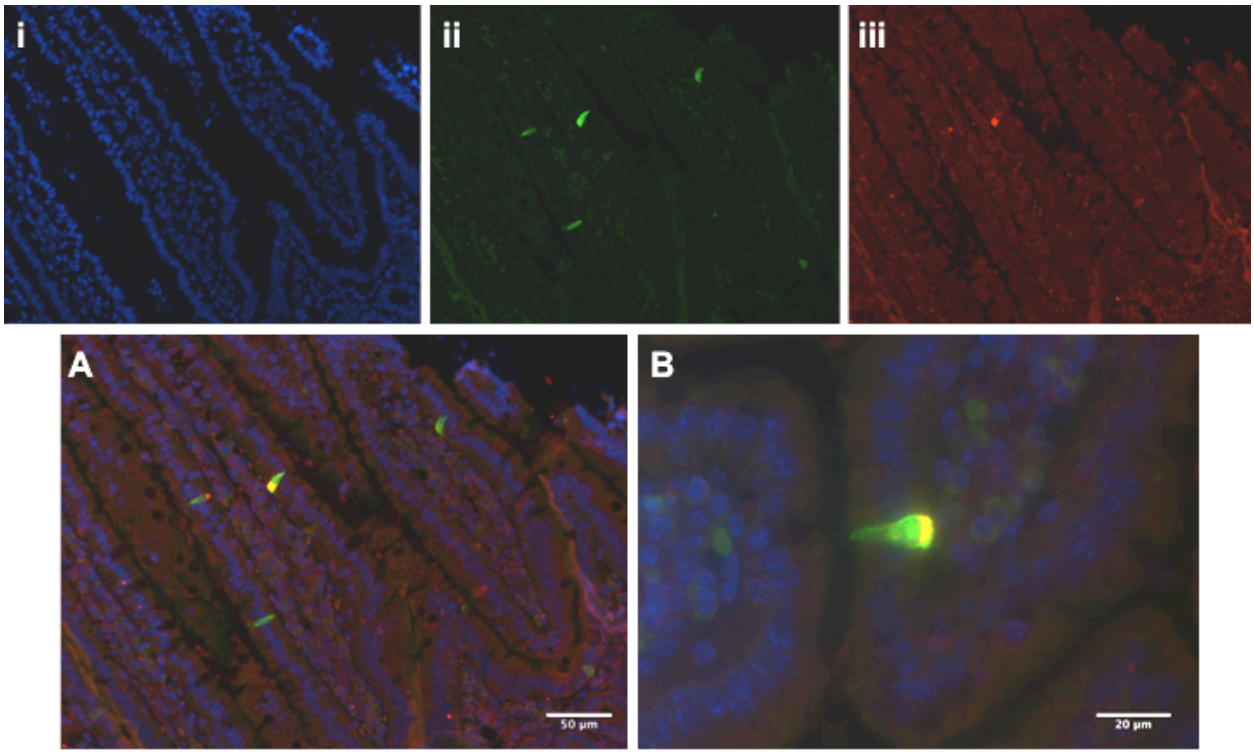


Figure 3.5. Representative images of anti-PYY and anti-eGFP immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Sections (4µm) were immunostained with anti-PYY and anti-eGFP antisera. i) Blue channel depicts nuclei staining with Hoechsst 33452. ii) Green fluorescence represents anti-GFP and iii) red fluorescence represents labelling with anti-PYY antisera. 45% (±3 n=9 slides) eGFP positive cells (n=400) were immunostained for PYY and 32% (±3 n=9 slides) of PYY-positive cells (n=260) were not eGFP positive. Merge images of dual labeled cells A) 20X objective image, scale bar represents 50µm and B) 60X objective image, scale bar represents 20µm. Anti-PYY staining was strongest towards the basolateral membrane of eGFP positive cells. Images were taken on a snapshot widefield microscope.

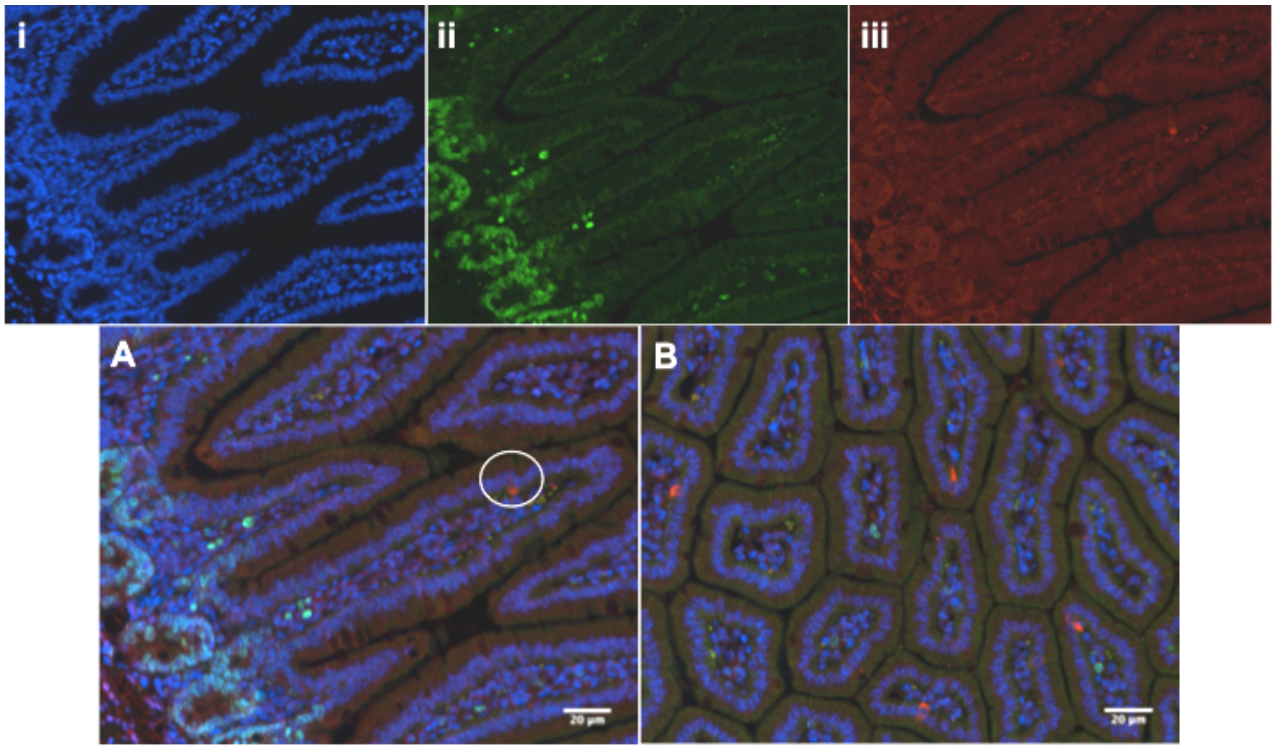


Figure 3. 6. Representative images of anti-PYY and anti-Ki-67 immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Sections (4µm) were immunostained with anti-PYY and anti-Ki-67 antisera.
i) Blue fluorescence shows nuclei staining with Hoechsst 33452. ii) Green fluorescence represents anti-Ki-67 label and iii) red fluorescence represents labelling with anti-PYY antisera. Cells labelled positively by anti-Ki-67 were concentrated within the intestinal crypts and a small number were seen along the villi. Anti-PYY positive cells were equally distributed between the crypt and villi regions. A) Crypt villi orientation, the white circle indicates a cell stained for PYY and B) transverse section of villi. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

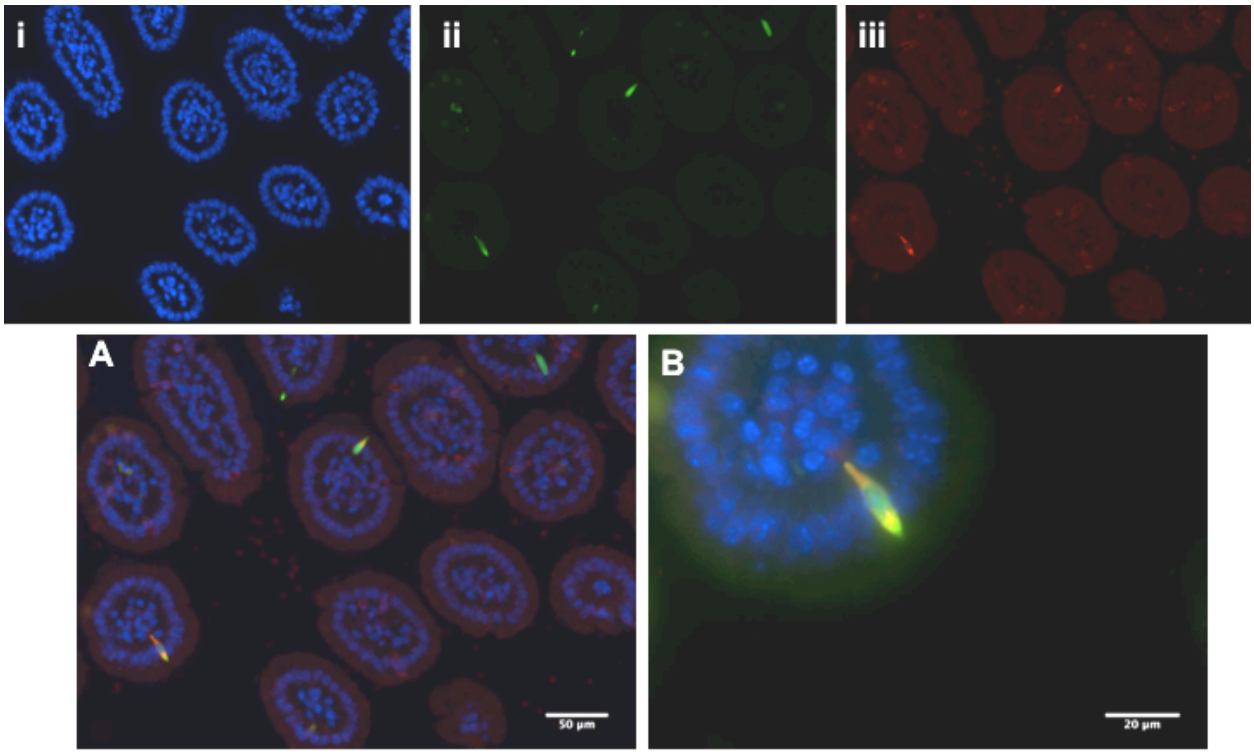


Figure 3.7. Representative images of anti-GIP and anti-eGFP immunostaining of paraffin embedded duodenum from eGFP-CCK mice.

Duodenal sections (4µm) were immunostained with anti-GFP and anti-GIP antisera. i) Blue fluorescence depicts nuclei staining with Hoechsst 33452. ii) Green fluorescence represents anti-eGFP label and iii) red fluorescence represents labelling with anti-GIP. 37% (±1.9 n=10 slides) eGFP positive cells (n=600) were immunostained for GIP and 18% (±3 n=10 slides) GIP positive cells were not eGFP positive.

Merge images of cells dual labelled for anti-GIP and anti-GFP. A) 20X objective image, scale bar represents 50µm and B) 60X objective image, scale bar represents 20µm. GIP staining was strongest towards the basolateral membrane of eGFP

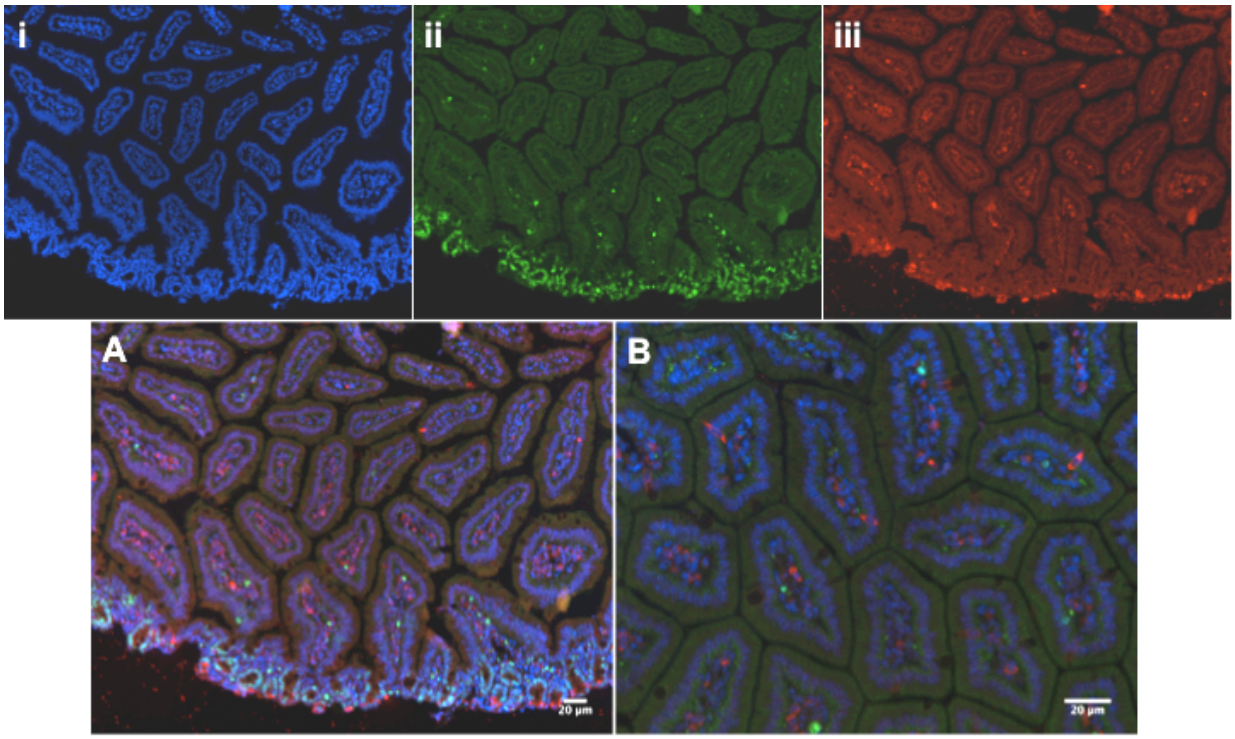


Figure 3.8. Representative images of anti-GIP and anti-Ki-67 immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Sections (4µm) were immunostained with anti-GIP and anti-Ki-67 antisera. i) Blue fluorescence shows nuclei staining with Hoechsst 33452. ii) Green channel represents anti-Ki-67 and red channel represents labelling with anti-GIP antisera. Cells labelled positively by anti-Ki-67 were concentrated within the intestinal crypts and a small number were seen along the villi. Anti-GIP positive cells were equally distributed between the crypt and villi regions.
Merge images displaying A) crypt-villi orientation, 10X objective and B) transverse section of villi, 20X objective. Images were taken on a snapshot widefield microscope. Scale bar represents 20µm.

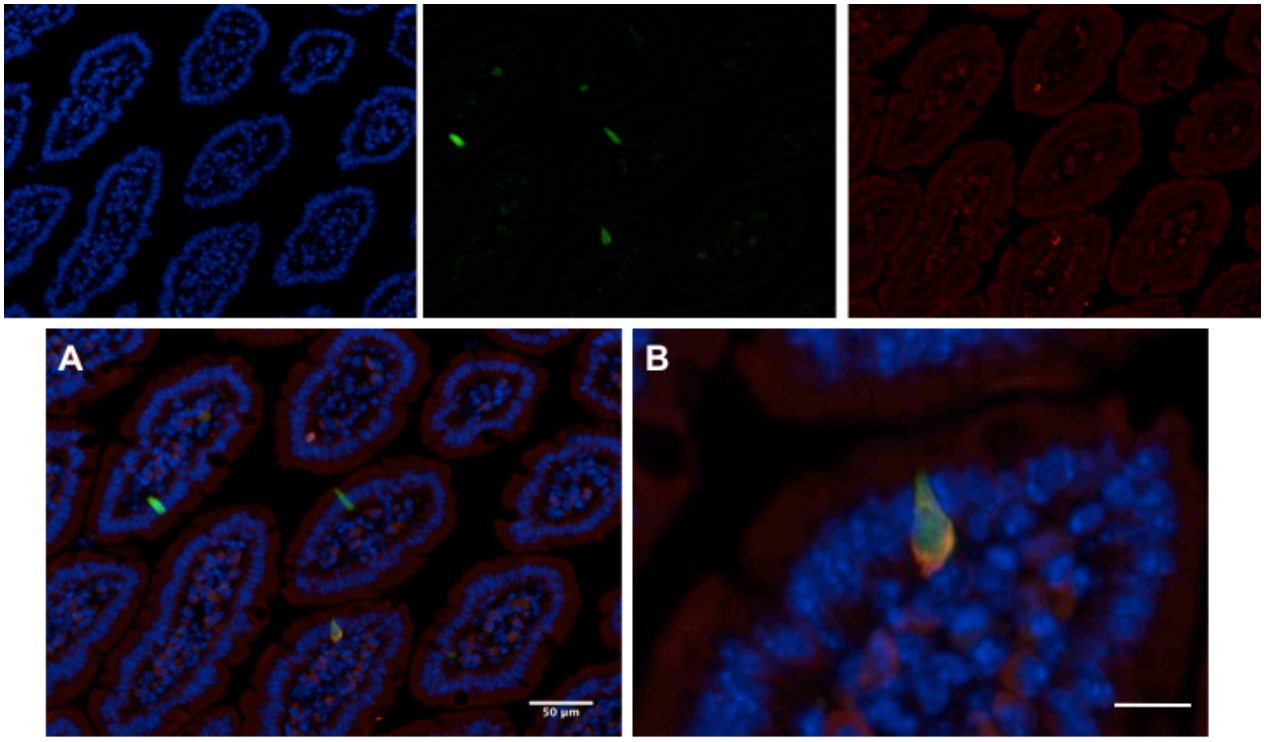


Figure 3. 9. Representative images of anti-proglucagon and anti-eGFP immunostaining of paraffin embedded duodenum from eGFP-CCK mice.

Sections (4µm) were immunostained with anti- proglucagon and anti-GFP. i) Blue fluorescence shows nuclei staining with Hoechsst 33452. ii) Green fluorescence represents anti-eGFP and iii) red fluorescence represents labelling by anti-proglucagon. 14% (±2 n=10 slides) eGFP positive cells (n=624) were immunostained for proglucagon. Antibody staining was strongest towards the basolateral membrane of eGFP cells.
Merge images of dual staining experiments for anti-proglucagon and anti-GFP. A) Merge image displaying one dual-labelled cell, 20X objective (scale bar represents 50µm) and B) High magnification image of a dual-labelled cell, 60X objective (scale bar represents 20µm). Images were taken on a snapshot widefield microscope.

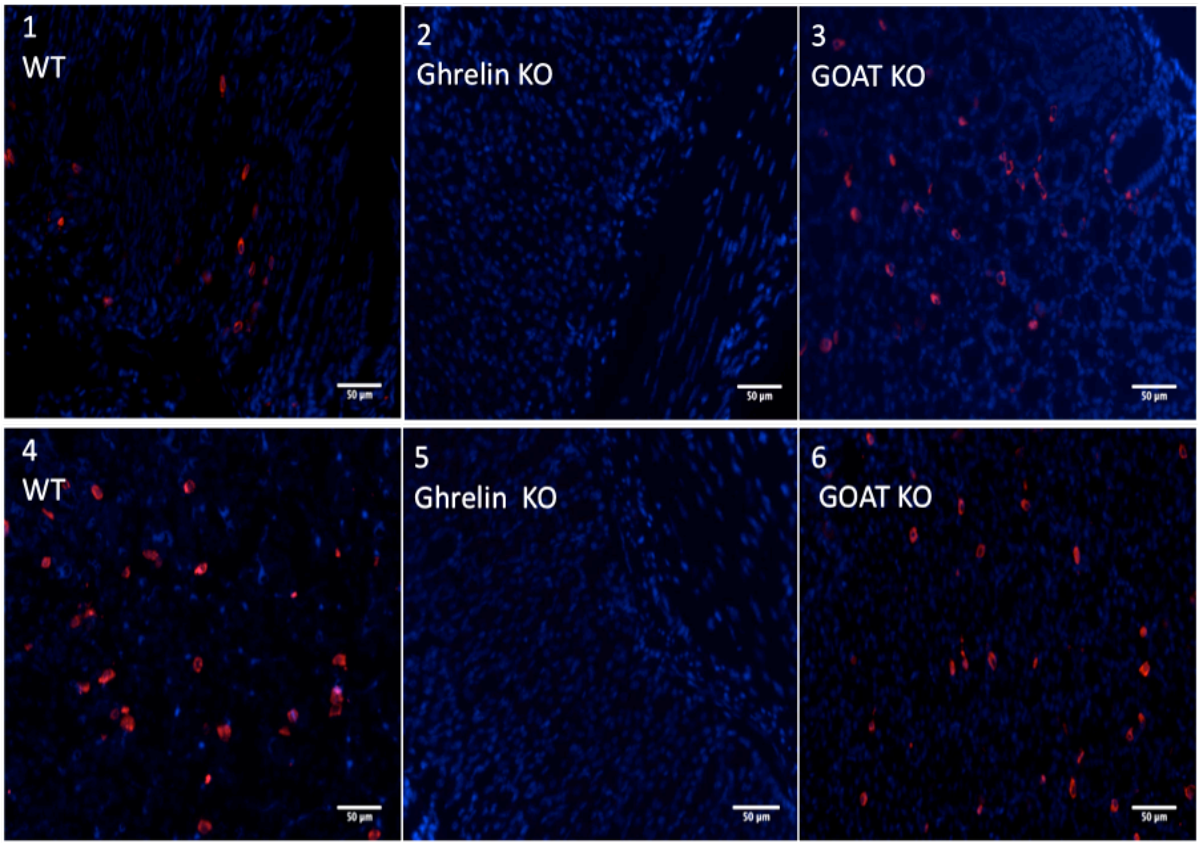


Figure 3. 10. Testing of ghrelin antisera on cryosections prepared from stomachs of wild type, Ghrelin KO and GOAT mice.

Anti-ghrelin antiserum (GA1) (1-3) Anti-ghrelin antiserum (1882) (GA2) (4-6) and were applied to cryosections prepared from Wild type (1,4), Ghrelin KO (2,5), and GOAT KO mice (3,6). Blue fluorescence represents nuclear staining with Hoechsst 33452 and red fluorescence represents anti-ghrelin labelling. GA1 and GA2 antisera both stained a large number of immunoreactive cells in wild type and GOAT KO stomachs. Absence of antisera labelling was seen in ghrelin KO tissue for both antisera. Images were taken on a snapshot widefield microscope. Scale bar represents 50µm.

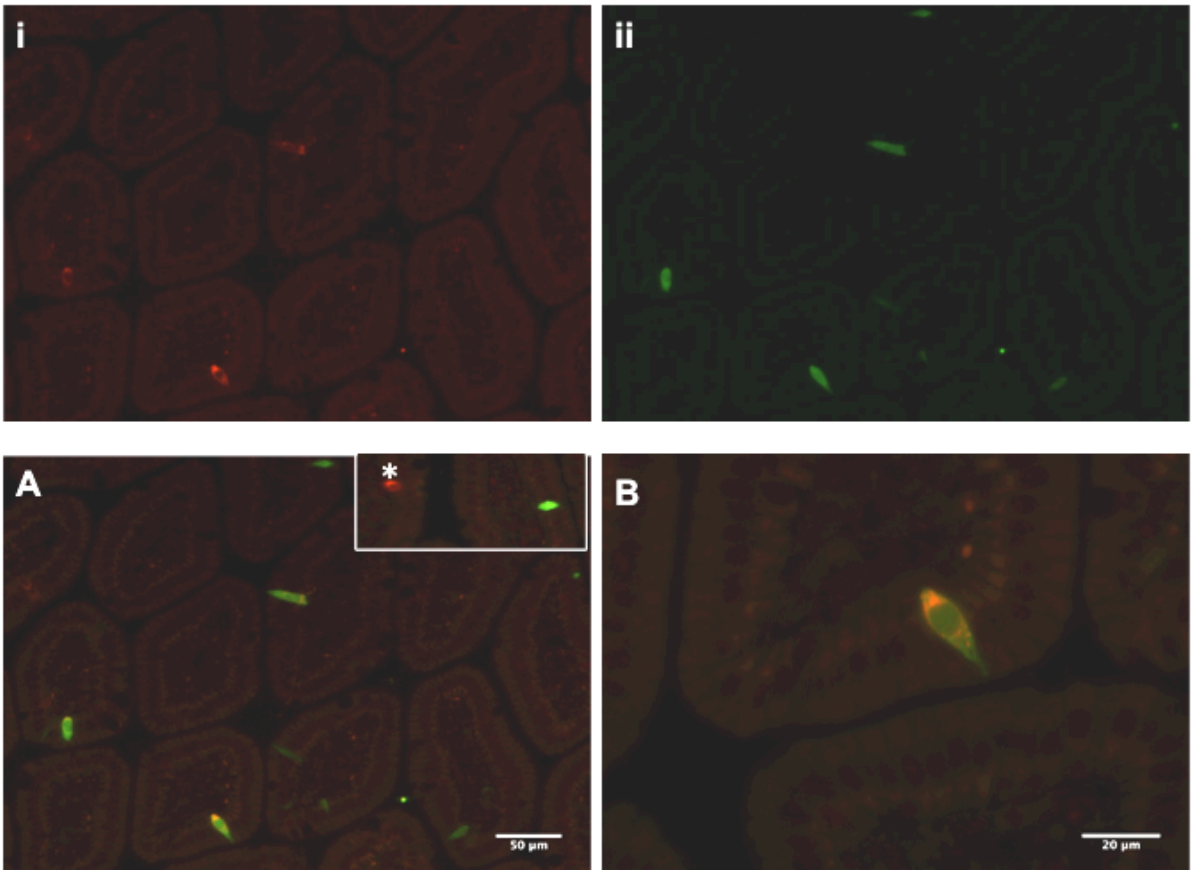


Figure 3.11. Representative images of anti-ghrelin (GA1) and anti-eGFP immunostaining of paraffin embedded duodenum from eGFP-CCK mice.

Sections (4µm) were immunostained with an anti- ghrelin and anti-eGFP antisera. i) Green fluorescence represents anti-eGFP and ii) red fluorescence represents labelling with anti-ghrelin antiserum (GA1). 50% (±1.7 n=3353) eGFP positive cells were immunostained for ghrelin.
Merge images of cells dual-labelled for ghrelin and eGFP; A) 20X objective image with inset: asterisk depicts staining of closed-type ghrelin cell, scale bar represents 50µm and B) 60X objective, scale bar represents 20µm. Anti-ghrelin staining was strongest towards the basolateral membrane of eGFP-cells. Images were taken on a snapshot widefield microscope.

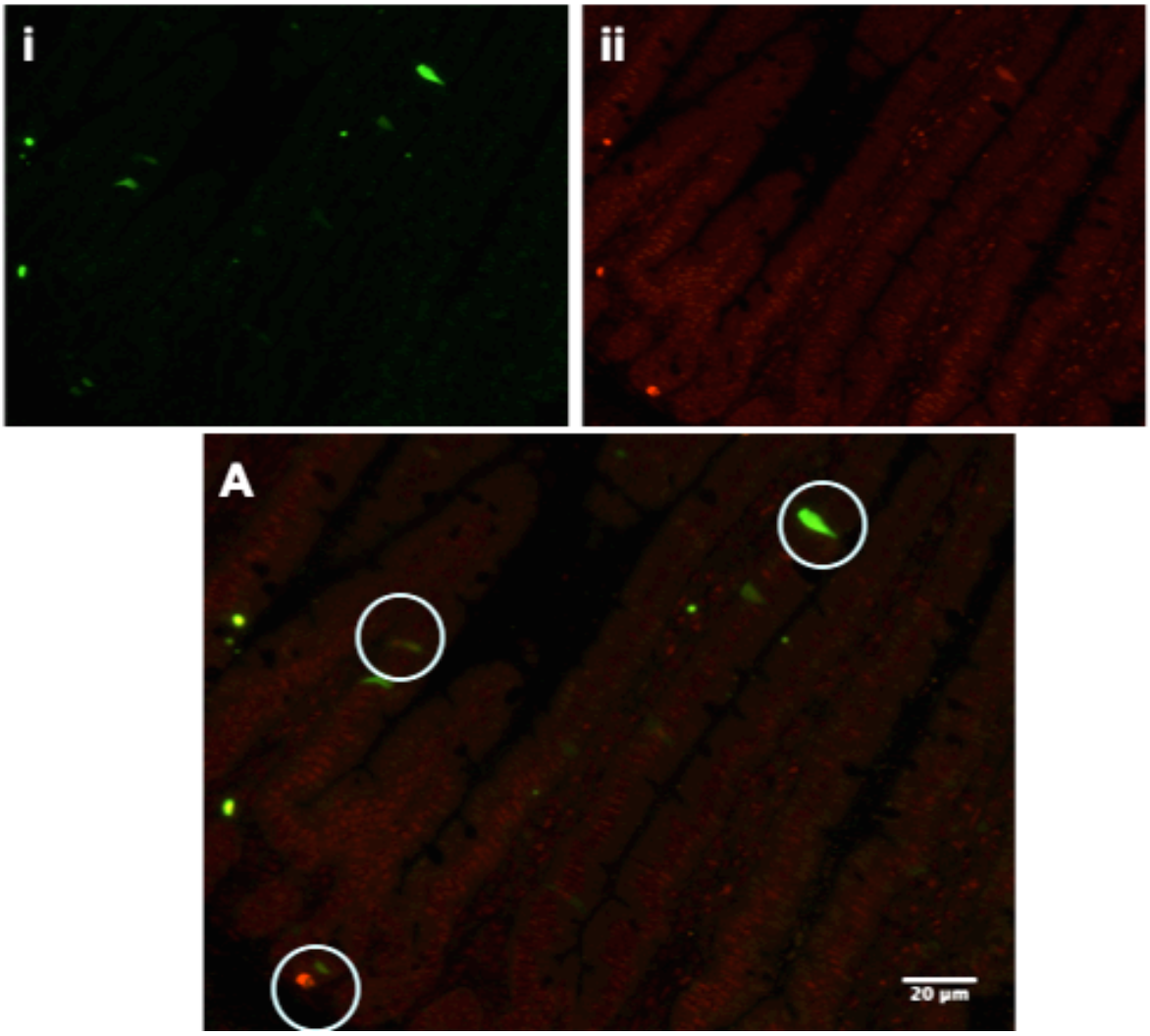


Figure 3.12. Cells dual stained with anti-ghrelin (GA1) and anti-eGFP antisera were located in the crypt and along the villi in duodenum of eGFP-CCK mice.

Representative image of duodenal sections dual labelled for anti-GFP and anti-ghrelin antisera. Dual labelled cells were seen located in crypts and along the villi. i) Green channel represents eGFP label and ii) Red channel depicts anti-ghrelin label. A) Merge image from red and green channels. Depicts dual-labelled cells in the crypt and the villus. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

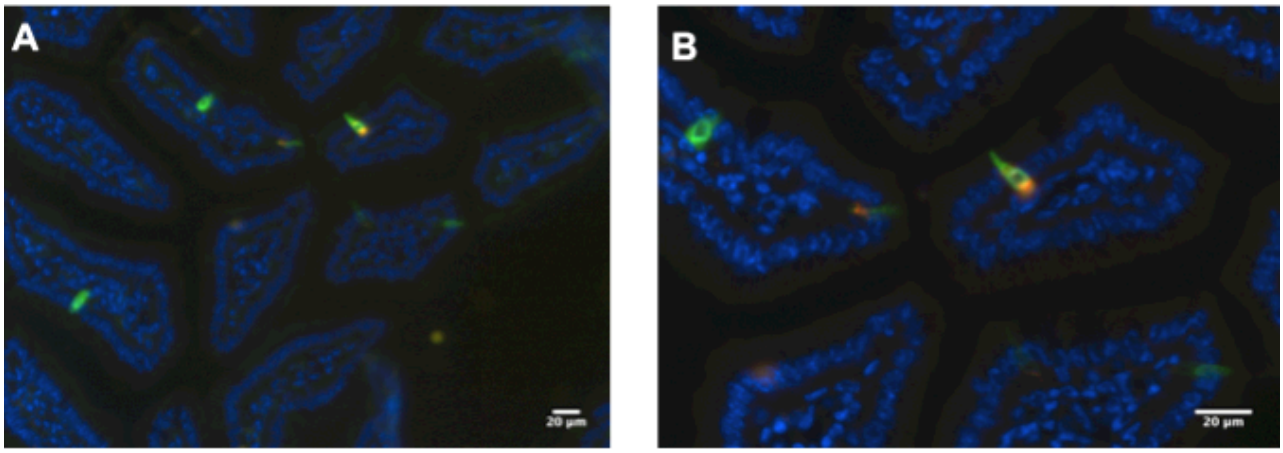


Figure 3.13. Representative images of anti-ghrelin (GA2) and anti-eGFP immunostaining of paraffin embedded duodenum from eGFP-CCK mice.

Re-affirmation of data confirms anti-ghrelin and anti-eGFP staining of cells in duodenum through use of anti-ghrelin antisera - GA2. Duodenal sections (4µm) were immunostained with anti-ghrelin (GA2) and anti-eGFP antisera. Green fluorescence represents anti-eGFP and red fluorescence represents labelling with anti-ghrelin. Merge images of cells dual-labelled for ghrelin and eGFP A) 20X objective B) 40X objective. Anti-ghrelin staining was strongest towards the basolateral membrane of eGFP-cells. Images were taken on a snapshot widefield microscope. Scale bar represents 20µm.

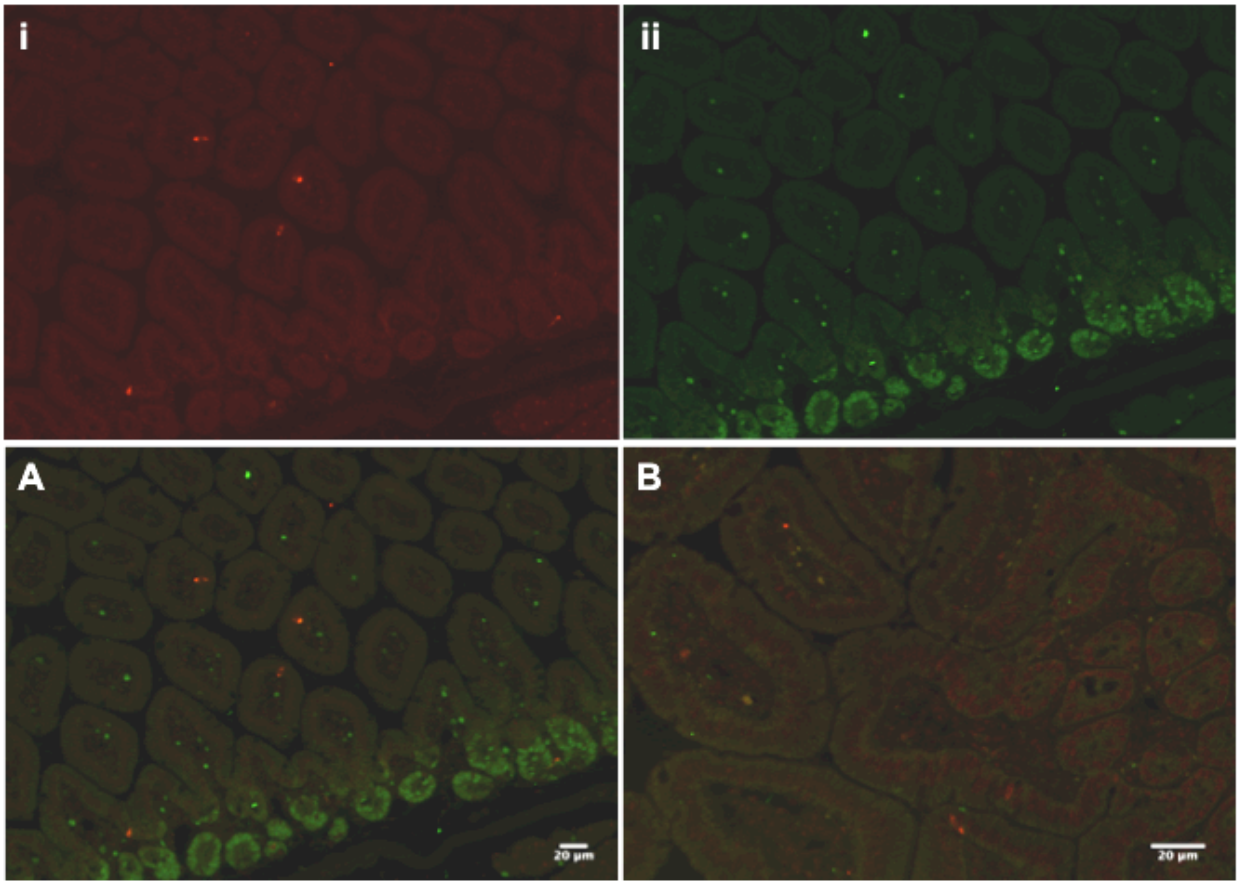


Figure 3.14. Representative images of anti-ghrelin and anti-Ki-67 immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Duodenal sections (4µm) were immunostained with anti-ghrelin (GA1) and anti-Ki-67 antisera. i) Green fluorescence represents anti-Ki-67 and ii) red fluorescence represents labelling with anti-ghrelin antisera. Cells labelled positively by anti-Ki-67 were concentrated within the intestinal crypts and a small number were seen along the villi. Anti-ghrelin positive cells were equally distributed between the crypt and villi regions. Merge images of A) Crypt villi orientation of positively stained cells, 20X objective B) Fab fragment control image demonstrating an absence of green fluorescence due to omission of the anti-Ki-67 antiserum, 40X objective. Images were taken on a snapshot widefield microscope. Scale bar represents 20µm.

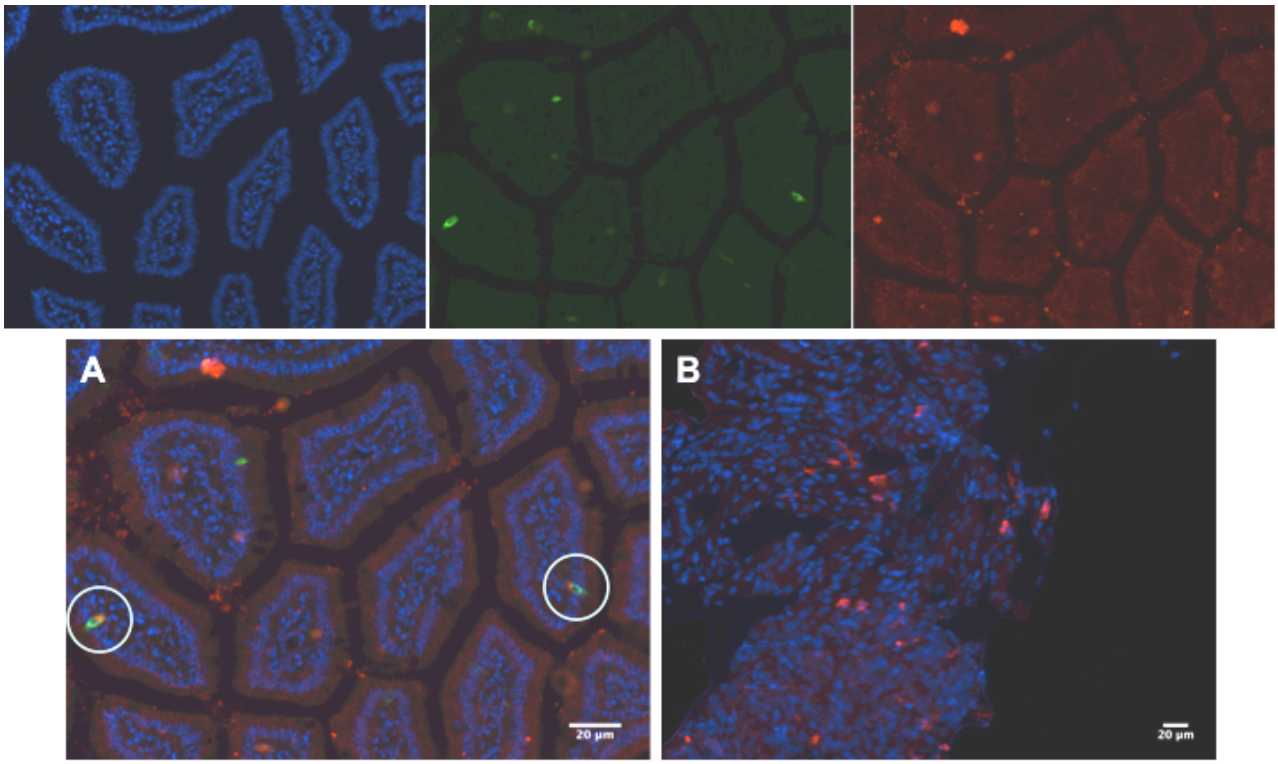
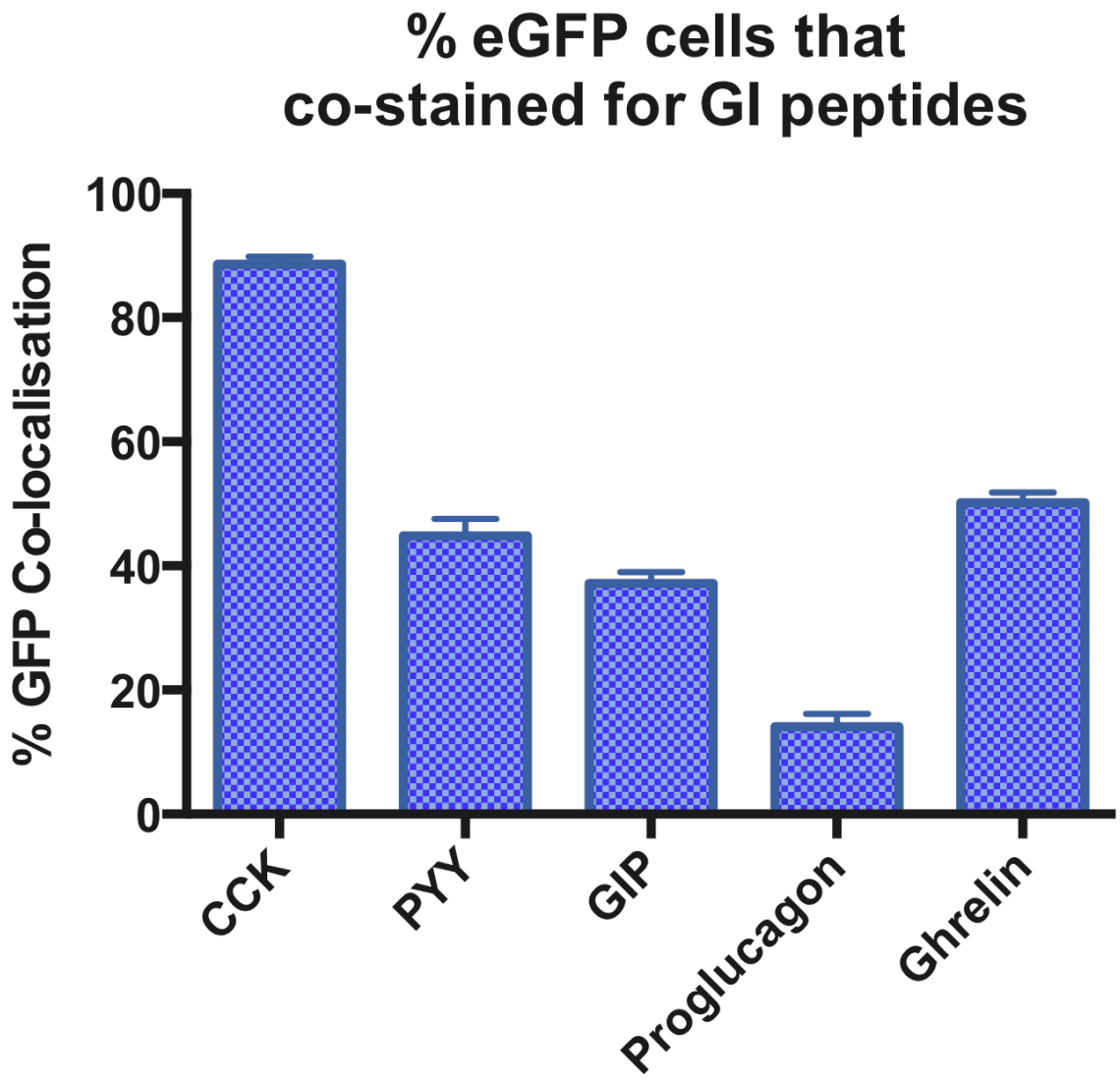


Figure 3.15. Anti-obestatin and anti-eGFP immunostaining of paraffin embedded tissue from mid-duodenum and stomach of eGFP-CCK mice.

Sections (4µm) were immunostained with anti-obestatin and anti-eGFP. i) Blue fluorescence depicts nuclei staining by Hoechsst 33452. ii) Green channel represents anti-eGFP label and iii) red channel represents labelling by anti-obestatin antiserum. A) Merge image of obestatin eGFP staining of duodenal tissue. A number of eGFP positive cells were positive for obestatin. Antibody staining was strongest towards the basolateral membrane of eGFP cells B) Obestatin staining in stomach tissue (positive control), many cells were positively labelled by anti-obestatin serum. Images were taken on a snapshot widefield microscope. Scale bar represents 20µm.



Graph 3.1. The percentage of eGFP cells labelled positively for CCK, PYY, GIP, Proglucagon and Ghrelin.

Immunohistochemistry analysis mid-duodenal sections of 3 transgenic mice showed eGFP cells to contain CCK 89% (±1.25 n=19 slides), PYY 45% (±2.75 n=9 slides), GIP 37% (±1.85 n=10 slides), proglucagon 14% (±2.14 n=10 slides) and ghrelin 50% (±1.71 n=25 slides). Sections were taken from 3 transgenic mice
A cell was considered to be positively labelled when fluorescence intensity exceeded a pre-defined threshold.

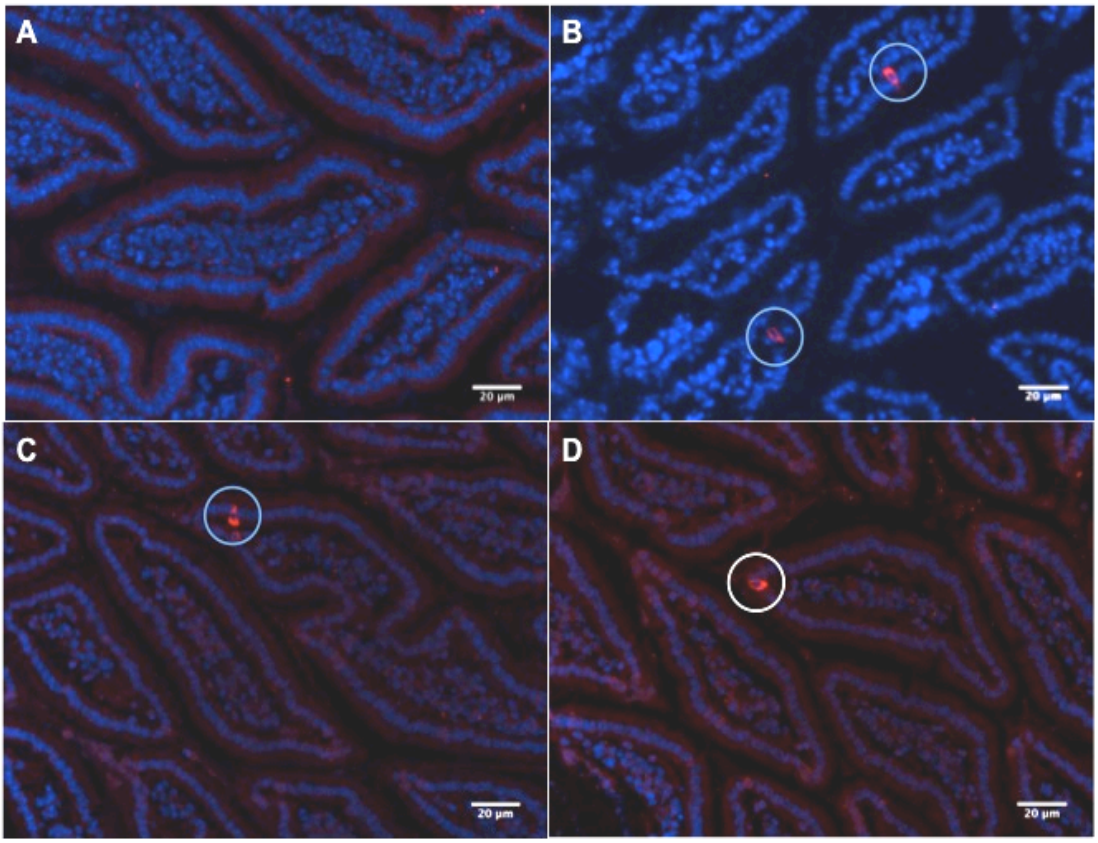


Figure 3.16. Mid-duodenum tissue sections of CCK^{LacZ} mice were immunostained with antisera specific for an array of GI peptides.

Representative images of duodenal sections (4µm) from CCK^{LacZ} mice immunostained against a selection of antisera. Red fluorescence represents labelling with antisera against each peptide. Blue fluorescence depicts nuclear staining by Hoechsst 33452. A) Application of anti-proCCK antisera demonstrates an absence of staining. Whereas B) anti-ghrelin GA1 antisera C) anti-PYY antisera and D) anti-glucagon antisera depict cells that are positively labelled by respective antisera. Images were taken on a snapshot widefield microscope, 20X objective. Scale bars represent 20µm.

3.3. Do enteroendocrine cells in mouse duodenum co-express CCK alongside other peptides?

Double-staining of duodenal sections for CCK and other GI peptides.

To further confirm the existence of CCK expressing cells that also express other gut hormones, double staining experiments were performed using established antisera against key gut hormones. Double staining was performed using CCK alongside GIP, PYY or ghrelin. In all experiments green fluorescence represents labelling by anti-proCCK and the red fluorescence represents labelling by antisera targeted to GIP, PYY or ghrelin. Processed sections retained no endogenous eGFP fluorescence after antigen retrieval and therefore eGFP fluorescence did not influence results (See Appendix A.1).
Experiments were performed using duodenal sections from 3 eGFP-CCK transgenic mice.

3.3.1. Co-expression of CCK and PYY in cells of mouse duodenum.

3.3.1.1. Dual-labelling anti-proCCK and anti-PYY antisera

Paraffin embedded sections from mid-duodenum were dual-stained by anti-proCCK and anti-PYY antisera (Figure 3.17). Cells that were co-stained for both peptides were seen in the crypts and along the length of the villi. Cells that labelled for CCK or PYY exclusively were also present. This demonstrates that CCK and PYY can be expressed in the same, or separate cells.

3.3.1.2. Analysis of anti-CCK and anti-PYY labelling within positively stained cells

High magnification micrograph images were analysed to ascertain the expression pattern of antisera staining within dual-labelled cells (Figure 3.18). A spectrum of

staining intensities was observed corresponding to red and green staining patterns. Representative micrograph images of 3D projection of maximal intensity of six individual cells are shown in Figure 3.18 (A-F).

In all images (A-F) green and red fluorescence can be seen to localise to very small structures within the cell, indicative of vesicles. Peptide localisation is observed around the nuclear area (the area inside each cell where staining was absent). A spectrum of fluorescence staining can be seen. Image A shows the highest quantity of red and green co-localisation that was calculated at 25.9% using the co-localisation package of the LAS-AF software, (Pearson's correlation value= 0.75). This cell displays a staining pattern that is distributed around the nucleus with a higher concentration toward the apical side of the nucleus. Image B shows a higher concentration of PYY red staining to the basolateral edge of the nucleus whilst CCK is distributed more evenly around the area. Images C and D have a much higher expression of CCK yet there are some staining of individual vesicles with red fluorescence that characterises PYY staining. Some cells stained for only one peptide; either anti-proCCK (E) or anti-PYY (F). This demonstrates that the pattern of co-localisation is variable. The data indicate that within dual-labelled cells, a small proportion of intracellular labelling is co-localised to the same vesicles. The majority of peptide labelling appears to be located within distinctly separate vesicular structures.

3.3.2. Co-expression of CCK and GIP in mouse duodenum

3.3.2.1. Dual-labelling using anti-proCCK and anti-GIP antisera

Mid-duodenal sections were dual-stained using anti-proCCK and anti-GIP antisera (Figure 3.19). Cells of duodenum were dual-stained for CCK and GIP that had shape and frequency characteristic of EECs. Dual-labelled cells were seen in the crypt and along the length of the villi (Figure 3.19). Some cells stained for CCK or GIP alone, which suggests co-expression in some cells and mutually exclusive expression in others.

3.3.2.2. Analysis of anti-CCK and anti-GIP labelling within positively stained cells

High magnification micrograph images of cells that were dual-labelled for CCK and GIP were analysed to visualise the distribution of peptide expression within cells (Figure 3.20). A spectrum of corresponding red (GIP) and green (CCK) staining patterns were seen in the cells analysed. Co-localisation analysis was performed to demonstrate variability in the expression of these peptides within dual-labelled cells. Five representative micrograph images of 3D projection of maximal intensity are shown in Figure 3.20 (A-E).

In all images (A-E) staining within cells can be seen to localise to very small structures within the cell indicative of vesicles, and achieved a similar pattern of staining as for CCK/PYY. A spectrum of fluorescence staining can be seen. Image A showed the highest rate of red and green co-localisation where 24.23% of pixels were measured to have co-localised fluorescence (Pearson's correlation = 0.8). This was clearly localised at the basolateral pole of the cell, with fainter staining around the side of the nucleus. Images B and C show peptides to be in a larger proportion of the cell although this may be due to the orientation of the slices compared with image A. Image C shows very intense staining throughout the cell, so much so the flask-shaped morphology of the EEC is apparent. Image D shows a cell that contains mostly CCK yet some red fluorescence (GIP) can be seen in defined vesicles, with a co-localisation value of 4.9% (Pearson's correlation = 0.5). Image E depicts a green (CCK) and a red (GIP) cell, the red cell was analysed and the pixel co-localisation value was 0.32%.

3.3.3. Co-expression of CCK and ghrelin in cells of mouse duodenum.

3.3.3.1. Dual-labelling using anti-proCCK and anti-ghrelin antisera

Mid-duodenal sections were dual-stained using anti-proCCK and anti-ghrelin antisera (Figure 3.21). Some cells were dual-stained for both CCK and ghrelin.

Dual-labelled cells were seen in the crypt and along the length of the villi. Some cells were seen that stained for CCK or ghrelin alone which demonstrates that the two peptides were also expressed in separate cells.

As anti-proCCK and anti-ghrelin antisera were both raised in the same species, fab fragments were employed to control respective secondary antibody binding.

As explained previously, fab fragments were used to block exposed IgG antigenic sites after application of the first primary and secondary antisera combinations. This ensured that labelling by the second primary antiserum is specific. Figure 3.22 depicts the fab control image for this experiment where anti-proCCK has been omitted from the experiment. This should ensure that the second secondary antiserum applied (anti-rabbit Alexafluor488) has no epitope with which to bind and should therefore provide no signal. In figure 3.22-A and 3.22-B a cell that displays strong labelling for anti-ghrelin, depicted by red fluorescence, possesses no green fluorescence. This indicates that all IgG sites were blocked following application of the first secondary antibody and thus validates these data.

3.3.3.2. Analysis of anti-CCK and anti-ghrelin labelling within positively stained cells

Six representative micrograph images of 3D projection of maximal intensity are represented in Figure 3.23. In all images (A-F) staining within cells can be seen to localise to very small structures within the cell, representative of vesicles. Staining patterns within cells is similar to that previously described and again a spectrum of fluorescence staining can be seen. Image A shows two cells, each dual-stained for ghrelin and CCK, displaying the highest rates of co-localisation at 20.6% (L=left) (Pearson's correlation = 0.75) and 19.9% (R=right) (Pearson's correlation = 0.8). This staining is ubiquitous throughout the cells, with the Left-cell appearing to display more red fluorescence than green. However, as this is a 3D projection of maximal intensity the green fluorescence may be resting at a different plane to the red and at a lower intensity. The right-cell shows a high degree of co-localisation with red fluorescence being more concentrated towards the basolateral pole. Image B shows an interesting circular pattern of red and

green staining, possibly explained by the orientation of the cells within this tissue section. This image depicts two cells in close proximity that have been sliced transversely through the nucleus, thus producing a ring-like pattern. Image C shows very intense staining throughout the cell, although by eye it looks to be highly co-localised red/green, analysis of the z-stack demonstrates that only 12.8% (Pearson's correlation = 0.7) pixel fluorescence was calculated to be co-localised. This suggests the fluorescence for each channel may be more intense on different planes of the z-stack. Image D shows a cell with more intense staining achieved by anti-proCCK with only a small proportion of red fluorescence that represent ghrelin that can be seen in defined vesicles with only 11% co-localisation rate recorded (Pearson correlation = 0.5). Image E shows two cells with close proximity, one of which is dual labelled and another, which is stained for proCCK (green) alone. The green cell was analysed and had a co-localisation rate of 0.01% (Pearson's correlation = 0.01). Image F shows a cell that has labelled for ghrelin alone.

3.3.4. Co-expression of CCK and proglucagon in cells of mouse duodenum.

3.3.4.1 Dual-labelling anti-proCCK and anti-proglucagon antisera

Mid-duodenal sections were dual-stained using anti-proCCK and anti-proglucagon antisera. Some cells of mid-duodenum were dual-stained for both proCCK and proglucagon. Dual-labelled cells were seen in the crypt and along the length of the villi. As the two antisera were raised in the same species fab fragments were used to block antigenic site to ensure specific secondary antibody binding. Fab controls were included to demonstrate that remaining antigenic sites had been successfully blocked. For this experiment it was clear that this blocking step had not been successful. Therefore these samples were not used for further for analysis. Data not shown.

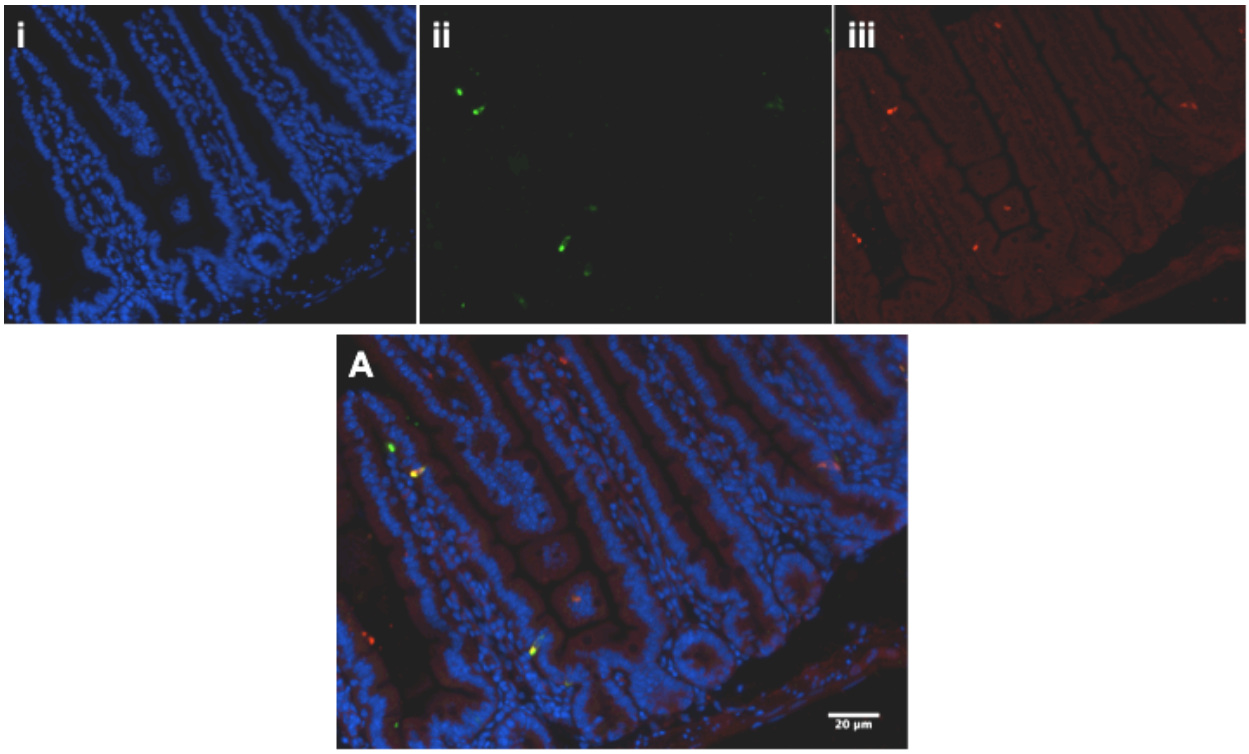


Figure 3.17. Representative images of anti-PYY and anti-proCCK immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Sections (4µm) were dual-labelled using anti-proCCK and anti-PYY antisera. i) Blue fluorescence depicts nuclear staining by Hoechsst 33452. ii) Green fluorescence represents anti-proCCK and iii) Red fluorescence represents labelling with anti-PYY antisera. A) Merge image depicting co-stained cells along the length of the villi. Some cells displayed green or red fluorescence alone. Positive labelling by antisera was restricted to epithelial cells with strongest staining towards the basal membrane. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

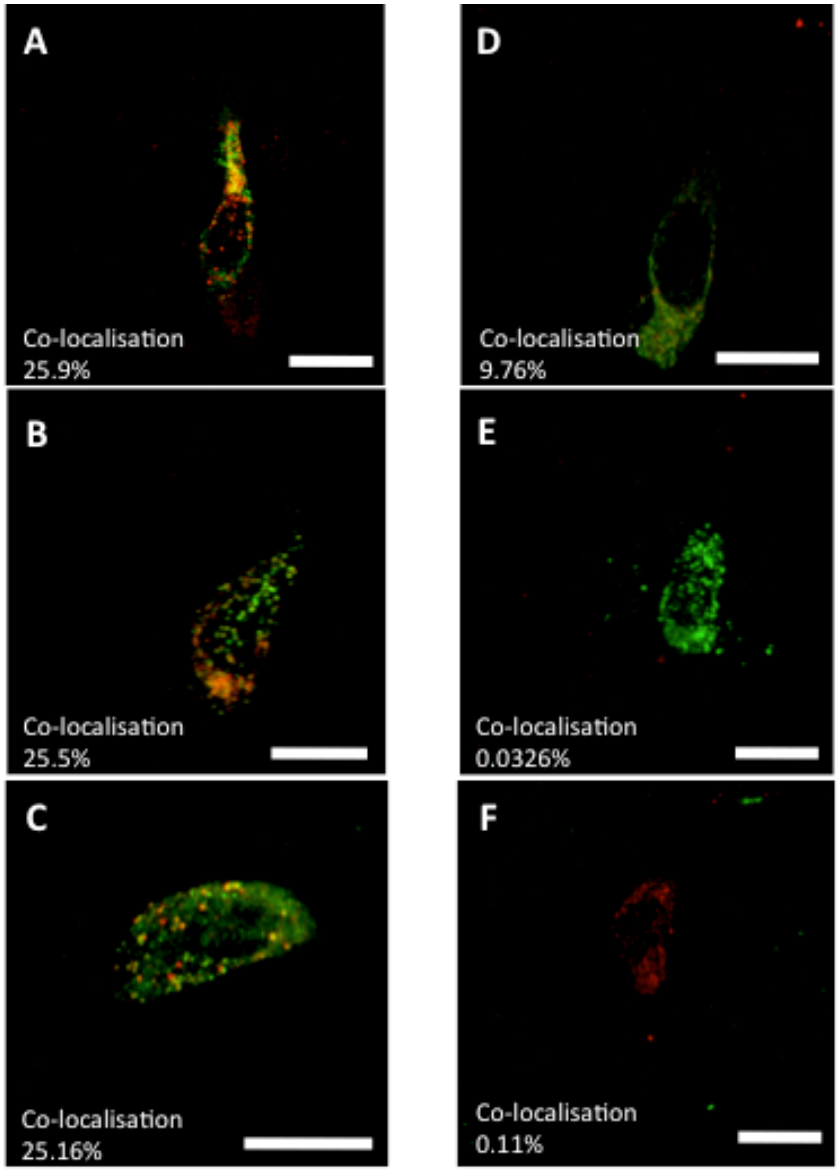


Figure 3.18. Images of anti-PYY and anti-proCCK immunostained cells of paraffin embedded mid-duodenum.

Sections (4μm) were immunostained with anti-proCCK and anti-PYY antisera. Positively labelled cells were analysed at high magnification to visualise staining localisation within cells. Green fluorescence represents anti-proCCK and red fluorescence represents labelling with anti-PYY antisera. Images (A-F) represent 3D maximal projection image of positively labelled cells. A spectrum of staining by anti-proCCK anti anti-PYY was seen. Images A-D show cells that have dual-labelled for PYY and CCK. E) Shows a cell which has been stained by anti-proCCK only F) Shows a cell which has been stained by anti-PYY only. The rate of co-localisation was calculated according to pixel intensity after subtraction of background values. Fluorescence was considered co-localised if pixel intensity of green and red was ≥50%. Images were captured on a Leica SP5 inverted microscope, 63X objective with optical zoom, and analysed using Leica LAS-AF software. Scale bar represents 10μm.

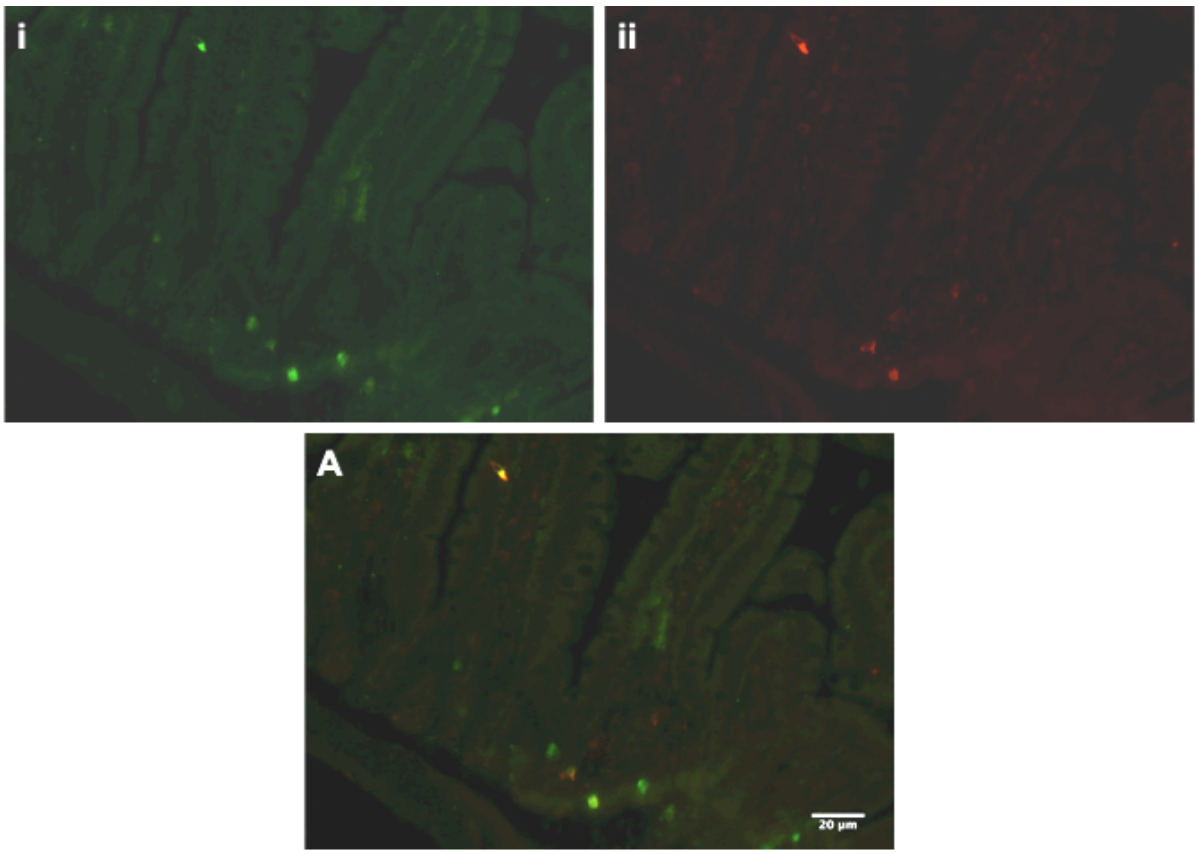


Figure 3.19. Representative images of anti-GIP and anti-proCCK immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Sections (4µm) were dual-labelled using anti-proCCK and anti-GIP antisera. i) Green fluorescence represents anti-proCCK and ii) red fluorescence represents labelling with anti-GIP antisera. A) Merge image depicting dual-labelled cells expressed in the crypts and along the length of the villi. Some cells displayed green or red fluorescence alone. Positive labelling by antisera was restricted to epithelial cells with strongest staining towards the basal membrane. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

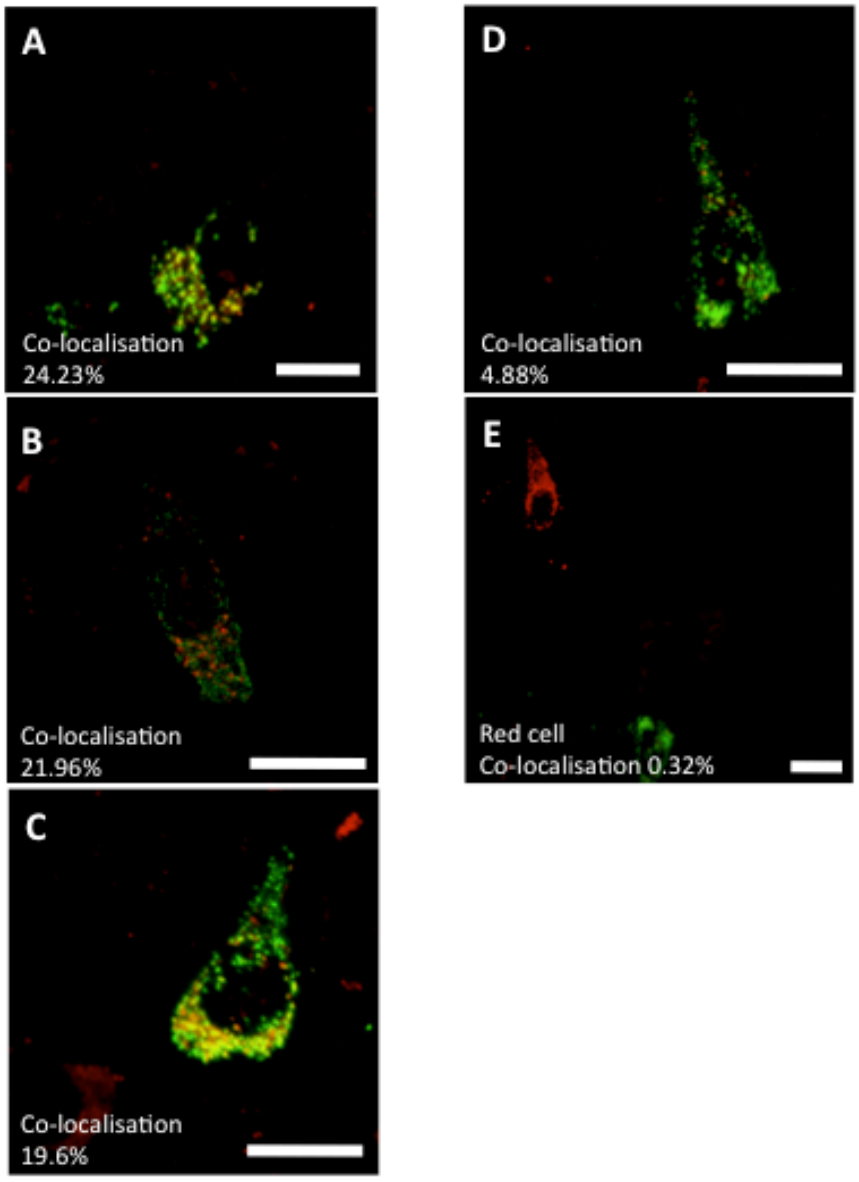


Figure 3. 20. Images of anti-GIP and anti-proCCK immunostained cells of paraffin embedded mid-duodenum.

Sections (4µm) were immunostained with anti-proCCK and anti-GIP antisera. Positively labelled cells were analysed at high magnification to visualise the localisation of staining within cells. Green fluorescence represents anti-proCCK and red fluorescence represents labelling with anti-GIP antisera. Images (A-E) represent 3D maximal projection image of positively labelled cells. A spectrum of staining by anti-proCCK anti anti-GIP was seen. Images A-C show cells that have dual-labelled for GIP and CCK. D) Shows a cell that has been stained by anti-proCCK only E) Depicts a cell that has been stained by anti-GIP exclusively. Co-localisation rate was calculated according to pixel intensity after subtraction of background values. Fluorescence was considered co-localised if pixel intensity of green and red was ≥50%. Images were captured on a Leica SP5 inverted microscope, 63X objective with confocal zoom and analysed using Leica LAS-AF software. Scale bar represents 10µm

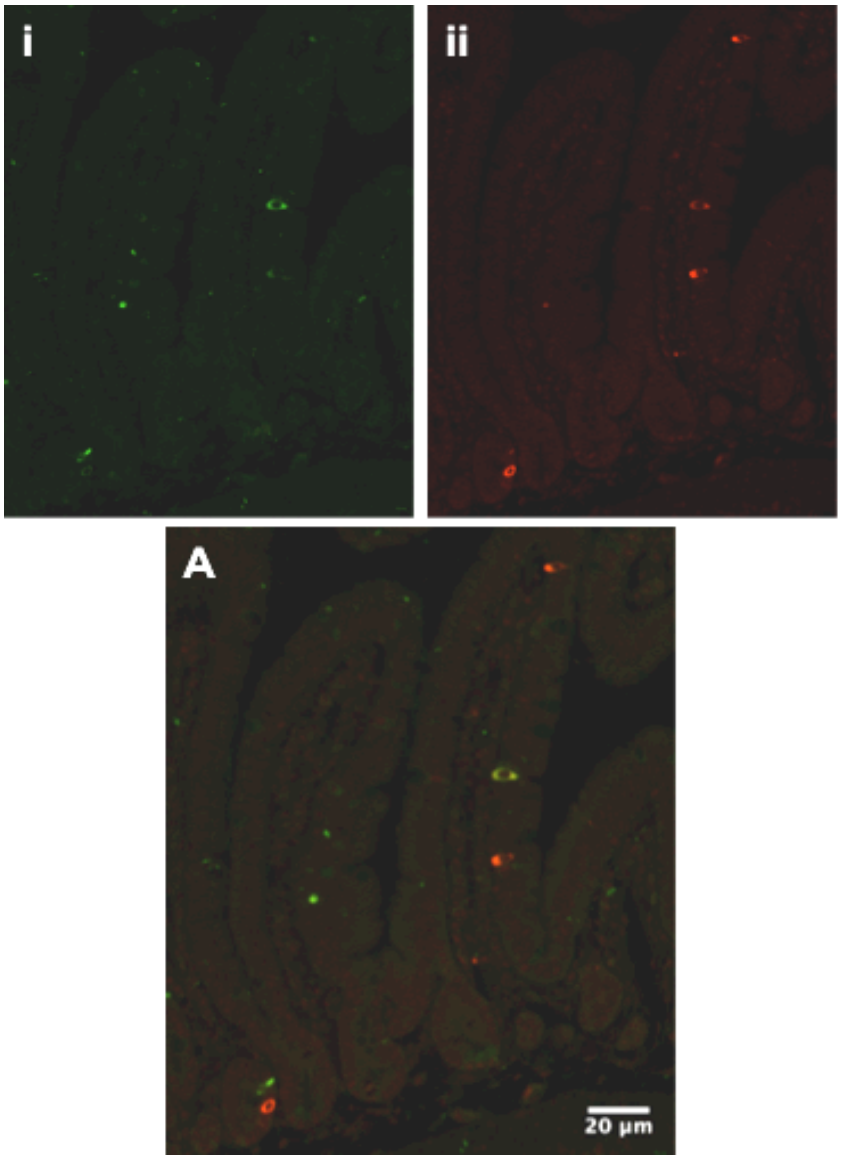


Figure 3.21. Representative images of anti-ghrelin and anti-proCCK immunostaining of paraffin embedded mid-duodenum of eGFP-CCK mice.

Sections (4µm) were dual-labelled using anti-proCCK and anti-ghrelin antisera. i) Green fluorescence represents anti-proCCK and ii) red fluorescence represents labelling with anti-ghrelin antisera. A) Merge image with co-stained cells displaying red and green fluorescence were seen along the length of the villi. Some cells displayed green or red fluorescence alone. Positive labelling by antisera was restricted to epithelial cells with strongest staining towards the basal membrane. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

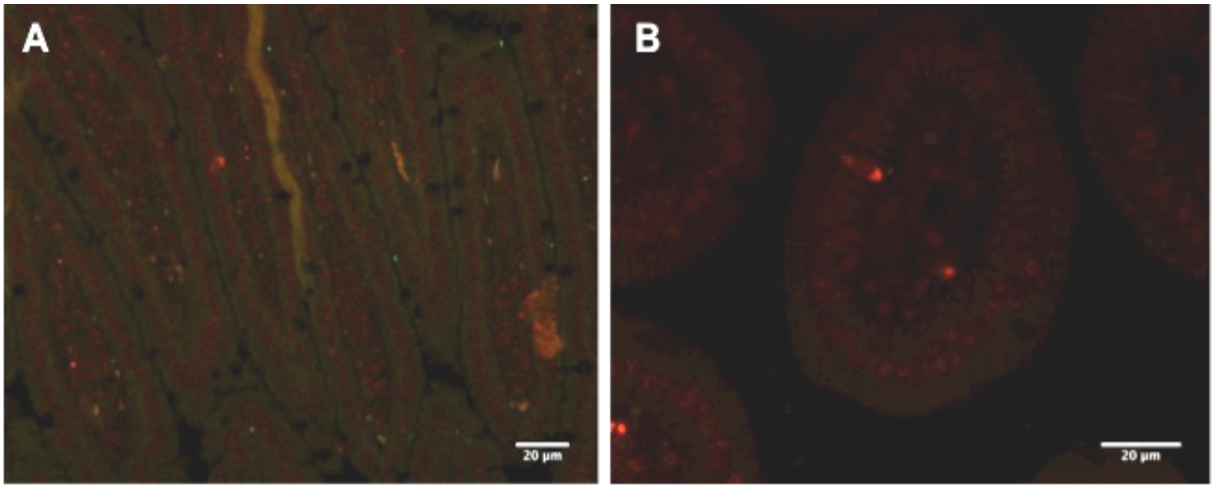


Figure 3.22. Fab Fragment control images for immunostaining experiment using two anti-rabbit antisera; anti-proCCK and anti-ghrelin.

Sections (4µm) were dual-labelled using anti-proCCK and anti-ghrelin antisera. Green fluorescence represents anti-proCCK and red fluorescence represents labelling with anti-GIP antisera. Fab fragment control shows sections where anti-proCCK has been omitted. Images clearly show absence of green fluorescence. confirming blocking of proCCK antibody epitopes. A) 20X objective B) 60X objective. Images were taken on a snapshot widefield microscope. Scale bar represents 20µm.

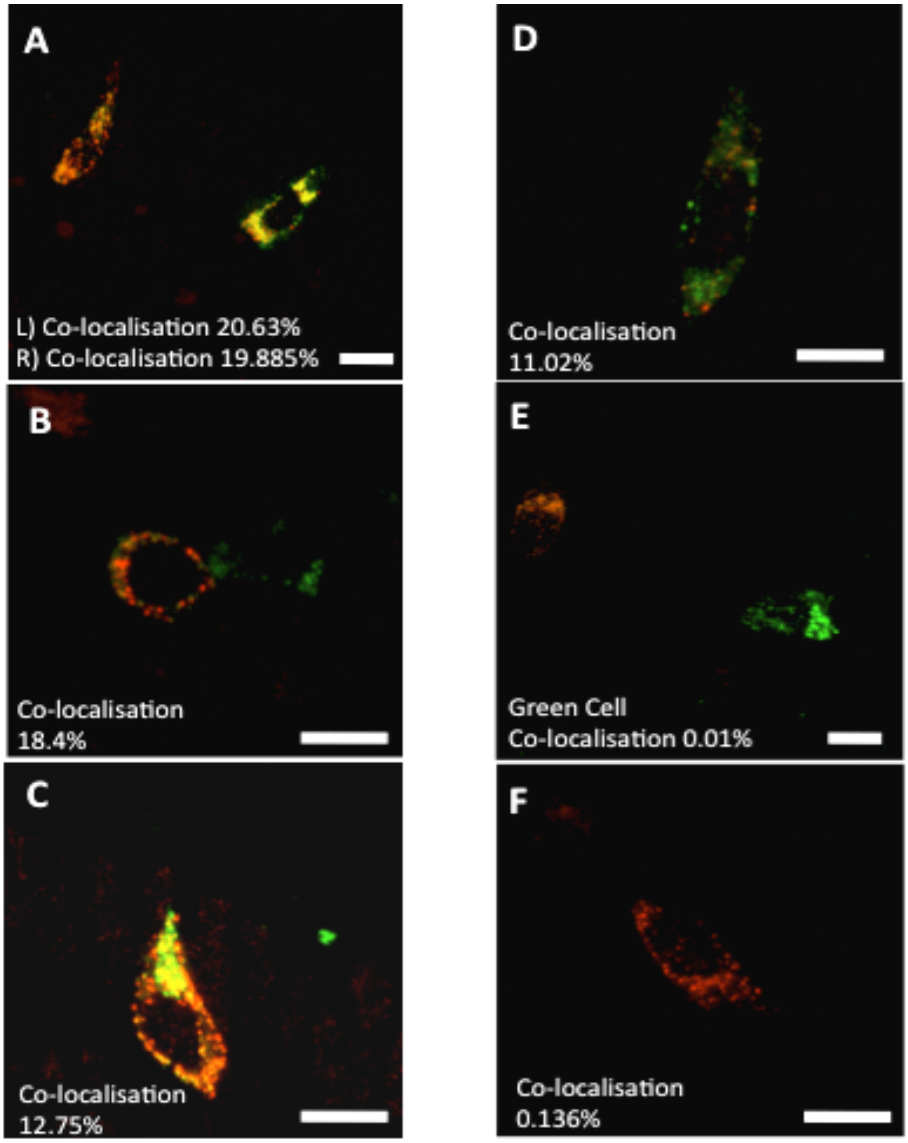


Figure 3. 23. Images of anti-ghrelin and anti-proCCK immunostained cells of paraffin embedded mid-duodenum.

Sections (4µm) were immunostained with anti-proCCK and anti-ghrelin antisera. Green fluorescence represents anti-proCCK and red fluorescence represents labelling with anti-ghrelin antisera. Images (A-F) represent 3D maximal projection image of positively labelled cells. A spectrum of staining by anti-proCCK anti anti-ghrelin was seen. Image A) 'L' and 'R' represent co-localisation data for the cell on the 'left' or 'right' respectively. Images A-E show cells dual-labelled for ghrelin and CCK. E) Depicts a cell that has been stained by anti-proCCK only. F) Shows a cell that has been stained by anti-ghrelin exclusively. Co-localisation rate was calculated according to pixel intensity after subtraction of background values. Fluorescence was considered co-localised if pixel intensity of green and red was ≥50%. Images were captured on a LeicaSP5 inverted microscope, 63X objective with confocal zoom, and analysed using Leica LAS-AF software. Scale bar represents 10µm

3.4. Discussion

This chapter analyses the validity of the eGFP-CCK transgenic mouse as a model to study CCK-cells in the SI. Additionally, immunohistochemistry techniques were employed using tissue sections from eGFP-CCK transgenic mice to target the null hypothesis that EECs that express CCK in the SI of eGFP-CCK mice, express only CCK. To do this a series of dual-immunostaining techniques were carried out to identify expression of GI peptides within eGFP-cells and then to determine co-expression of peptides within cells.

The eGFP-CCK transgenic mouse strain was developed with the aim of facilitating research on cells that express CCK. These BAC transgenic mice were engineered to express an eGFP reporter driven by the *CCK* gene promoter. Primarily these mice were made as part of the GENSAT project at Rockefeller University with the aim of mapping CCK expressing neurones in the brain, however, since CCK is also expressed in other tissues, the CCK-containing 'I-cells' in the small intestine should also be labelled. The eGFP-CCK transgenic mouse strain has been successfully used in other laboratories to research intestinal CCK-cells by combining cell isolation using fluorescent FACS sorting to obtain relatively pure populations of CCK-cells for molecular analysis (Samuel et al., 2008, Egerod et al., 2012) or to perform secretion studies (Liou et al., 2011c, Liou et al., 2011b, Liou et al., 2011a) as well as immunohistochemistry techniques on extracted cell and tissue samples (Chandra et al., 2010, Sei et al., 2011).

The eGFP-CCK transgenic mouse strain was established in Manchester from breeding pairs bought in from the MMRRC. It was therefore essential to the success of the work described in this thesis to confirm that the Manchester breeding stock faithfully expressed eGFP in CCK-expressing cells. Therefore, a preliminary aim of this chapter was to confirm the eGFP-CCK mouse model as a robust model to identify and sort SI I-cells. To do this it was necessary to firstly confirm that epithelial cells of the small intestine expressed the eGFP protein, and that these cells contained CCK. Small intestinal eGFP-cells could then be considered to represent CCK-cells.

3.4.1. Validation of the eGFP-CCK transgenic mouse model.

Tissue sections from 3 eGFP-CCK mice were analysed for expression of eGFP. The green fluorescent protein (GFP) tag is easily detected and thus used widely in many studies of gene expression and protein localisation (Li et al., 1998). GFP is a very stable molecule. It has a robust and compact 6 α-helix structure with an 11-stranded β-barrel that together protect the central fluorophore (Li et al., 1998). Despite this, the immunostaining protocol employed utilised a heat treatment step to uncover antigenic sites and through this, it was thought caused loss of eGFP fluorescence possibly through heat-induced denaturation of the endogenous eGFP fluorescence within tissue sections. In some instances, high temperatures can cause the GFP scaffold to rupture which renders the inner fluorophore non-fluorescent. This has been documented as an issue for some immunohistochemistry techniques that have involved this fluorescent tag (Ward and Bokman, 1982, Bokman and Ward, 1981, Nakamura et al., 2008). However, this problem was easily overcome by using an anti-GFP antiserum to label the cells, as the eGFP epitope was empirically found to remain intact.

3.4.1.1. eGFP cells were present in tissues of the small and large intestine

Immunostaining employing a anti-GFP antisera established that eGFP-positive cells were expressed in the duodenum, jejunum, ileum and colon.

The eGFP-CCK transgenic mouse model is designed to drive the expression of eGFP in all cells in which the CCK promoter is active. It is interesting that eGFP cells were found in all portions of the small and in the large intestine as specific CCK expression was previously believed to be exclusively located to the proximal SI (Roth et al., 1990). However, more recently, CCK expressing EECs have been reported throughout the SI in other studies using the eGFP-CCK mouse model (Liou et al., 2011b, Sei et al., 2011, Egerod et al., 2012). In addition, several studies have reported that cells expressing CCK are present along the length of the SI (Liou et al., 2011b, Sei et al., 2011, Egerod et al., 2012). Furthermore, Habib and colleagues, analysed the hormonal content of fluorescently tagged GIP-cells

and proglucagon-cells of ileum and colon, and found CCK mRNA expression within these cells (Habib et al., 2012). The expression of CCK in the distal SI, recorded by Habib et al. supports the expression of eGFP that is reported in the present study.

The presence of eGFP along the length of the SI is also supported by studies that have demonstrated CCK secretion from cells of duodenum, jejunum and ileum (Rehfeld et al., 2001, Chandra and Liddle, 2007). Release of CCK from cells in these regions subsequently enters the circulation or interacts with receptors expressed on local afferents to exert effects on target tissues nearby, such as the pancreas or gall bladder. The close proximity of these tissues enables the onset of effects to be rapid, and thus initiates release of pancreatic enzymes and bile to promote TG hydrolysis in the SI. Furthermore, a SI paracrine role of CCK has recently been suggested in which it may exert a modulatory function upon neighbouring enterocytes. Conversely, colonic expression of CCK, colonic secretion of CCK or a possible function for CCK expression in colon to date remains obscure. Clearly, the fact that CCK is expressed in colon suggests that it serves a function, however it is maintained that only a very small number of eGFP cells were observed in this region.

Along the GI tract, eGFP-positive cells localised to the crypt and villus areas. This was further supported in tissue sections dual-stained for eGFP alongside the proliferative marker Ki-67. Ki-67 is expressed in the nucleus of proliferating cells and for this reason is predominantly expressed in the crypt regions where proliferative stem cells reside. This pattern of eGFP expression reflects that observed by Sei et al., (2011) and Egerod et al., (2012) who also saw green fluorescing cells in crypt and villus in a eGFP-CCK transgenic mouse model. This is reasonable when considering the differentiation patterns of EECs, which originate in the intestinal crypts and migrate upwards along the length of the villi (Aiken et al., 1994, Roth and Gordon, 1990). Furthermore, a small proportion of stem cells that terminally differentiate into EECs, migrate downwards towards the bottom of the crypts (Bjerknes and Cheng, 1981, Formeister et al., 2009). Some of the eGFP-cells observed to be residing deep in the crypts might be a result of this property.

The eGFP-cells identified in the duodenum displayed a 'flask-shaped' morphology'. This is a typical characteristic of duodenal EECs which have a narrow apical membrane in contact with the lumen and a broader bulb shaped basolateral membrane where secretory granules are concentrated (Chandra et al., 2010). Progressing distally along the small intestine, eGFP-cells retain a 'flask-like' shape yet become slightly elongated and thinner, merging into a 'spindle-like' shape that is also a commonly accepted characteristic of EECs, particularly of the lower SI (Sutherland et al., 2007, Bohorquez et al., 2011). The relative change in EEC shape and anatomical characteristics according to location is a typical property of EECs. This is presumably an adaption that reflects a shift in the predominant processes acting in different regions of the small and large intestine (Bohórquez and Liddle, 2011). Qualitatively, eGFP staining within EECs was at a similar intensity in duodenum, jejunum and ileum, with less intense cellular staining in the colon.

eGFP-cells represented a very small number of cells that were evenly distributed and restricted to the cells lining the intestinal epithelium. Additionally, green fluorescence was not observed in neighbouring cells. These are both well-documented characteristics of EECs and notch signalling maintains this property amongst intestinal epithelial cells (Artavanis-Tsakonas et al., 1999, Rindi et al., 2004, Jensen et al., 2000). Quantitative FACS data from our laboratory have demonstrated eGFP-CCK cells represent approximately 0.4% of isolated duodenal cells (Sykaras et al., 2012). Qualitatively the number of eGFP-cells appeared to decrease moving distally along the tract. This agrees with the expression of CCK previously reported (Rindi et al., 2004, Habib et al., 2012).

Importantly, eGFP expressing cells were confirmed along the length of the GI tract and these cells showed typical EEC morphology and characteristics to EECs of the GI epithelium. The next step was to ensure that eGFP-cells represent CCK-containing 'I-cells'. To do this, duodenal tissue sections were probed with an established and well-characterised anti-proCCK antiserum (Habib et al., 2012, Sykaras et al., 2012).

3.4.1.2. eGFP-cells in duodenum labelled for proCCK

Immunostaining was used to confirm that eGFP expressing cells of the proximal SI represented CCK-cells. Duodenal sections were dual-stained with anti-GFP and anti-proCCK antisera. 89% of green fluorescing cells were co-stained for CCK for which staining was strongest at the basolateral membrane. Secretory granules containing packaged peptide are typically concentrated towards the basolateral pole of an EEC. The basolateral pole of EECs is in close proximity to capillaries and innervating neurones, which transmit signal of peptide release to target tissues around the body. Localisation of secretory granules towards the basal pole of EECs is a physiological advantage to enable rapid release of peptide and a rapid onset of effects.

It is important to note that all the cells that were labelled for CCK also contained eGFP. This indicates successful tagging of the CCK protein as there were no cells that contained CCK without eGFP. Specificity of anti-proCCK antiserum was confirmed using tissue isolated from CCK KO mice.

In the eGFP-CCK mouse model, the CCK promoter drives eGFP expression. This infers that if a cell contains eGFP it must also express CCK. However, these data show that ~10% of eGFP cells did not label for CCK. This suggests these cells express eGFP without CCK. Chandra et al. (2010) reported a similar degree of co-staining with the eGFP-CCK mouse model and this has been validated in other studies (Chandra et al., 2010, Liou et al., 2011b, Liou et al., 2011c). In fact in other fluorescently labelled EEC-models, such as the GLU-Venus mouse model in which proglucagon gene is tagged with Venus, Venus-fluorescing cells were only 70-80% positive for glucagon in upper and lower small intestine respectively. Furthermore, in GIP-Venus mice, Venus fluorescing cells were 90% positive for GIP, yet only 30% of GIP-positive cells contained Venus (Habib et al., 2012). Unfortunately, this is a characteristic of transgenic labelling models, which commonly do not achieve 100% representation of native protein expression.

A major factor that may have influenced the lack of CCK labelling in ~10% eGFP-cells is limitations of the antiserum. Insufficient antiserum binding may produce a negligible signal, especially when probing a cell with possible low expression of

epitope. In addition, non-specific binding of antisera is also a factor in that this creates a background staining that renders low-level positive signals to be indiscernible. Control experiments where primary antiserum has been omitted enables validation of non-specific 'background' signal provided by the secondary antiserum. Control images are provided in the appendices that depict non-specific staining to be marginal yet background auto-fluorescence from tissue sections is apparent and may detract from low-level positive signals that would result in the interpretation of a negative result (Habib et al., 2012). Alternatively it is also possible that the anti-GFP antiserum achieved more effective binding than the anti-CCK antiserum and could therefore account for a lack of positive labelling within some eGFP- cells. Considering these limitations, it is possible that although ~10% eGFP-cells did not label for CCK, they may still contain CCK protein albeit at a low concentration below the detection limits of the antiserum and immunostaining technique.

In some cases of fluorescent tagging, expression of the fluorescence tag without the labelled protein is potentially a result of ectopic expression of the fluorescent protein (Li et al., 1998). However, in this instance analysis of eGFP-cell properties enabled the conclusion that eGFP-cells were confined to EECs. Therefore ectopic expression of eGFP does not appear to be a feature within the eGFP-CCK mouse model.

Taking into consideration the EEC characteristics displayed by eGFP-cells and the high proportion of eGFP-cells that positively labelled for CCK, eGFP-cells in the small intestine of eGFP-CCK mice can be considered to represent CCK-cells. Therefore the null hypothesis 'eGFP-cells in eGFP-CCK transgenic mice do not contain CCK' can be rejected.

The availability of this transgenic mouse model has substantially increased the potential for research into I-cells. The next aim of the current work was to characterise the hormonal content of eGFP-cells.

3.4.2. Hormonal Characterisation of Duodenal eGFP-cells

The total EEC population represents less than 1% of the GI epithelium. For many years it has been widely acknowledged that within this population each EEC subtype stores and releases peptides that are derived from one peptide precursor (Liddle, 1997, Rindi et al., 2004, Egerod et al., 2012). However, recently several groups have reported co-expression of GI peptides within EECs (Egerod et al., 2012, Habib et al., 2012).

The duodenum is known to be the principle site of CCK-cell expression. For this reason, the hormonal content of eGFP-cells of mouse duodenum was analysed using immunohistochemistry techniques. This chapter presents data that demonstrates eGFP-cells from duodenum of eGFP-CCK mice, which represent CCK-cells, actually contain a broad repertoire of hormones.

3.4.2.1. eGFP-cells in duodenum express CCK, PYY, GIP, Proglucagon and Ghrelin.

Immuno-staining experiments that employed dual-labelling techniques demonstrated that 89% eGFP-cells contained proCCK, this established eGFP-cells to represent CCK-cells. 45% of eGFP-cells also contained PYY and 37% of eGFP-cells contained GIP protein. A smaller proportion of eGFP-cells in the duodenum were also observed to contain proglucagon (14%). What was more astonishing was that analysis of duodenal eGFP-cells using anti-ghrelin antisera showed that 50% of total eGFP-cells in duodenum labelled for ghrelin. Interestingly 32% of PYY-cells did not contain eGFP. Furthermore, 18% of GIP-cells did not contain eGFP and there were no cells in the duodenum that stained for proglucagon alone. This indicates that there were relatively few hormonal cells, of the selection of peptides that were probed, which were not eGFP-positive. Unfortunately it was not possible to acquire these data for ghrelin-eGFP co-staining as ghrelin is also expressed in closed-type EECs. Qualitatively, these data suggest that CCK is present in EECs that contain other gut peptides. To confirm the co-expression of hormones within duodenal EECs dual labelling was

performed using anti-proCCK and antisera against either GIP, PYY or ghrelin and dual-labelled cells were observed in all cases. This further validated peptide co-expression within EECs.

A caveat to the conclusions from the data of the current work, that more than two hormones are co-expressed in a single EEC, is that only dual-immunostaining was utilised. Triple staining was attempted, but was not successful. However, transgenic mouse models of targeted cell ablation support the expression of more than one hormone within an EEC. Ablation of secretin cells (Rindi et al., 1999) and ablation of glucagon cells (Egerod et al., 2012) affected the expression of many other hormone expressing cells in the GI tract, indicating that multiple promoters are active within the same EECs.

Multiple hormone expression in eGFP-CCK cells has been recorded previously. The laboratory of Schwartz saw that eGFP-CCK cells from mouse duodenum firstly confirmed highest expression levels of CCK mRNA as expected but also found GIP, ghrelin, glucagon and PYY mRNA within eGFP-cells (Egerod et al., 2012). High levels of neurotensin and secretin were also detected within eGFP-cells, however these peptides were not analysed in the current study. These data are reflective of unpublished data conducted by Sykaras and colleagues whereby high expression of CCK, GIP, secretin, proglucagon, PYY, ghrelin and neurotensin was detected in CCK-cells isolated from eGFP-CCK mice (Unpublished, Sykaras et al. 2013). In addition, Habib and colleagues have conducted protein and mRNA analysis of the hormonal content of Venus-tagged K-cells (GIP) and L-cells (proglucagon/PYY) from the small intestine, showed these EEC subtypes also contained a vast array of GI hormones (Habib et al., 2012). These data indicate that a multiple hormone complement appears to be a typical feature of many EEC subtypes.

Co-expression of GI peptides within the same EEC was first observed nearly three decades ago when the co-localisation of PYY and proglucagon gene products within EECs became an established property of L-cells back in 1986 (Bottcher et al., 1986). Since then, co-localisation has also been demonstrated for GLP-1 and GIP expression in porcine, rat and human small intestine (Mortensen et al., 2003).

Though these data indicated there to be a relationship between EEC subtypes this was not acknowledged as a typical characteristic until use of transgenic mouse models, such as the eGFP-CCK model described in this thesis, that have highlighted EEC peptide co-expression data.

Intestinal cell line models, typically derived from endocrine tumours, have been consistently shown to express many different hormones. Examples of this exist for models of pancreatic endocrine tumours (Larsson et al., 1975), the GLUTag cell line (Brubaker et al., 1998) and the STC-1 cell line which has been widely used as a model of I-cells (Rindi et al., 1990, Kieffer et al., 1995, Chang et al., 1994). In fact the only studies to date that have measured cellular secretion of mixed peptides were conducted on STC-1 cells. As such, STC-1 cells have been demonstrated to contain and secrete a mixture of CCK, PYY and GIP (Hand et al., 2012) a hormone complement mirroring that observed for CCK-cells detailed in this thesis.

Due to the predominant expression of CCK, STC-1 cells have been widely used as a model of I-cells and the expression of other hormones within the cell were considered a limiting factor of the model. However the data presented in this thesis and in recent studies is providing evidence that these cell-line models may in fact be a more accurate model of EECs than was previously thought. STC-1 cells are known to express CCK, multiple products of the proglucagon gene, pancreatic polypeptide, neurotensin, secretin, GIP, PYY and undoubtedly other peptides (Rindi et al., 1990, Geraedts et al., 2009). Given the newly founded similarity between hormone expressions in STC-1 cells and primary 'I-cells' some of the *in vitro* work upon STC-1 cells could be considered more reflective of the relative activity of the native I-cell than previously believed.

The data presented demonstrate that eGFP-cells from eGFP-CCK mice contained CCK, GIP, PYY, proglucagon and ghrelin. Therefore the null hypothesis 'duodenal eGFP-cells in eGFP-CCK mice do not express a repertoire of anorectic GI peptides' can be rejected. Furthermore the null hypothesis 'duodenal eGFP-cells in eGFP-CCK mice do not express the orexigenic peptide ghrelin' can also be rejected.

The data in this thesis and in other recent studies have demonstrated that isolated EEC subtypes have multiple hormone expression. Before interpreting the implications of peptide co-expression it is firstly important to reflect whether this is an attribute of developing EECs or mature EECs. This is important to determine the relative functionality of co-expressing cells.

3.4.2.2. Are multiple hormones expressed in EECs located in crypts and villi?

The functional properties of EECs vary according to their location within the cross-section of the intestinal mucosa. Importantly, hormonal cells found in the crypt regions can represent differentiating stem cells, which have not yet migrated towards the villus. Alternatively these cells may also reflect a small sub-population of EECs that are known to differentiate whilst migrating downward deep into the crypt where they remain as quiescent stem cells (Bjerknes and Cheng, 1981, Potten et al., 1997). In either scenario, EECs within the crypts typically have stem cell properties with a high rate of differentiation and proliferation yet these cells also possess properties of endocrine cells; containing the secretory marker chromogranin A and the expression of GI peptides (Barker et al., 2007, Sei et al., 2011). Although endocrine cells located within the crypt possess the hormonal properties of an EEC they do not function as a typical EEC as they are not exposed to the intestinal lumen. Alternately, EECs located in the villi are exposed to nutrients within the intestinal lumen. Nutrient stimulation of EECs induces the release of bioactive peptide directly into the neural or capillary network situated beneath the intestinal epithelium in the core of the villus. Therefore, broadly speaking, cells that are situated along the villus axis are considered mature and functional EECs.

The data presented in this thesis clearly demonstrate that cells co-expressing gut peptides were located in both the crypt and villi regions. These data correlate to that presented by Egerod et al., (2012) who demonstrated peptide co-expression within EECs isolated from crypt and villi fractions. Not only this but CCK-cells of

the villi were deemed more highly enriched in secretin, PYY and neurotensin expression than CCK-cells of the crypts (Egerod et al., 2012). In a similar study by Sykaras and colleagues, polymerase chain reaction (PCR) analysis of isolated CCK-cells from intestinal villi and crypt fractions observed an enrichment of ghrelin mRNA in villus CCK-cells compared with crypt CCK-cells (Unpublished, Sykaras et al. 2013).

These data are in contrast to the conclusions made by Wank and colleagues who stated that CCK-cells co-expressing other GI peptides were an exclusive characteristic to CCK-cells located in the crypt regions. The study by Wank analysed CCK-cells from intestinal crypt preparation isolated from eGFP-CCK mice. Immunohistochemistry analysis of crypt CCK-cells found that 40% CCK-cells contained secretin and 80% CCK-cells contained GIP. Furthermore, ghrelin protein was detected in at least 30% of CCK-cells of the crypts (Sei et al., 2011). Peptide co-expression was not recorded in EECs located in the villus. CCK-cells within the crypt also contained the stem cell marker Lgr5 thus together established the conclusion that co-expressing EECs were not representative of functional EECs (Sei et al., 2011). These data clearly differ to the data presented in this thesis where CCK-cells containing GIP, PYY, proglucagon or ghrelin were observed in both crypt and villi regions and were therefore deemed to represent functional EECs.

Peptide localisation data were further validated by confirming the typical distribution and proliferative activity of PYY-, GIP- or ghrelin-cells through application of anti-Ki-67 antisera that stains the nucleus of highly proliferative cells. Proliferating cells would broadly incorporate stem cells due to their highly proliferative nature, although not exclusively. To this end, Ki-67 staining was dependable within the crypt areas and scattered sporadically within the villi regions. These experiments validated that PYY-, GIP- and ghrelin-cells were expressed within the highly proliferative crypt regions and also identified these cells to be distributed in non-proliferative cells in the villus.

Collectively, these data enable the null hypothesis ‘duodenal eGFP cells do not express multiple hormones throughout their life span’ to be rejected.

Taken together, the presented data refute the conclusions of Sei and colleagues that EECs that co-expressed GI peptides were exclusive to crypts and had a stem cell phenotype. Interestingly Sei et al. observed an absence of anti-Ki-67 staining in a small number of crypt CCK-cells, these cells expressed the secretory marker chromogranin A and indicated that these hormonal cells were terminally differentiated. The question was raised that cells such as these may represent functional hormone-secreting cells. Further work is required to determine the exact phenotype and role of CCK-cells within different regions of the crypt-villus axis. This could be conducted by gene expression analysis to determine differences in the molecular machinery within the cells or alternately to investigate the secretory profile of these cells.

3.4.2.3. Peptide co-expression – a possible artefact of genetic tagging?

A factor to consider when analysing these data is whether hormone co-expression could be a phenomenon introduced via BAC-transgene labelling. Additionally 11% of eGFP-cells were not labelled for CCK, which presented a relatively minor element of reservation towards the mouse model. Egerod and colleagues debated this potential limitation of the model, and therefore subsequently employed a BAC-transgene induced ghrelin-hrGFP mouse model to validate their findings. GFP-cells isolated from stomach of the ghrelin-hrGFP mouse model contained no other hormone type. This validated that the hormone co-expression 'phenomena' was not a side effect of the BAC transgene system. The study conducted by Egerod et al. also demonstrated GI peptide co-expression in human duodenum, which was also independent of the potential interference of the transgene (Egerod et al., 2012).

3.4.2.4. Does peptide co-expression reflect peptides derived from a shared cell lineage?

The data presented in this chapter have demonstrated that a portion of CCK-cells co-express PYY, GIP, proglucagon and ghrelin and that these cells are distributed within the crypt and villus regions. Studies have suggested that shared cell lineage pathways could explain the co-expression of GI peptides, particularly for cell types with similar distribution and functions. It has been postulated that EECs that share differentiation pathways exert a low level of broad transcriptional activity prior to their terminal differentiation. This may enable an EEC to retain the ability to transcribe a variety of gut peptides through its life cycle. This raises the suggestion that an EEC subtype may be able to modulate its profile of expression of peptides according to integrated systemic signals.

Intestinal epithelial cell types are derived from pluripotent stem cells that reside deep within the intestinal crypts. Differentiating cells migrate up the crypt-villus axis, during which they mature and commit to their terminal cell fate. The differentiation process is dependent upon a cascade of transcription factors and cellular signalling pathways that determine final cell outcome. EEC subtypes are very closely linked until the final stages as they share a common transcriptional pathway.

Mouse gene knockout studies have reinforced the notion of a close relationship between EEC subtypes. The first demonstration of this was through using the targeted ablation of secretin cells. Secretin cell ablation was achieved through transgenic expression of the herpes simplex virus thymidine kinase under the control of the secretin gene promoter. This was selectively switched on upon application of the nucleoside analogue ganciclovir (Rindi et al., 1999), which caused a total ablation of secretin cells coupled with a near total ablation of CCK-, glucagon- and PYY-cells. This highlighted a common link between L-, I- and S-cells (Figure 3.24) (Rindi et al., 1999). Equally, Egerod and colleagues developed a GLP-1 knockout model that introduced a human diphtheria toxin receptor under the control of the proglucagon promoter. Subsequent administration of diphtheria toxin induced a near total ablation of ileal GLP-1- and PYY-cells within 24hours, and this was coupled with a vast reduction in ileal CCK-, secretin-,

neurotensin- and GIP-cells. This mouse model enabled a direct demonstration that the proglucagon promoter is also active in L-, I-, S-, N-, and K-cells (Figure 3.24). (Egerod et al., 2012). A similar model, which ablated GIP-cells, again using a diphtheria toxin ablation technique, demonstrated an ablation of GIP-cells (K-cells) and reduction in CCK-cells (I-cells) (Althage et al., 2008, Brubaker, 2012). Furthermore, other studies involving the transgenic KO of specific transcription factors (TF) resulted in a similar effect; NeuroD KO mice lacked I- and S-cells and deficient development of the endocrine islets of the pancreas (Naya et al., 1997); and again Pdx1 KO mice lacked I- and S-cells as well as G-cells (Gannon et al., 2008). These studies have collectively demonstrated that both KO of TFs and cell ablation models targeting specific peptide promoters affect multiple EEC subtypes. This immediately refutes the long-standing characteristic of EECs that stated each cell subtype possessed the transcriptional activity of just one peptide precursor.

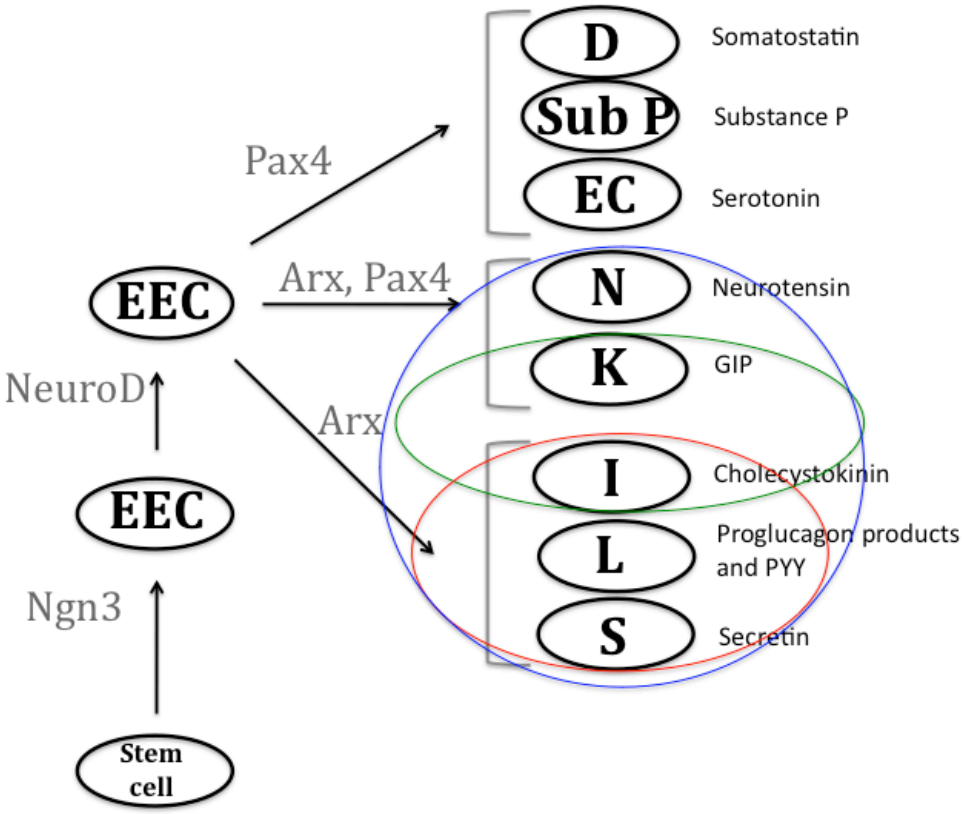


Figure 3.24. Example of EECs that share cell lineage pathways.

Cells of the intestinal epithelium are derived from pluripotent stem cells. The transcription factor (TF) Neurogenin3 (Ngn3) initiates differentiation towards an EEC lineage and NeuroD further drives EEC fate. Additional TFs determine terminal EEC subtype. This includes Pax4 that controls development of D-, Substance P, and EC-cells, a combination of Pax4 and Arx is required for differentiation into N- and K-cells and Arx is require for development of I-, L- and S-cells. Shared cell lineage pathways have been identified through models of targeted cell ablation that affected multiple EEC subtypes, demonstrating that more than one peptide precursor is active in an EEC. The affects of three such examples of targeted cell ablation are depicted. Red circle links EEC types affected in targeted cell ablation driven by the secretin promoter. Green circle links EECs affected through targeted cell ablation controlled by the GIP promoter. Blue circle links EEC types affected in targeted cell ablation driven by the proglucagon promoter. Adapted from (Brubaker, 2012).

An interesting point of reflection is that upon analysis of the data that characterises both the hormonal content of fluorescently tagged EEC subtypes and the models of targeted EEC ablation, it is clear that CCK is a predominant feature. In the studies described above, targeted cell ablation of SI EECs subtypes consistently disrupted the expression profile of CCK-cells. Furthermore, in transgenic models of fluorescently tagged-EECs, cellular CCK expression is consistently high whether probing I-, K-, or L-cells (Egerod et al., 2012, Brubaker, 2012, Habib et al., 2012). This suggests CCK expression is highly promiscuous in the proximal SI, which is probably reflective of the dominant role of CCK in this region. The data of the current study demonstrate that of the selection of peptides probed, only a small number of the duodenal EECs labelled were not CCK-cells. It has been postulated that these data collectively advocate that CCK-cells may act as a precursor for development of other EEC types (Brubaker, 2012). The CCK^{LacZ}/CCK KO model employed in the current work is genetically designed to specifically disrupt the CCK gene; exerting no effect upon additional gene or peptide expression. Immunostaining was carried out on duodenal sections of CCK^{LacZ} mice demonstrated that cells in the duodenum of KO mice to stain positively for PYY, proglucagon or ghrelin. Therefore this demonstrates that the transcriptional ability of other GI hormones was not reliant upon production of CCK.

Duodenal EECs that dual-stained for CCK and other key GI peptides, demonstrated that intracellular hormone expression existed at a spectrum of intensities. Furthermore, whilst difficult to interpret due to limitations of the technique, co-localised peptides appeared to label either the same or distinctly separate vesicles within the cell. Whilst co-expression data provide no detail of peptide co-release or differential release, the co-expression of two peptides within the same EEC suggests physiological significance.

GI hormones exert a spectrum of functionally important effects, several of which are overlapping in function. These include an array of effects within the SI to enhance the digestion and absorption. An exciting proposition is that hormone co-expression within EECs enables a physiological advantage to potentiate, expand or even regulate the relative roles of GI peptides.

3.4.2.5. What are the implications for the co-expression of GI peptides within EECs?

The data presented in this thesis demonstrate that CCK-cells contain a variety of GI hormones including GIP, PYY, proglucagon and ghrelin. The data from this study and others refute the long-standing hypothesis that individual EECs express GI peptides encoded by one peptide promoter. Furthermore, distribution of dual-labelled cells along the length of the villi indicates these cells are functional EECs. The functional significance for the co-expression of multiple hormones in individual EECs of the duodenum is open to several interpretations.

In the case of CCK co-localisation with PYY and GIP, these hormones have a repertoire of overlapping effects. These are anorectic peptides, which signal to the hypothalamic regions in the brain to limit further food intake. Furthermore, these peptides have local effects on GI functioning that include delays in gastric emptying and the release of gastric and pancreatic enzymes as well as bile (Talsania et al., 2005). These three peptides are all released upon detection of fatty acids, and to a variable degree to glucose; PYY release is also stimulated by bile acids whereas GIP and CCK release is also simulated by amino acids. Furthermore GIP has additional roles in glucose homeostasis and FA metabolism (Zhou et al., 2005). The individual characteristics relating to peptide release and function are comparable for each peptide and would enable an EEC to produce an additive effect if these peptides were released simultaneously. It is easy to comprehend how this may be advantageous to an EEC. Ingested food exists as a complex mix of nutrient types that are delivered simultaneously into the duodenum as chyme. These nutrient types require activation of separate processes to ensure their breakdown. Stimulation of individual EECs to release a cocktail of peptides would optimise the enteroendocrine system. Additionally, immediate release of more than one peptide enhances the EEC communication-loops connecting portions of the SI. In this instance GIP has been identified to function as a feed-forward loop for information of incoming nutrients to distal regions of the SI (Buchan, 1999, Kellett et al., 2008).

Alternatively the low proportion of duodenal CCK-cells co-stained with proglucagon correlates with the long-established knowledge that proglucagon cells are characterised with highest expression in the ileum and colon. However, like for PYY and GIP, proglucagon products have a similar secretory profile and functional effects as CCK (Cummings and Overduin, 2007, Reimann et al., 2008, Poreba et al., 2012).

On the other hand, the implications for the co-localisation of CCK with ghrelin are comparatively complex, and this will now be discussed.

3.4.2.6. Occurrence of CCK and ghrelin – a peptide functionally distant from the other anorectic hormones

Of the peptides for which CCK-cells were probed, the co-localisation of ghrelin alongside CCK was the most surprising and novel finding of this study. This co-localisation was unexpected for the foremost reason that ghrelin is a potent orexigenic peptide whereas CCK is a potent anorexigenic peptide.

Searches of the literature have unearthed only two studies that have reported co-localisation of CCK and ghrelin. The study conducted by Sei and colleagues demonstrated co-localisation of CCK and ghrelin through immunostaining analysis of primary intestinal crypt preparations from eGFP-CCK mice. However cells co-expressing CCK and ghrelin were exclusively located deep within the crypt areas and thus concluded to be functionally insignificant (Sei et al., 2011). The data presented in the current work strongly refute this conclusion because CCK and ghrelin co-expression was observed in cells along the entire length of the crypt-villus axis. This is confirmed in unpublished work where PCR analysis of CCK-cells isolated from duodenal crypts or villus fractions of eGFP-CCK mice, observed enrichment of ghrelin transcripts within CCK-cells of the villi compared to crypt (Unpublished, Sykaras et. al. 2013). An additional study by Egerod et al., also utilised the eGFP-CCK mouse model, and found ghrelin mRNA to be expressed within villus eGFP-CCK cells yet further analysis of tissue sections using immunohistochemistry, concluded that the mature ghrelin protein was not

expressed in these CCK-cells (Egerod et al., 2012). Whilst the detection of ghrelin mRNA supports the presented data, the absence of ghrelin protein does not.
In a related study, upper SI L-cells that were isolated from a GLU-Venus mouse found a small amount of ghrelin mRNA within L-cells. Notably, isolated L-cells in this region contained CCK as the second predominant message after GIP. Whilst the presence of transcripts for the two peptides correlates with the present data, the proportions do not mirror the 50% co-localisation eGFP with ghrelin recorded in this study. Again expression of ghrelin protein within L-cells was not noted (Habib et al., 2012). It should also be considered that the laboratory of Schwartz, have a GFP-ghrelin (ghrelin-hrGFP) mouse model at their disposal, yet to date have not reported co-expression of CCK and ghrelin in duodenum. It is possible that these experiments have not been considered. However, in view of the focus of this lab on I-cell research, it is more probable that notable results have not been found (Egerod et al., 2012).

The co-expression of CCK with ghrelin is a puzzling finding. As a potent orexigenic hormone that is released prior to food intake, ghrelin classically has completely contrasting actions to CCK. Ghrelin and CCK-cells are also typically distant in terms of the differentiation pathways they follow. This distance extends from initial stages of EEC differentiation whereby CCK-cells are dependent on the TFs; Ngn3 and Nkx2.2, whereas ghrelin-cells require neither. (Lee et al., 2002a, Jenny et al., 2002, Desai et al., 2008). This makes the co-expression of ghrelin in CCK-cells intriguing. Not only this, but the proportion of CCK-cells labelled for ghrelin was higher than that recorded for the co-localisation of CCK with any of the anorectic GI peptides tested. The detection of obestatin within eGFP-cells further validates these findings, as obestatin is a product of the preproghrelin gene, which is the gene precursor for ghrelin.

Expression of ghrelin cells along the length of the intestine has previously been characterised. Ghrelin is expressed in closed-type EECs, reflective of the typical (X/A) ghrelin-cell found in the stomach, and within open-type EECs (Sakata et al., 2002). This mirrors the pattern of ghrelin expression of the current study whereby a number of closed-type EECs were stained for ghrelin. These were

small and round in shape and did not extend to the intestinal lumen. Closed-type EECS are the typical cell-type attributed to ghrelin cells as they respond to mechanical, hormonal or neuronal stimuli. Distinctively, luminal nutrients do not stimulate secretion of active ghrelin as for other GI peptides. Incidentally the presence of nutrients in the intestine have been shown to suppress ghrelin release (Williams and Cummings, 2005). Nutrient-stimulated peptides such as CCK and other anorectic peptides are exclusively expressed in open-type EECs and as such CCK-cells that were co-stained by the ghrelin antisera exclusively represented open-type EECs.

To validate these data, the efficacy of both anti-ghrelin antisera (GA1 and GA2) were tested by application of the antisera to ghrelin KO and GOAT KO stomach tissue. These experiments firstly reinforced the specificity of the antisera for the ghrelin epitope and secondly demonstrated that neither antiserum was specific for the active form of ghrelin. Active ghrelin contains an octanoylated reside on serine-3 that is essential for binding its receptor GHS-R (Yang et al., 2008). Octanoylation is catalysed by the ghrelin o-acyl transferase GOAT enzyme, thus GOAT KO mice do not have the capacity to synthesise 'active' ghrelin. Ghrelin that has not been octanoylated is termed des-acyl ghrelin (DAG) and this has been long considered the 'inactive' form of ghrelin. Application of ghrelin antisera to control tissue therefore demonstrated that the pattern of ghrelin expression recorded in the current study was representative of total ghrelin expression and was not specific for either active ghrelin or DAG.

The expression of ghrelin cells within the GI tract that was mapped by Sakata et al. achieved an expression pattern reflective to that observed in this study in that both open- and closed-type EECs were positively labelled for ghrelin. Sakata and colleagues stated this pattern of expression was specific for 'active' ghrelin as the antisera applied was generated against the ghrelin epitope including the octanoylated residue (Sakata et al., 2002). Incidentally the anti-ghrelin antiserum (GA1) employed in the current study was generated with specificity for acyl-ghrelin, however application to GOAT KO tissue proved this specificity was not manifested. The study by Sakata and colleagues did not demonstrate antiserum specificity (Sakata et al., 2002) and therefore it can be postulated that the

expression pattern of ghrelin cells achieved may have also been reflective of total ghrelin. Therefore, the form of ghrelin detected in the current study is unknown. This is a significant element of consideration and can now be open for interpretation.

Examination of literature published within the past decade has debated the relative roles of active ghrelin and DAG, as both forms of the peptides have been ascribed an array of functional differences. Ghrelin has been well characterised for a number of decades as the exclusive orexigenic peptide within the GI tract with highest circulating levels during fasting and decreased levels postprandially (Tschop et al., 2001, Cummings et al., 2001). Ghrelin also has a role in modulation of GI functions such as stimulation of intestinal motility, and suppression of GI peptide and insulin release (Gibson et al., 2008, Fujimiya et al., 2012, Tong et al., 2010). Conversely, although DAG has been recognised to exist as the dominant form of circulating ghrelin for many years, it was previously deemed the 'inactive' form of the peptide due to its inability to bind GHSR-1a. Due to this property, until just over a decade ago, DAG was believed to exert no physiological role (Hosoda et al., 2000). DAG was considered a peptide awaiting an activation signal, that enabled tight control over the bioavailability of active ghrelin. In this way GOAT was believed to act as a 'nutrient sensor' as it utilised dietary MCFA to octanoylate awaiting DAG and thus relate active ghrelin concentrations directly to the availability of dietary nutrients (Kirchner et al., 2009).

DAG has now been identified to exert its own specific effects within the body, namely exerting an anorectic action and effects to enhance digestive functions (Gibson et al., 2008). In addition to these effects, DAG has also been identified to exert a direct inhibitory action upon active ghrelin (Asakawa et al., 2005, Toshinai et al., 2006, Inhoff et al., 2008a). These inhibitory effects are particularly robust when the two forms of the peptide are co-administered, which depicts an antagonistic role. This inhibition is not believed to exist as competitive inhibition due to the inability of DAG to bind the GHSR-1 (Kojima et al., 1999, Inhoff et al., 2008a). However this inhibitory role has caused speculation that an additional receptor may exist that is shared by ghrelin and DAG (Gauna et al., 2006, Delhanty et al., 2012).

Whatever its mode of action, DAG has been shown to counteract the roles of ghrelin in many instances (Inhoff et al., 2008b). DAG inhibits the activation of hypothalamic neuronal activity by ghrelin. This not only influences food intake but also relays affects on GI functions such as gastric emptying (Asakawa et al., 2005, Fujimiya et al., 2010). Furthermore, DAG counteracts the inhibitory actions of ghrelin on GI peptide secretion; DAG promotes release of glucagon, somatostatin, pancreatic polypeptide (PP) and insulin (Fujimiya et al., 2010). In this way, DAG also antagonises the pancreatic effects of ghrelin, influencing blood glucose homeostasis through the manipulation of insulin release (Broglio et al., 2004, Delhanty et al., 2012, Kumar et al., 2010, Fujimiya et al., 2010, Tong et al., 2010).

Likewise, obestatin also has opposing functions to those of active ghrelin. Obestatin is an anorectic peptide that relays effects to delay gastric emptying whilst inhibiting GI motility (Zhang et al., 2005, Gibson et al., 2008). Obestatin also increases the release of pancreatic juice enzymes into the duodenum (Kapica et al., 2007) and inhibits glucose-induced insulin secretion (Ren et al., 2008). It has also been stated to have an antagonistic effect against ghrelin although this has not been as thoroughly characterised (Gibson et al., 2008).

These characteristics portray both DAG and obestatin as peptides with vastly similar functions to CCK. For this reason it would be simple to assume co-expression of ghrelin alongside CCK within open-type EECs, could reflect ghrelin in the form of DAG. The co-expression and potential co-release of DAG alongside CCK may implicate an additional dimension of control over GI functions and satiety. Firstly, evidence in the literature suggests these peptides exert similar effects within the GI tract and upon satiety and therefore may be able to potentiate hormonal effects. Secondly, the antagonistic action of DAG upon ghrelin enables speculation that DAG may exert a regulatory role within the enteroendocrine system. In this way, co-release of DAG and CCK could not only potentiate peptide activity, but also inhibit the opposing actions of ghrelin that may remain in circulation from a pre-prandial state. The half life of ghrelin is calculated at approximately 30 minutes (Hillman et al., 2011). With this in mind, it would be advantageous for the enteroendocrine system to be able to 'switch off' the activity of ghrelin prior to this time-point. In this way the involvement of DAG

could be an exciting mediator to control gut hormone signalling between pre- and post-prandial status.

As antisera specificity was evidently non-specific for active ghrelin or DAG, it is probable that a portion of the ghrelin epitope detected must also represent expression of active ghrelin. Closed-type cells that stained for ghrelin did not contain eGFP-CCK. Furthermore closed-type cells are characterised to elicit the classical orexigenic roles of ghrelin as these respond to mechanical, hormonal or neuronal stimuli and are not stimulated by nutrients. It is speculated that this expression may be reflective of active ghrelin although it appears it is difficult to reliably confirm this using ghrelin antisera alone. Identification of expression of GOAT within these cells would be a more accurate measure to determine the form of ghrelin.

Expression of CCK with active ghrelin in open-type EECs would be more difficult to interpret. As described previously, ghrelin and CCK cells do not typically share cell lineage pathways therefore residual expression is unlikely to convey historical expression of the protein. It is difficult to envisage a physiological advantage for the simultaneous release of CCK and active ghrelin due to their classically opposing functions and opposing secretory profiles. Co-localisation data of cells dual-labelled for CCK and ghrelin, demonstrated that labelling within cells co-localised in up to 20% of antisera signal. This indicates that the majority of antisera labelling is localised to distinctly separate structures (vesicles) within the cell, indicating the majority of co-expressed peptide are differentially packaged within the cell. In this instance a cell may be able to control the selective release of peptides according to alternate stimuli. Alternatively, it may be the case that a cell may be able to adjust its dominant functions according to significant systemic changes such in extreme diets or illness.

Ultimately, due to the functional and stimulatory differences between open- and closed-type EECs, the most simplistic explanation would be that ghrelin contained within closed-type EECs is likely to reflect 'active' ghrelin. Furthermore, due to the functional similarities of the two peptides, it is speculated that ghrelin expression in CCK-cells represents DAG. However this

requires confirmation through development and application of a sensitive GOAT antiserum to probe the expression of the enzyme within eGFP-CCK cells.

EECs classically undergo constant regeneration, which enables EECs to possess a higher degree of plasticity and acute reactivity to respond to demands (Barker et al., 2008). This is supported by the variable patterns of intracellular peptide expression observed in all instances of dual-labelled peptides that was observed in the current study. It is becoming increasingly clear that an EEC can possess the molecular machinery to transcribe multiple peptides. Variable intensity of peptide expression indicates EECs are individual and subtypes cannot be assumed to exist with identical characteristics. It is probable that EEC subtypes may still exist but these may be easier defined according to the dominant hormone it contains. An EEC may be able to adjust the protein complement according to systemic signals and nutrient demands as well as in reflection of the location of the cell.

The co-expression of peptides indicates a cell may be able to co-release more than one peptide. This may have a role to potentiate the corresponding effects upon GI functions as well as influencing the ability of a cell to respond to variable stimuli. Although the co-release of peptide from an individual EEC has not yet been demonstrated, the data indicate peptides are contained in the same, and in separate vesicles, which may enable a cell to control release according to stimuli. This would implicate enhanced regulation and control over the system, which will now be discussed.

3.4.2.7. Intracellular localisation of hormone labelling – determining the packaging or secretion of similar or separate vesicles

The evidence gathered from dual staining for two GI hormones shine light on a complex co-packaging network within EECs. Co-localisation analysis of dual-labeled cells established fluorophore co-localisation patterns in cells labeled with

CCK/GIP or CCK/PYY or CCK/ghrelin. The degree of co-localised staining ranged from cells that had a large amount of one peptide with smaller amount of the second peptide, to cells that appeared to co-stain for the two peptides more equally. Co-staining within the cell appeared to localise to the same vesicular structures in up to 25% of intracellular staining, however some dual-labelled cells demonstrated only a very small % of co-localised labelling within the cell. Intracellular localisation of peptides is difficult to discern definitively due to potential overlap of signal and limitations in antisera binding. More sensitive techniques such as immuno-gold labelling and imaging using electron microscopy may validate the current conclusions.

The data acquired in the current study indicate that the majority of the peptides co-expressed within EECs are packaged within separate vesicles. This indicates a cell may be able to control the differential release of each peptide according to cell signals.

It is known that two or more peptides can be co-packaged within a cell; this encompasses peptides that are packaged separately or within the same secretory granules (Fumagalli and Zanini, 1985, Ishibashi and Shiino, 1989). This lends to the understanding that peptides can be simultaneously released, but does a cell possess the machinery to release granules separately and in a controlled approach (Ishibashi and Shiino, 1989)?

It may be possible to model cellular co-packaging on peptides that are contained and released from neurones (Furness et al., 1989, Reti et al., 2008). Neurones can contain more than one neuropeptide; typically these are packaged within the same vesicles. When two or more neuropeptides are released simultaneously they are termed co-transmitters. However neurones can also contain neuropeptides that are packaged into separate vesicles. In this way a neurone possesses advanced versatility in its signalling ability. To this end, variable stimulation at synaptic terminals can induce the selective release of neuropeptides, allowing for alterations in signalling properties (Sossin and Scheller, 1991, Bartfai et al., 1988).

It would be advantageous for an EEC to be able to control differential peptide release in this way. This could occur at the level of nutrient detection and activation of cell signaling pathways, intracellular nutrient metabolism or from systemic signals providing information of infection or energy status. Exocytosis of GI peptides has been demonstrated to involve a series of both related and separate intracellular signaling pathways. An important example of this is the involvement of cellular Ca^{2+} signalling that is essential to initiate CCK and GIP release (Takahashi et al., 2000, Kieffer et al., 1994) but is not required for PYY release (Ballantyne et al., 1993, Hand et al., 2012). Additionally, the synthesis and secretion of CCK, GIP and PYY involve cell signalling pathways for protein kinase A (PKA) (Kieffer et al., 1994, Chisholm and Greenberg, 2000, Deavall et al., 2000) and protein kinase C (PKC) (Takahashi et al., 2000, Chisholm and Greenberg, 2000, Parker et al., 2009). Recent secretion studies on STC-1 cells and a newly derived cell-line pGIP/Neo STC-1, which constitutes STC-1 cells that have been genetically manipulated to be enriched in GIP, replicated these data demonstrating the Ca^{2+} dependence and PKA activity yet did not see evidence for the involvement of PKC in the secretion of CCK, GIP or PYY (Hand et al., 2012).

Taken together, it can be speculated that intestinal secretory cells, which express more than one hormone, may have the ability to differentiate between secretory granule exocytosis. Under this hypothesis, an EEC may be able to control differential peptide release according to stimuli or signal intensity. In this instance this may occur at the level of nutrient detection and activation of cell signaling pathways, but could also involve signals from intracellular nutrient metabolism or systemic sources providing information of infection or energy status.

It is important to remember that the collective data from the secretion studies mentioned were acquired from a population of cells. Therefore they provide no information on a single cell basis to demonstrate the individual characteristics of GI peptide release. To test these theories, single cell analysis could be probed using fluorescent labelling and live-cell confocal imaging. However it is difficult to isolate and probe a live secretory cell without affecting the inner machinery. Secretion studies using isolated pools of primary EECs would be informative yet

an extensive range of potential GI peptides to measure against numerous stimuli may be problematic on such a sparse cell population.

3.4.2.8. Multiple Hormone co-expression within EECs – established property or representative of a sub-population?

It is difficult to ascertain whether GI peptide co-expression encompasses cells that 1) contain a single peptide alone 2) contain two GI peptides or 3) a proportion of cells that contain a large number of different peptides.

Unfortunately, it was not possible to obtain quantitative data for dual-hormone-labelled cells due to limitations with antiserum efficiency and labelling procedure. However the quantitative data obtained examining the hormonal expression of eGFP-CCK cells indicated a vast potential overlap of hormones co-expressed within EECs.

The multi-hormone complement of fluorescently tagged CCK-cells isolated from eGFP-CCK mice were probed using quantitative PCR. This found only half the cells analysed expressed the genes encoding more than one hormone. Furthermore, the expression of more than two hormones within a cell appeared to be a rare occurrence (Egerod et al., 2012). Although these findings support the co-expression data presented in this thesis they do not correlate with the quantitative data detailed. The number of cells that co-express CCK alongside GIP or ghrelin is stated to represent only a small number of cells (Egerod et al., 2012). This is much lower than the relative co-localisation recorded in this thesis whereby GIP or ghrelin co-localised with 37% and 50% total eGFP-cells respectively. However, these two data sets represent analysis of mRNA expression and protein expression respectively, so cannot be taken as a direct comparison.

Studies that have analysed the expression of hormonal mRNA within EECs have demonstrated that hormones are co-expressed within a population of EECs, however this provides no details on a single cell basis. Analysis of single cells using immunohistochemistry is problematic when probing for numerous targets

simultaneously. One of the reasons for this is the limitation in antisera affinities. Firstly peptide epitopes are generally considered to be in close proximity, with relative expression within vesicles of the same cell. This can cause insufficient antisera binding that would fail to generate a signal due to competition for binding sites. Additionally, there are only a finite number of secondary antisera labelling systems that can be used in one experiment as these need to be clearly distinct in order to distinguish between signals. Accounting for the limitations of the procedure, a reasonable assumption using the quantitative data obtained for eGFP staining suggests there is a significant overlap in GI peptide protein expression. However it is difficult to confirm this further.

3.4.3 De-bunking the one-cell one-hormone dogma.

The data presented in this thesis contradict the long standing 'one cell one hormone' hypothesis whereby EEC subtypes were believed to express peptides encoded by only one peptide promoter (Creutzfeldt, 1970). The newly established properties of the enteroendocrine system demonstrate that there is a high degree of crossover between EEC subtypes. This property may enable an EEC to be highly adaptive and respond to environmental and systemic signals to alter peptide expression levels within the cell (Egerod et al., 2012). Classically, EECs undergo constant turnover with an average life span of ~4 days, and therefore have the capacity to enforce a degree of plasticity (Barker et al., 2008). Adaptations can therefore be initiated during cell differentiation where multi-faceted signals determine the generation of each EEC type. This enables EEC characteristics to be able to adapt according to prolonged changes in diet, pathology or other systemic changes.

It is clear that the location of an EEC appears to be a dominant factor in determining the hormonal complement within a cell (Brubaker, 2012). The differences in the hormonal complement of EECs of different regions of the SI have been analysed by Egerod and colleagues. eGFP cells isolated from

duodenum, jejunum and ileum of an eGFP-CCK mouse model demonstrated a significant difference between the hormonal content of cells from different regions of the SI. Most notably this was reflected in the vast decrease in CCK expression in these 'CCK-tagged' cells, which was approximately 5-fold lower in the jejunum compared to the duodenum (Egerod et al., 2012). The expression of secretin, ghrelin, glucagon, neurotensin and PYY were notably different in eGFP-positive cells from each of the three small intestinal regions. The only peptide that displayed any consistency was GIP that had similar level of expression in eGFP cells from duodenum compared with the jejunum. However GIP expression then significantly decreased when analysing ileal eGFP-cells (Egerod et al., 2012). These data are reflected in the shift in the hormonal content of fluorescently tagged cells isolated from different SI regions from GIP-Venus and GLU-Venus mice, representing K- and L-cells respectively. Using these two mouse models it was concluded that 'L-cells' of the proximal SI had more in common with 'K-cells' of the proximal SI than 'L-cells' of the distal SI despite theoretically representing the same EEC phenotype (Habib et al., 2012). These data sets clearly demonstrate that differences in EEC phenotype may be more accurately applicable in comparing EECs of different regions. This is an obvious advantage when considering the variable functions of various regions of the small and large intestine, particularly concerning the nutrients and breakdown products a cell may be exposed to.

The ability to classify a typical and defined EEC subtype may be non-existent. What may exist instead is a hormone complement within EECs that is defined by localisation within the GI tract coupled with the integration of systemic and environmental signals. In this way, EECs may possess the molecular machinery to express a combination of GI peptides according to demands thus demonstrating plasticity of the system with the ultimate end-point; to optimise the digestion and absorption nutrients.

Adaptations in EEC expression and behaviour have been demonstrated in extreme or prolonged changes in diet such as cases of high and low fat diets. These both alter EEC distribution patterns as well as the profile of circulating hormones. This has been closely demonstrated in cases of obesity and in

monitoring subjects following bariatric surgery. In both instances a dramatic alteration in the GI peptide milieu is apparent (Moran et al., 2008) and suggest that the EEC network has the potential to undertake a complete and rapid remodelling (Tschop and DiMarchi, 2011).

It would be interesting to determine relative alterations in cellular hormone complement. This would help establish whether the enteroendocrine system has the capacity to acutely modulate hormone expression according to short-term or prolonged diet changes. Alternatively it would be fascinating to monitor the change in intracellular peptide co-localisation to determine the degree of plasticity within the system. This would provide invaluable information to understanding the behaviours of the enteroendocrine system in cases of over- and under-eating.

The majority of limitations of the presented data originate in using IHC techniques to definitively characterise the properties of a cell population. Further quantitative data are required to define the true scope of the enteroendocrine system in terms of hormone overlap within single EECs. Furthermore quantitative data concerning changes hormone complement through the portions of the small and large intestine would be beneficial. This could be used to demonstrate physiological changes and adaptation in hormonal cells according to prolonged environmental changes. Ultimately immunostaining data can be combined with data obtained from molecular analysis of single cell populations to further characterise the properties of I-cells. However, the most informative source of data would be to perform functional experiments, which to date have been difficult to perform on primary cells.

3.4.4. Summary

The valuable new tool for EEC research, in the form of fluorescently tagged transgenic mice has ushered in a new era of enteroendocrine hormone research and proves that is not possible to define these cells quite so simply.

This chapter has demonstrated that the eGFP-cells from the eGFP-CCK mouse model are a sufficient representation of CCK-cells. Data have been presented that established CCK-cells to co-express a number of anorectic GI peptides and the orexigenic peptide ghrelin. Dual-labelled cells for CCK alongside other GI peptides demonstrated that peptide co-localisation existed in a spectrum of intensities and gave detail to vesicle labelling inside the cell. This observed co-expressed peptides to be contained within the same and distinctly separate vesicle. These data provide evidence that the enteroendocrine system is more adaptive than previously thought.

Chapter Four

Modulation of Enterocyte Fatty Acid Uptake by Gut Hormones

4.1. General Overview

The GI peptide, GLP-2 has been demonstrated to increase FA absorption through up-regulating the expression and activity of the membrane transport protein CD36 (Hsieh et al., 2009). Conversely GLP-1, a product of the same gene actively decreases intestinal FA absorption (Mellitzer and Gradwohl, 2011, Hsieh et al., 2009). The ethanolamide lipid, OEA has also been reported to modulate FA uptake by enterocytes (Yang et al., 2007) however, the archetypal satiety hormone, CCK, has not been tested.

Therefore, the aim of this chapter was to test the null hypothesis that CCK exerts no effect upon FA absorption in intestinal cells. To test this null hypothesis a fluorescent FA analogue was used to assess FA uptake. Firstly, a robust methodology was developed that enabled FA uptake into primary intestinal cells to be measured. This methodology was used to determine the effects of CCK upon FA uptake by enterocytes. Experiments were also performed to determine the involvement of CCK-expressing EECs in modulating cellular FA uptake.

The specific aims of the work presented in this chapter were to:

- Develop a robust method to measure enterocyte FA uptake
- Determine whether CCK modulates FA uptake by enterocytes
- Determine the basic mechanism of CCK-induced enhancement of enterocyte FA uptake
- Determine whether CCK expressing EECs influence FA uptake in enterocytes

The null hypotheses were as follows:

- Incubation with CCK does not enhance FA uptake in enterocytes
- Carrier-mediated transport is not implicated in CCK-induced enterocyte FA transport
- Ligand-stimulated release of CCK from CCK-cells does not induce FA uptake.

4.2. Results

4.2.1 Development of a method to measure Bodipy-FA uptake by primary enterocytes

The Bodipy-FA uptake protocol described by (Yang et al., 2007), (Lynes et al., 2011) and (Gimeno et al., 2003) was adapted to the protocol described in section 2.6. Adaptations included development of a robust method for single cell isolation. The protocol described by Sykaras and colleagues has been demonstrated to successfully isolate intestinal epithelial cells whilst maintaining cell viability (Sykaras et al., 2012). Reagents and equipment were adapted to those available and to maintain feasibility of the protocol. Preliminary experiments were necessary to determine the optimal conditions for experiments using fluorescently labelled Bodipy FA analogues (Bodipy-FA).

4.2.1.1. Time And Dose Responses

The first step in developing the methodology to measure Bodipy-FA uptake was to determine an optimal Bodipy-FA concentration and incubation time period with which to use. These were important because FA uptake into cells relies on facilitative diffusion, thus it was necessary to guard against saturation of the transport process and equilibration of FA across the cellular membrane. Optimal FA concentration represented a concentration that was ideally half the apparent V_{max}, thus giving a dynamic range over which to observe changes. Equally, the optimal incubation time was deemed a period of time during which cells had not reached equilibrium and also a period of time that balanced logistical experimental constraints. To determine the optimal conditions, cells were incubated with Bodipy-FA at concentrations ranging from 2.5 – 50μM and at incubation time periods ranging between 0.5-30mins. Cells were pelleted and washed in 'stop' solution and the cell fluorescence measured using FACS analysis as described in section 2.6.1.

The plot of Bodipy-FA uptake versus Bodipy-FA incubation time showed a curve typical of diffusional flux of FA (Graph 4.1-A). There was a steady increase in cellular Bodipy-FA uptake between incubation periods of 0.5 to 10mins. The uptake then levels off, with only a slight increase in mean cell fluorescence recorded for incubation times of 10 and 30mins. This suggests that by 30mins Bodipy-FA uptake approaches equilibration. The equilibration time point (V_{max}) was calculated at 740R.A.U (Relative Arbitrary Units) using the Michaelis-Menten kinetics equation (calculated using GraphPad Prism software) yet for 30mins incubation with Bodipy-FA a mean cell fluorescence of 565R.A.U was recorded. This indicates incomplete saturation, however the negligible differences in mean fluorescence recorded for incubation periods of 10 or 30mins suggests otherwise. Statistical analysis showed T_{50} for half equilibration occurred at 7.9mins. Therefore an incubation time of this length would ensure ample headroom before equilibration. From a logistical standpoint a period of 2mins was deemed most suitable in terms of the overall length of the experiment and in order to limit any differences due to deterioration of the primary cells.

A standard dose-response curve was constructed by plotting mean cell fluorescence against Bodipy-FA concentration for increasing concentrations of Bodipy-FA when incubated for 2mins (Graph 4.1-B). The resultant plot showed an initial steep increase in mean cell fluorescence recorded between 2.5 - 10μM Bodipy-FA. The graph then flattened off at 25μM and remains level at 50μM Bodipy-FA. V_{max} was calculated at 256RAU, indicating Bodipy-FA uptake had reached saturation. The calculated value for half the maximal FA uptake was 128RAU, corresponding to a Bodipy-FA concentration of 6.3μM. Based on these data, a concentration of 5μM was subsequently taken forward for future experiments.

In consideration of feasible experimental design and to enable a dynamic response in Bodipy-FA uptake to be measured, a 2mins incubation period and a concentration of 5μM Bodipy-FA were taken forward for future experiments.

4.2.1.2. Dose response for established stimulators of fat uptake

OEA and GLP-2 are known modulators of FA uptake in enterocytes (Yang et al., 2007, Hsieh et al., 2009). These compounds were used as positive controls in Bodipy-FA uptake experiments. Dose response experiments were performed to establish effective concentrations with which to use these hormones.
To determine statistical significance one-way ANOVA tests with a Bonferroni multiple comparison test were used.

OEA dose response

Intestinal cells were incubated with OEA at concentrations ranging from 10nM to 50μM. Only at a concentration of 100nM was a significant increase in Bodipy-FA uptake above control levels observed ($p>0.05$, Graph 4.2-A). At 100nM Bodipy-FA uptake increased 3-fold compared to control. At 10nM there was a small but statistically none significant increase in cellular Bodipy-FA uptake. At a concentration of 1μM OEA induced a small but statistically non-significant increase and at higher concentrations OEA did not elicit an increase in Bodipy-FA uptake compared to control. Therefore, as 100nM OEA produced the only statistically significant result this concentration was used in subsequent experiments.

GLP-2 dose response

Incubation of primary intestinal cells *in vitro* with GLP-2 had a biphasic dose dependent effect of Bodipy-FA uptake. Addition of GLP-2 to cells at a concentration of 10pM increased Bodipy-FA uptake more than 3-fold (Graph 4.2-B). At higher concentrations, GLP-2 induced small increases in Bodipy-FA uptake that did not reach statistical significance until a concentration of 10nM was reached. At 10nM and 100nM, GLP-2 caused a significant increase in Bodipy-FA uptake compared to control ($p>0.01$). The lowest concentration that stimulated a significant increase in Bodipy-FA uptake was 10pM and this concentration was chosen to be used in future experiments.

Qualitative comparison of the dose response described by OEA to that observed for GLP-2 revealed a considerable difference in the overall dose responses; OEA only stimulated Bodipy-FA uptake to a statistical significant level at a concentration of 100nM whereas GLP-2 showed a biphasic effect on Bodipy-FA uptake.

CCK (Sulphated) dose response

Intestinal cells were incubated with CCK at concentrations ranging from 1pM to 10nM. CCK at a concentration of 10pM produced a statistically significant increase in Bodipy-FA uptake above control levels ($p>0.01$, Graph 4.3-A). At 10pM Bodipy-FA uptake doubled compared to control. At 1pM there was a small increase in Bodipy-FA uptake but this was not statistically significant. The Bodipy-FA uptake at 1pM was comparable to that observed at higher concentrations of 100pM, 1nM and 10nM CCK, all of which also failed to significantly increase uptake. In summary, CCK at a concentration of 10pM CCK caused a doubling in FA uptake. The dose response of the CCK effect showed a bi-phasic pattern. To further investigate the CCK response the active concentration of 10pM was used in subsequent experiments.

CCK Non-sulphated (CCK-NS) dose response

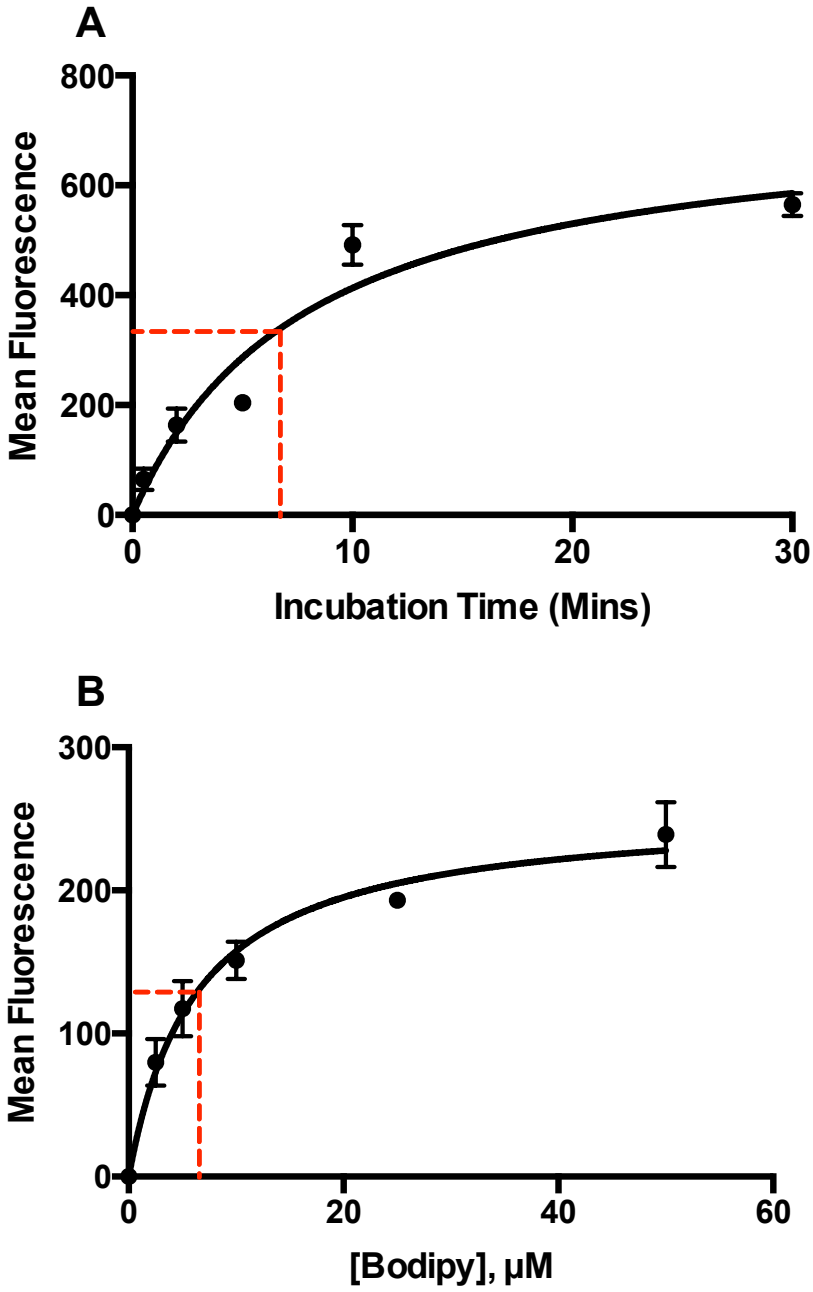
Intestinal cells were incubated with CCK-NS at concentrations ranging from 1pM to 10nM. In contrast to sulphated CCK, there were no statistically significant changes in cellular Bodipy-FA uptake within this concentration range. At a concentration of 10pM Bodipy-FA uptake increased almost 2-fold above control levels yet statistical analysis of the experiment as a whole revealed no statistically significant differences (Graph 4.3-B). At 1pM, 1nM and 10nM concentration of CCK-NS Bodipy-FA uptake was not different to control. Surprisingly, at 100pM, CCK-NS caused a marginal decrease in Bodipy-FA uptake compared to control values.

Bodipy-FA uptake for cells incubated with 10pM sulphated CCK was included as a positive control and a significant increase (of 3-fold) in Bodipy-FA uptake was observed.

4.2.1.3. Testing the effect of CCK to increase Bodipy-FA uptake in three different mice strains.

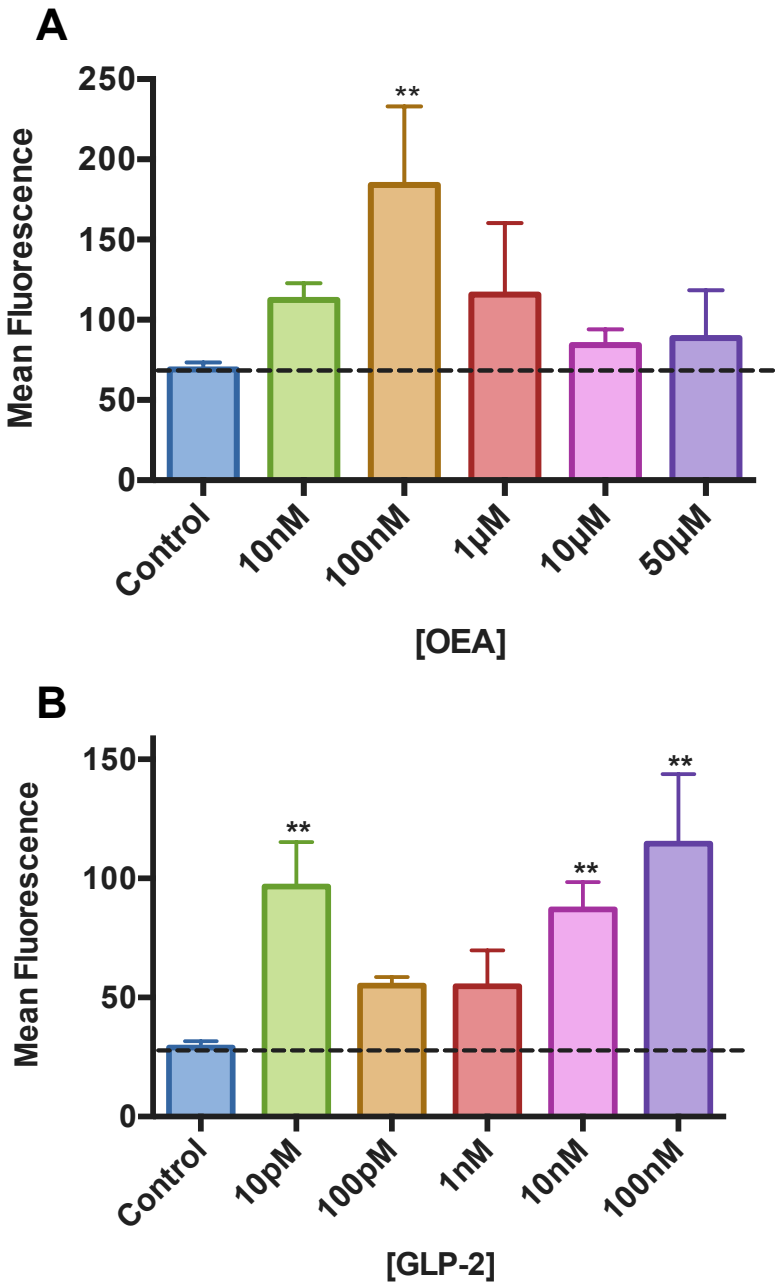
To ensure that the increase in FA uptake elicited by CCK was not dependent on the mouse strain employed Bodipy-FA uptake experiments were performed on intestinal cells from eGFP-CCK, CCK^{LacZ} and C57 WT mice and the results were compared. To enable meaningful comparisons to be made, data were normalised to the relevant mean control (Graph 4.4). For the cell population from each mouse strain, optimised concentrations of CCK (10pM) induced a significant increase ($p>0.01$) in Bodipy-FA uptake. Therefore the relative CCK-induced response on cellular FA-uptake appears to be universal and is therefore non-strain dependent.
Cells from all strains showed approximately a doubling of FA uptake when exposed to 10pM CCK.

In conclusion, CCK has the ability to increase Bodipy-FA uptake in intestinal cells, irrespective of mouse strain. Therefore the null hypothesis 'CCK does not have an effect upon FA uptake by enterocytes' was rejected.



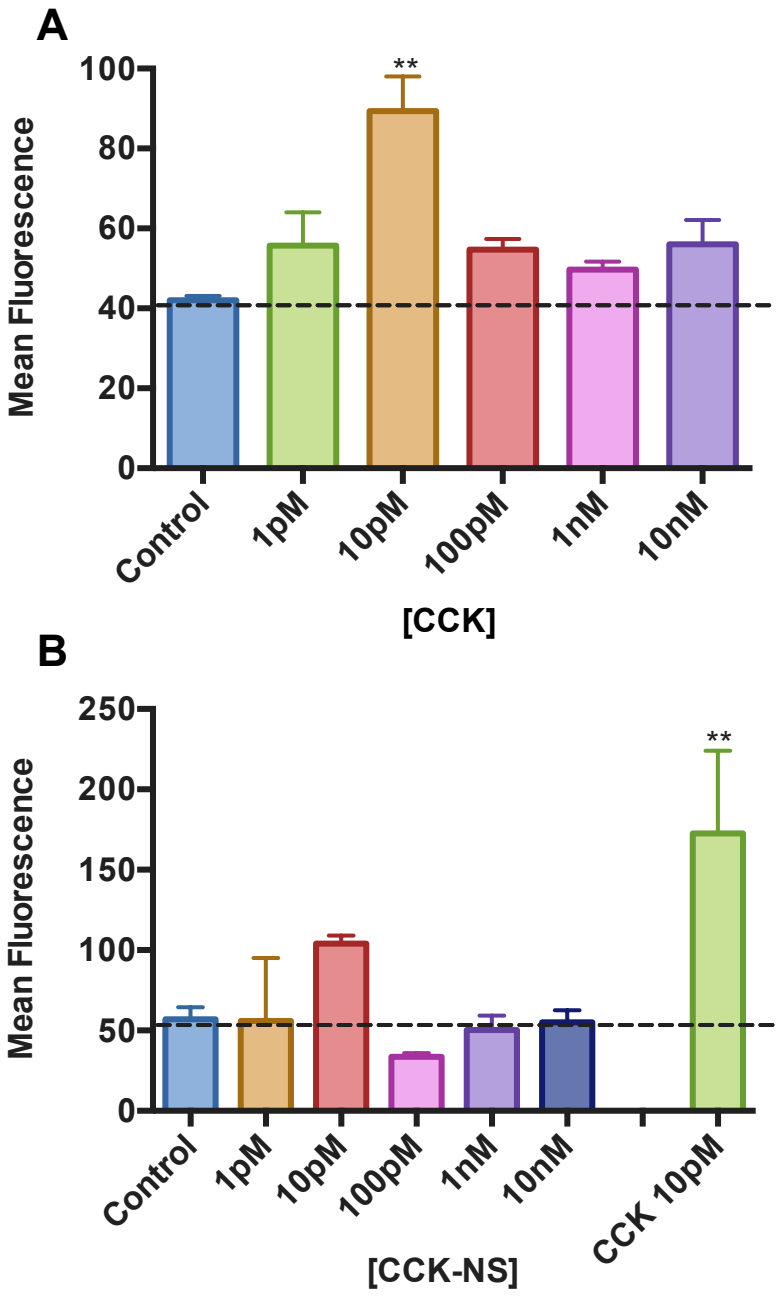
Graph 4.1. Determination of the optimal incubation time and concentration of Bodipy-FA for uptake studies.

A) Incubation time optimisation; mean cell fluorescence plotted against incubation time. Cells were incubated with Bodipy-FA for periods ranging from 0.5-30mins. The T_{50} was calculated at 7.9mins. B) Optimisation of Bodipy-FA concentration; mean cell fluorescence plotted against Bodipy-FA concentration. Cells were incubated in Bodipy-FA at concentrations ranging between 2.5–50µM. The EC_{50} was calculated at 6.3µM.
Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3). The red dash line indicates T_{50} and EC_{50} values.



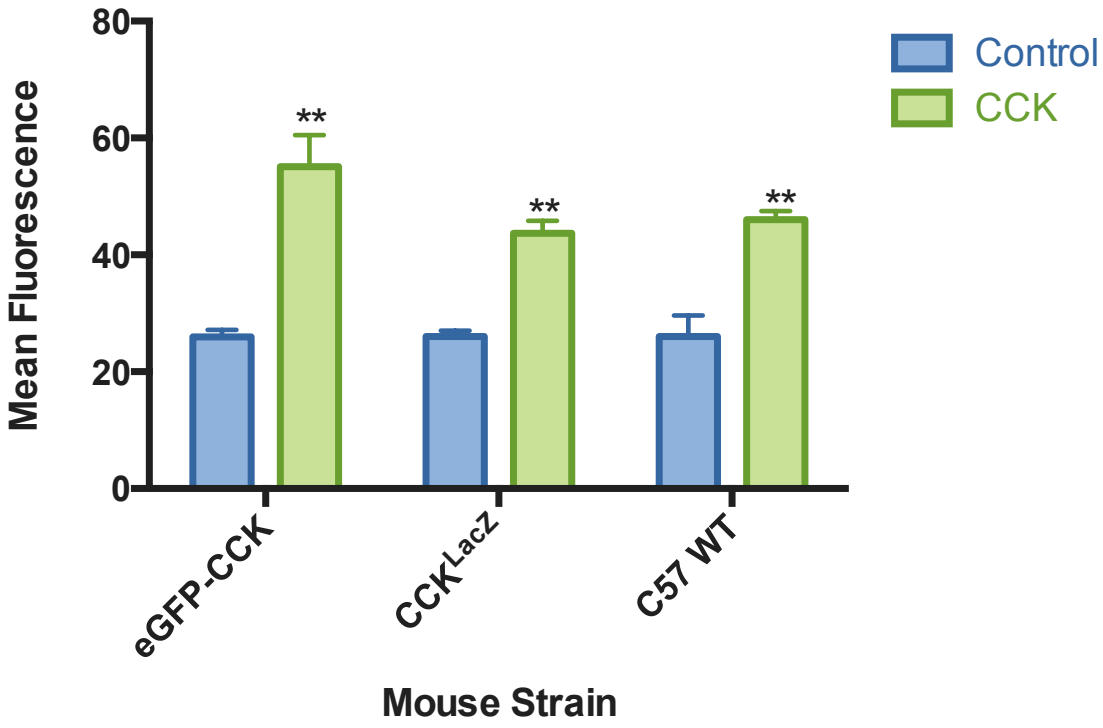
Graph 4.2. Dose response for OEA and GLP-2 on Bodipy-FA uptake.

A) OEA dose response curve. Intestinal cells were incubated for 15mins in concentrations of OEA ranging from 10nM to 50µM. B) GLP-2 dose response curve. Cells were incubated for 15mins in GLP-2 at concentration ranging from 10pM to 100nM. Following treatment, cells were incubated with Bodipy-FA for 2mins. Significant increases in fluorescence recorded at 100nM OEA; and at 10pM, 10nM and 100nM GLP-2.
Using FACS analysis to measure cellular fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared with control. Black dash line represents respective mean control value.



Graph 4.3. Dose Response for CCK (sulphated) and CCK-NS (non-sulphated).

A) CCK (sulphated) dose response curve. Intestinal cells were incubated for 15mins in concentrations of CCK ranging from 1pM to 10nM. B) CCK-NS dose response curve. Cells were incubated for 15mins in CCK-NS ranging from 1pM to 10nM. Cells were then incubated with Bodipy-FA for 2mins. Significant increases in cell fluorescence occurred for 10pM CCK whereas CCK-NS failed to produce a significant effect on Bodipy-FA uptake (positive control data included).
Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) **=p>0.01 compared to control. Black dash line represents respective mean control value.



Graph 4.4. Testing the CCK response in different mouse strains.

Intestinal cells from eGFP-CCK mice, CCK^{LacZ} mice or C57 (WT) mice were incubated with 10pM CCK. For each mouse strain cell population, a significant increase in Bodipy-FA uptake was observed following pre-treatment with CCK (10pM). Each data set has been normalised to respective control mean to enable direct comparisons.

Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Blue bars represent mean values of non-treated cells. Green bars represent cells incubated with CCK (10pM). Means represent triplicate values and error bars represent SEM (n=3). **=p>0.01 compared with control.

.2.2. Experiments to determine the basic mechanism of CCK-induced enterocyte FA uptake

4.2.2.1. The effect of loxiglumide on CCK-induced stimulation of Bodipy-FA uptake.

To test the involvement of the CCK-R_A in the CCK-induced response the CCK-R_A-specific inhibitor, loxiglumide, was employed. Cells were prepared as described and Bodipy-FA uptake measured in the presence of 100μM loxiglumide. Incubation of cells with 100μM loxiglumide alone caused no significant change in Bodipy-FA uptake. As before, incubation of intestinal cells with 10pM CCK caused the characteristic increase in Bodipy-FA uptake ($p>0.05$) when compared with control values (Graph 4.5). Addition of 100μM loxiglumide alongside CCK completely inhibited the CCK-induced increase in Bodipy-FA uptake ($p>0.01$). Loxiglumide therefore significantly blocked the stimulatory action of CCK on Bodipy-FA uptake.

4.2.2.2. The effects of phloretin on hormone stimulated Bodipy-FA uptake.

To determine if FA uptake was carrier mediated, two well-characterised inhibitors of membrane transporters were employed. Phloretin, a compound know to inhibit protein mediated FA transport, including that mediated by CD36 (Abumrad et al., 1981, Ibrahimi et al., 1996), or the CD36-specific inhibitor sulfo-N-succinimidyl oleate (SSO) (Coort et al., 2002, Schwenk et al., 2010, Pohl et al., 2005) were applied in addition to the proven stimulators of Bodipy-FA uptake presented in section 4.2.1.2. One-way ANOVA tests with a Bonferroni multiple comparison test were used to test for statistical significance.

Incubation of intestinal cells with OEA 100nM (Graph 4.6-A) or GLP-2 (Graph 4.6-B) as previously observed resulted in a significant ($p>0.05$ and $p>0.01$) increase in mean cell fluorescence when compared to control. Incubation of intestinal cells with 200μM phloretin alone significantly reduced control Bodipy-FA uptake ($p>0.01$), implying that under control conditions a substantial amount of FA

uptake is sensitive to phloretin and therefore likely to be transporter mediated. Incubation of cells with OEA or GLP-2 in the presence of phloretin abolished the hormone-stimulated increase in Bodipy-FA uptake and reduced FA uptake to values below the respective mean control ($p>0.01$). Therefore phloretin completely inhibited the stimulatory effects of OEA and GLP-2.

The effects of phloretin on CCK-induced Bodipy-FA uptake

Incubation of intestinal cells with 10pM CCK characteristically caused a significant increase in Bodipy-FA uptake ($p>0.01$, Graph 4.6-C). Incubation with phloretin decreased Bodipy-FA uptake. Incubation of cells with CCK and phloretin caused a significant ($p>0.05$) decrease in Bodipy-FA uptake compared to incubation with 10pM CCK alone. Therefore phloretin inhibited the stimulatory effects of CCK.

From these data it can be concluded that the observed increase in cellular Bodipy-FA uptake was sensitive to phloretin, a known inhibitor of membrane transporter proteins. Therefore the hormone-induced increase in Bodipy-FA uptake was likely to be mediated by membrane transporter proteins. Due to the non-specific inhibitory actions of phloretin it was deemed necessary to perform additional experiments to better identify the transporter protein mediating the Bodipy-FA effect. The membrane protein CD36 was a prime a candidate responsible for transmembrane FA movement. Therefore SSO, a potent and selective inhibitor of the FA translocase CD36, was employed.

4.2.2.3. The effects of SSO on hormone stimulated Bodipy-FA uptake

The effects of SSO on OEA or GLP-2 stimulated Bodipy-FA uptake

Incubation of intestinal cells with 100nM OEA (Graph 4.7-A) or 10pM GLP-2 (Graph 4.7-B) resulted in a significant ($p>0.05$) increase in Bodipy-FA uptake. Incubation of intestinal cells with 1μM SSO alone resulted in no change in the mean cell fluorescence compared to control values. Treatment of cells with either OEA or GLP-2 increased Bodipy-FA uptake 1.5-fold and 1.8-fold respectively. In comparison, incubation of intestinal cells with OEA or GLP-2 in the presence of 1μM SSO completely inhibited the hormone-stimulated uptake of Bodipy-FA. These data suggest that OEA or GLP-2 stimulated FA uptake is mediated by CD36. Interestingly, unlike phloretin, treatment with SSO did not cause Bodipy-FA uptake to decrease to below control levels

The effect of SSO upon CCK-stimulated Bodipy-FA uptake

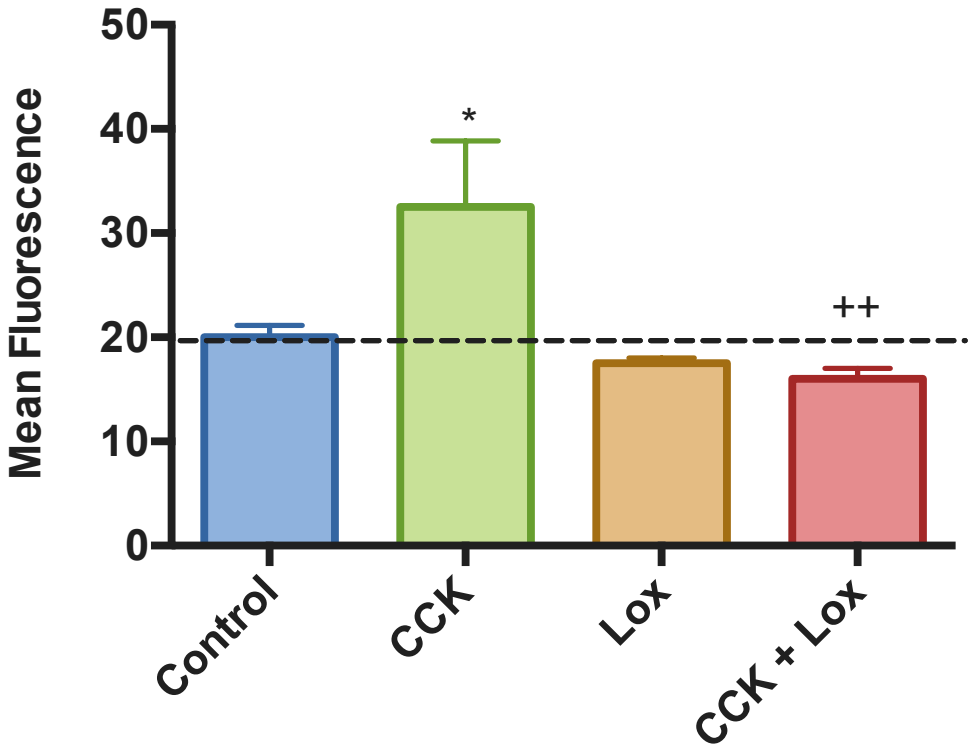
Incubation of intestinal cells with CCK 10pM induced a significant ($p>0.01$) increase in Bodipy-FA uptake (Graph 4.7-C). Incubation of cells with SSO alone had no effect on Bodipy-FA uptake. In comparison, SSO completely abolished the CCK-stimulated increase in Bodipy-FA uptake. From these results it was concluded that the CCK-induced increase in Bodipy-FA uptake was mediated by CD36.

Together these data showed that SSO inhibited the stimulatory effects of OEA, GLP-2 or CCK on cellular Bodipy-FA uptake and suggested that the membrane protein CD36 mediated this increase in FA uptake.

Summary

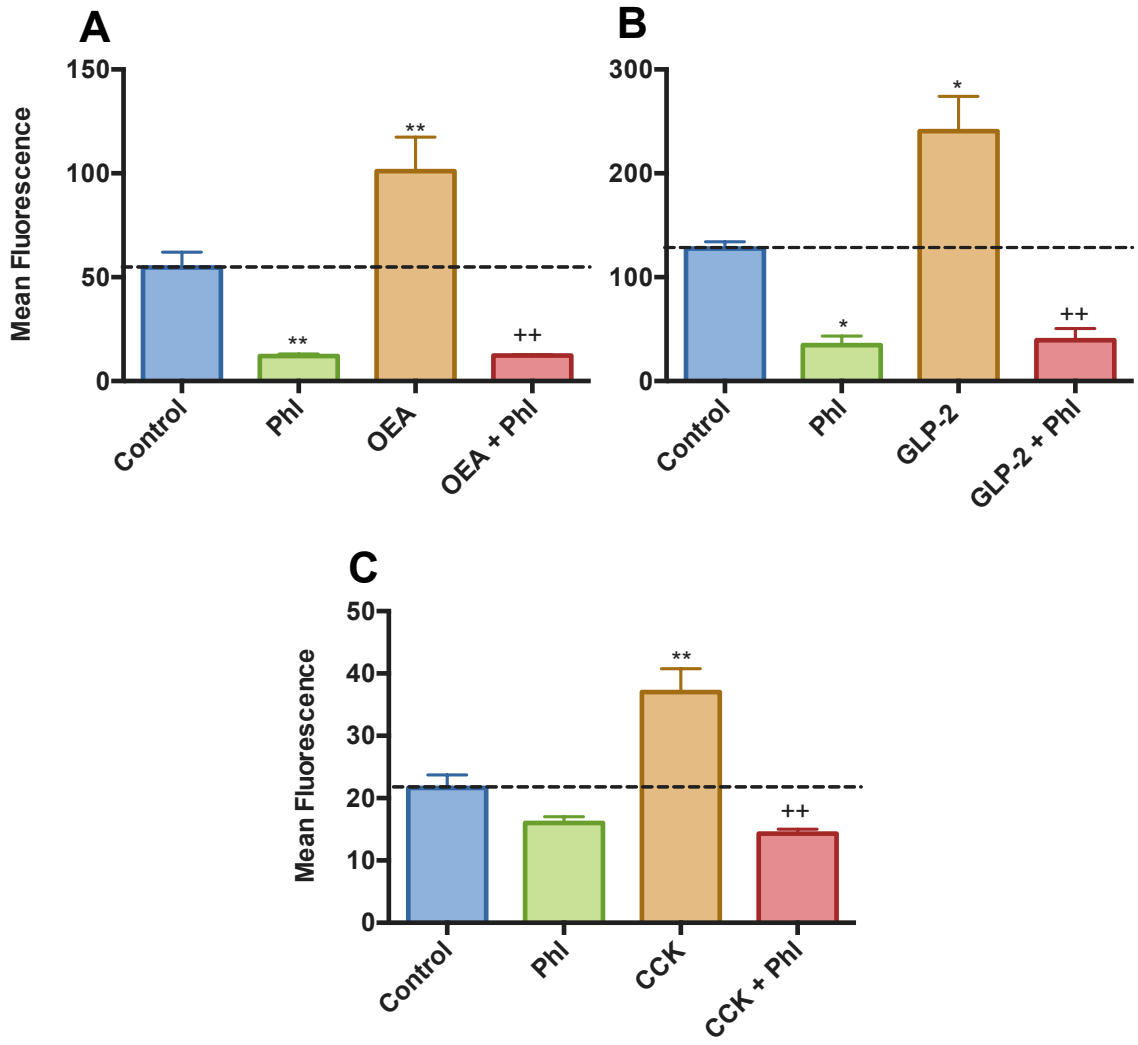
These experiments have collectively determined some of mechanisms involved in hormone-induced increase in FA uptake in intestinal cells. Use of the CCK-R_A antagonist demonstrated that CCK exerts these effects through interaction with CCK-R_A. Furthermore application of phloretin demonstrated that the increase in FA uptake was protein-mediated, and application of SSO demonstrated this was attributable to the fatty acid translocase protein CD36.

The subsequent aim of this study was to take one step back and implicate CCK-containing I-cells in this process.



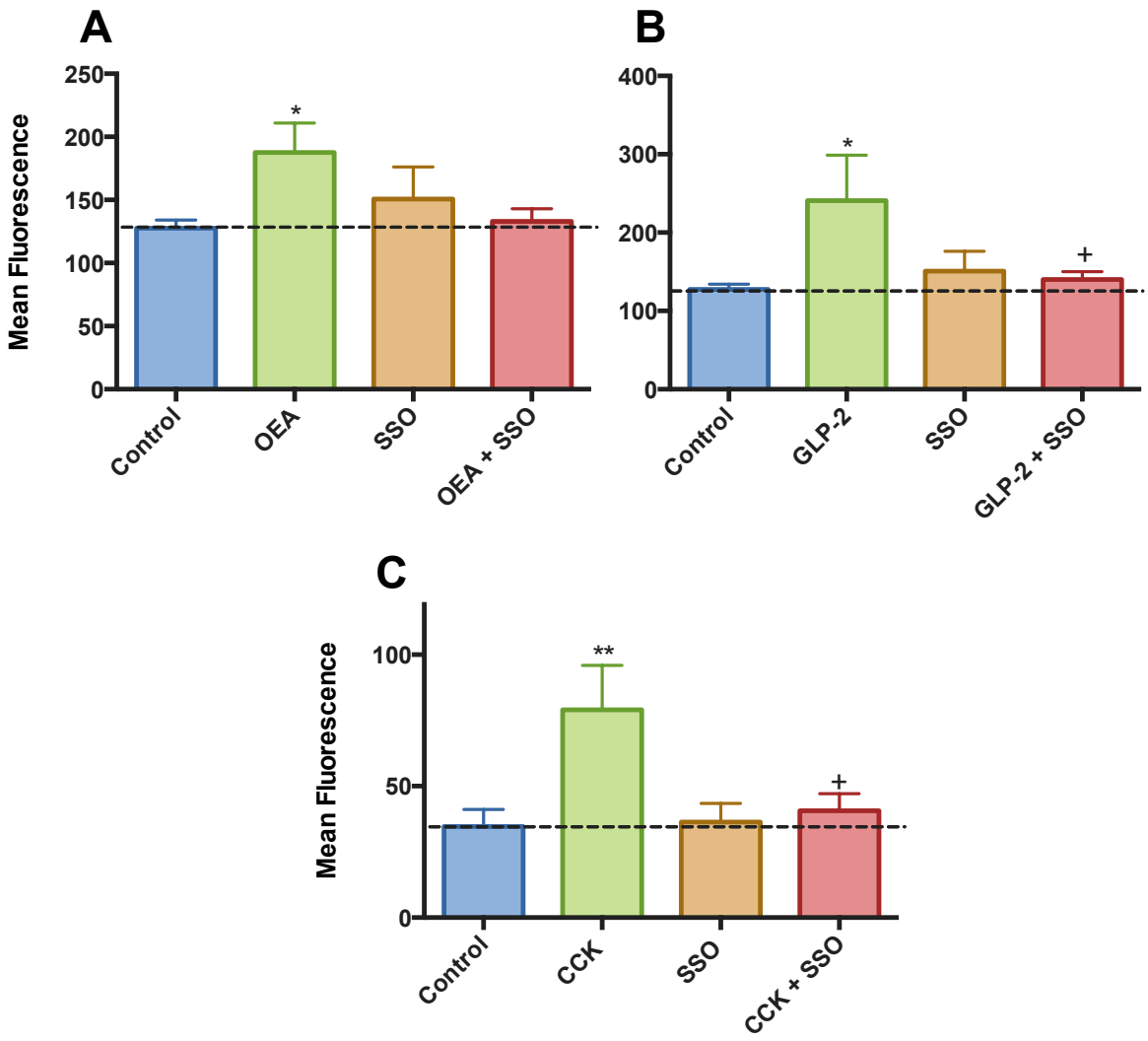
Graph 4.5. Loxiglumide inhibits the stimulatory effects of CCK on cellular Bodipy-FA uptake.

The stimulatory effects of CCK 10pM were significantly inhibited when applied alongside Loxiglumide (Lox) 100μM. Incubation with loxiglumide alone caused no significant change in cell fluorescence when compared with control. Cells were incubated for 15mins with peptides, before application of Bodipy-FA for 2mins.
Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent ± SEM (n=3) *=p>0.05 compared to control; ++=p>0.01 compared to cells pre-treated with CCK alone. Black dash line represents respective mean control value.



Graph 4.6. Phloretin inhibits Bodipy-FA uptake

Cellular Bodipy-FA uptake was significantly increased when incubated with peptides A) OEA B) GLP-2 and C) CCK, and significantly decreased when phloretin 200µM was added, in all instances. Cells were incubated for 15mins with peptides, followed by incubation with Bodipy-FA for 2mins.
Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared to control; ++=p>0.01 compared to cells pre-treated with stimulator. Black dash line represents respective mean control value.



Graph 4.7. SSO inhibits the stimulatory effects of OEA, GLP-2 and CCK upon Bodipy-FA uptake.

Cellular Bodipy-FA uptake was significantly increased when incubated with stimulatory peptides A) OEA B) GLP-2 and C) CCK. This increase was abolished in the presence of 1μM SSO. SSO alone had no effect on mean cell fluorescence compared with control.
Cells were incubated for 15mins with peptides, before application of Bodipy-FA for 2mins. Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared to control; +=p>0.05 compared to stimulator. Black dash line represents respective mean control value.

4.2.3. Experiments to determine whether CCK expressing EECs influence FA uptake in enterocytes.

The aim of this section was to establish the involvement of CCK-expressing EECs to elicit the CCK-induced effects upon FA uptake. Known stimulants of EEC secretion were applied and Bodipy-FA uptake was measured. CCK^{LacZ} mice were employed for comparison since these animals were null for CCK.

Stimulation of EECs by Bombesin

4.2.3.1. Bombesin dose response

Bombesin is a tetrapeptide that stimulates the release of CCK (Banks, 1980, Chen et al., 2006, Wang et al., 2011, Cuber et al., 1989). Intestinal cells were incubated with bombesin at concentrations ranging from 1pM to 1μM. Incubation with 100pM or 1nM bombesin caused a statistically significant increase ($p>0.01$) in Bodipy-FA uptake (Graph 4.8) in that cell fluorescence increased more than 2-fold compared to control values.
At all other concentrations tested bombesin did not cause a statistically significant change in Bodipy-FA uptake compared to control. Therefore, subsequent experiments utilised 1nM bombesin.

Comparison of the dose response curve described for CCK to that observed for bombesin revealed similarities. Both achieved significant stimulation of Bodipy-FA uptake at an optimal concentration and at other concentrations there was little or no effect. Whether this relationship is substantiated remains to be confirmed.

4.2.3.2. The effect of loxiglumide on bombesin-stimulated Bodipy-FA uptake

Incubation of cells with 100μM loxiglumide, the CCK-R_A-specific inhibitor, produced no change in mean cell fluorescence when compared with control

values. Incubation of intestinal cells with 1nM bombesin caused a significant ($p>0.01$) increase in cell fluorescence (Graph 4.9-A). Incubation of cells with 1nM bombesin and 100μM loxiglumide abolished the stimulatory effects of 1nM bombesin ($p>0.01$). In fact, incubation of cells with loxiglumide and bombesin caused a reduction in mean cell fluorescence compared with uptake in control untreated cells.

Therefore, these data suggest loxiglumide inhibits the stimulatory effects induced by bombesin, suggesting that bombesin's stimulatory effects are likely mediated by CCK and involve CCK-R_A.

4.2.3.3. The effect of phloretin on bombesin-stimulated Bodipy-FA uptake

Intestinal cells incubated with bombesin 1nM resulted in a significant increase in mean cell fluorescence ($p>0.05$) (Graph 4.9-B) compared with control values. Incubation of cells with phloretin 200μM alone caused significant decrease in Bodipy-FA uptake from control values ($p>0.01$). Phloretin also abolished bombesin-stimulated Bodipy-FA uptake ($p>0.01$). Mean cell fluorescence values for cells incubated with bombesin and phloretin were reduced to a level only marginally higher than that recorded for phloretin alone samples.

Therefore, as for the previous experiments, phloretin inhibited Bodipy-FA transport in intestinal cells even in the presence of bombesin thus phloretin can be considered to inhibit the stimulatory effect of bombesin via inhibition of FA transporters.

4.2.3.4. The effect of SSO on bombesin-stimulated Bodipy-FA uptake

SSO at 1μM did not affect basal Bodipy-FA uptake. As before, incubation of intestinal cells with 1nM bombesin caused a significant ($p>0.005$) increase in cell fluorescence (Graph 4.9-C) whereas incubation with bombesin and SSO together negated this response ($p>0.05$). Therefore, like that observed for CCK-stimulated

Bodipy-FA uptake, SSO inhibited the effects of bombesin and thus indicates that bombesin is stimulating FA uptake in a process mediated by CD36.

4.2.4. Stimulation of I-cells using nutrients

Fatty acids (McLaughlin et al., 1999, Feltrin et al., 2004) or amino acids (Meyer et al., 1976, Liou et al., 2011a, Liddle, 1995, Feltrin et al., 2004) are established inducers of CCK release, presumably from I-cells. It was deemed practically unfeasible to measure fluorescent-FA uptake in the presence of a transportable FA agonist because the unlabelled 'stimulatory FA' would alter the driving force for Bodipy-FA uptake and hence cause an apparent increase in Bodipy-FA uptake over and above any increase due to FA stimulatory effect. Although, scenarios were envisaged that might have enabled stimulation by FA to be tested, these were deemed far more complicated and susceptible to uncontrollable variability than stimulation using amino acids. Therefore, experiments using amino acids as a previously proven ligand to stimulate CCK release were undertaken.

4.2.4.1. Do amino acids stimulate Bodipy-FA uptake?

Intestinal cells were incubated with either the aromatic amino acids L-phenylalanine, or L-tryptophan or L-histidine, or the aliphatic amino acid L-alanine all at a concentration of 10mM. In addition, the aminoglycoside antibiotic neomycin was also tested at 100μM since this is a known agonist of the CaSR (Graph 4.10). The amino acids, L-phenylalanine, L-tryptophan and L-histidine caused a significant ($p>0.05$) increase in mean cell fluorescence compared to control values. Of these, L-phenylalanine induced the largest increase ($p>0.01$) in Bodipy-FA uptake, increasing cell fluorescence 4-fold compared to control values. L-tryptophan increased cell fluorescence 3-fold and L-histidine 2-fold above mean control values. Incubation with neomycin and L-alanine caused a small but statistically none significant increase in Bodipy-FA uptake.

Therefore, incubation of cells with the aromatic amino acids L-phenylalanine, L-tryptophan and L-histidine stimulated an effective increase in Bodipy-FA uptake. These data indicate that amino acids themselves are able to mediate the activation of cellular pathways involved in FA uptake.

4.2.5. Using CCK^{LacZ} mice as a model to exclude endogenous CCK expression

Intestinal cells isolated from CCK^{LacZ} mice were employed to investigate the effects of endogenous CCK expression for the Bodipy-FA uptake experiments presented. These cells represented a CCK KO model. The experimental procedure was followed as before.

4.2.5.1. CCK dose response in CCK KO cells

Intestinal cells from CCK^{LacZ} mice were incubated with CCK at a concentration range between 1pM to 10nM. CCK at a final concentration of 10pM produced the only statistically significant increase in Bodipy-FA uptake above control levels ($p>0.01$, Graph 4.11). At 10pM CCK, Bodipy-FA uptake increased four-fold. 1pM and 100pM CCK increased cell fluorescence yet to values not statistically significant. Furthermore, at 1nM and 10nM concentrations, mean cell fluorescence was comparable to that of control.

Comparison of this CCK dose response with observed in CCK-replete cells (Graph 4.3-A) reveals a similar dose response curve was achieved for both cell models and 10pM CCK was the only concentration of CCK that stimulated Bodipy-FA uptake. Therefore the absence of endogenous CCK did not affect the ability of the intestinal cells to respond to incubation with CCK.

4.2.5.2. Do CCK KO cells display an altered response to stimulants of Bodipy-FA uptake?

It was established that application of CCK induced a dose response of Bodipy-FA uptake in intestinal cells of CCK^{LacZ} mice. It was important to establish whether, in the absence of endogenous CCK, the mechanisms involved in modulating cellular FA uptake were still in place. Therefore CCK^{LacZ} cells were incubated with OEA, GLP-2 and bombesin to determine whether the lack of CCK expression affected the Bodipy-FA uptake response in these cells.
Incubation of CCK KO cells with 10pM GLP-2, elicited a significant increase in cell fluorescence ($p>0.05$) from control values (Graph 4.12). Additionally, incubation of cells with either 100nM OEA or 1nM bombesin also induced a significant increase in cell fluorescence ($p>0.01$) where values increased approximately two-fold compared to control values (Graph 4.12).

Therefore it can be concluded that lack of endogenous CCK did not affect the Bodipy-FA response to administration of OEA, GLP-2 or bombesin. Importantly, these data suggested that all the agents tested did not require endogenous CCK to elicit their stimulatory effects and that either they were mediated by causing the release of a different hormone or had a direct effect on transporting enterocytes. The finding that bombesin did not require endogenous CCK for its stimulatory effect confounded the premise of the experiment described in section 4.2.3. which aimed to use bombesin to stimulate CCK release from EECs.

4.2.5.3 Do amino acids stimulate Bodipy-FA uptake in cell populations from CCK KO mice?

Intestinal cells from CCK^{LacZ} mice were incubated with either the aromatic amino acids L-phenylalanine, or L-tryptophan or L-histidine, or the aliphatic amino acid L-alanine all at a concentration of 10mM. In addition, the aminoglycoside antibiotic neomycin, a known agonist of CaSR, was also tested at 100μM (Graph

4.13). The amino acids, L-phenylalanine, L-tryptophan and L-histidine stimulated significant increases in cell fluorescence ($p>0.01$), increasing cell fluorescence more than 3-fold above control values.

Incubation of CCK KO cells with neomycin also significantly increased ($p>0.05$) Bodipy-FA uptake. Furthermore, incubation of CCK KO cells with L-alanine caused an increase in Bodipy-FA uptake but this was not to a statistically significant value.

These data suggest that endogenous CCK is not essential for the amino acid mediated response to enhance Bodipy-FA uptake. This demonstrates amino acids are not a suitable ligand to establish a role for I-cells in FA uptake.

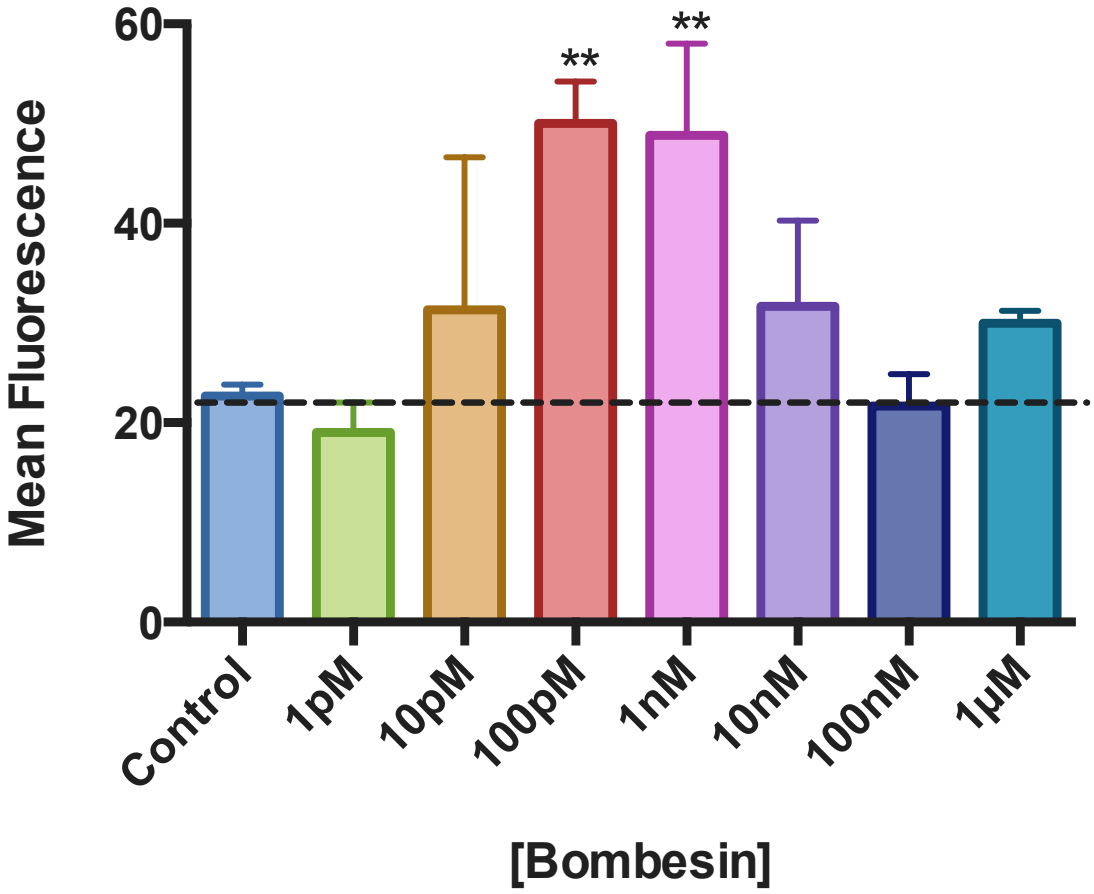
4.2.6. Using a eGFP minus cell population as a model of excluding native I-cells.

In many KO models, compensatory mechanisms are in place, which enable normal physiological processes to continue. Due to the fact that CCK^{LacZ} mice display a normal phenotype and do not appear malnourished it is probable that this may be the case. In this way the results acquired using CCK^{LacZ} cells could be deemed attributable to the affects of compensatory mechanisms that may include an altered or increased expression of other GI peptides that could exert effects similar to CCK or an altered stimulatory profile of these peptides. In this way stimulation of EECs by bombesin or amino acids could instigate release of other GI peptides that may have the ability to modulate FA absorption. In addition to this, it needs to be tested whether the effect exerted by bombesin or amino acids is at the level of directly interacting with enterocytes as opposed to indirectly through the release of GI hormones.

Therefore, it was decided to investigate the effect of the removal of CCK-expressing cells upon CCK or amino acid stimulated Bodipy-FA uptake. FACS cell sorting was used to sort cell populations from eGFP-CCK transgenic mice. Cells were sorted into eGFP+ and eGFP- populations. Using this approach it has been

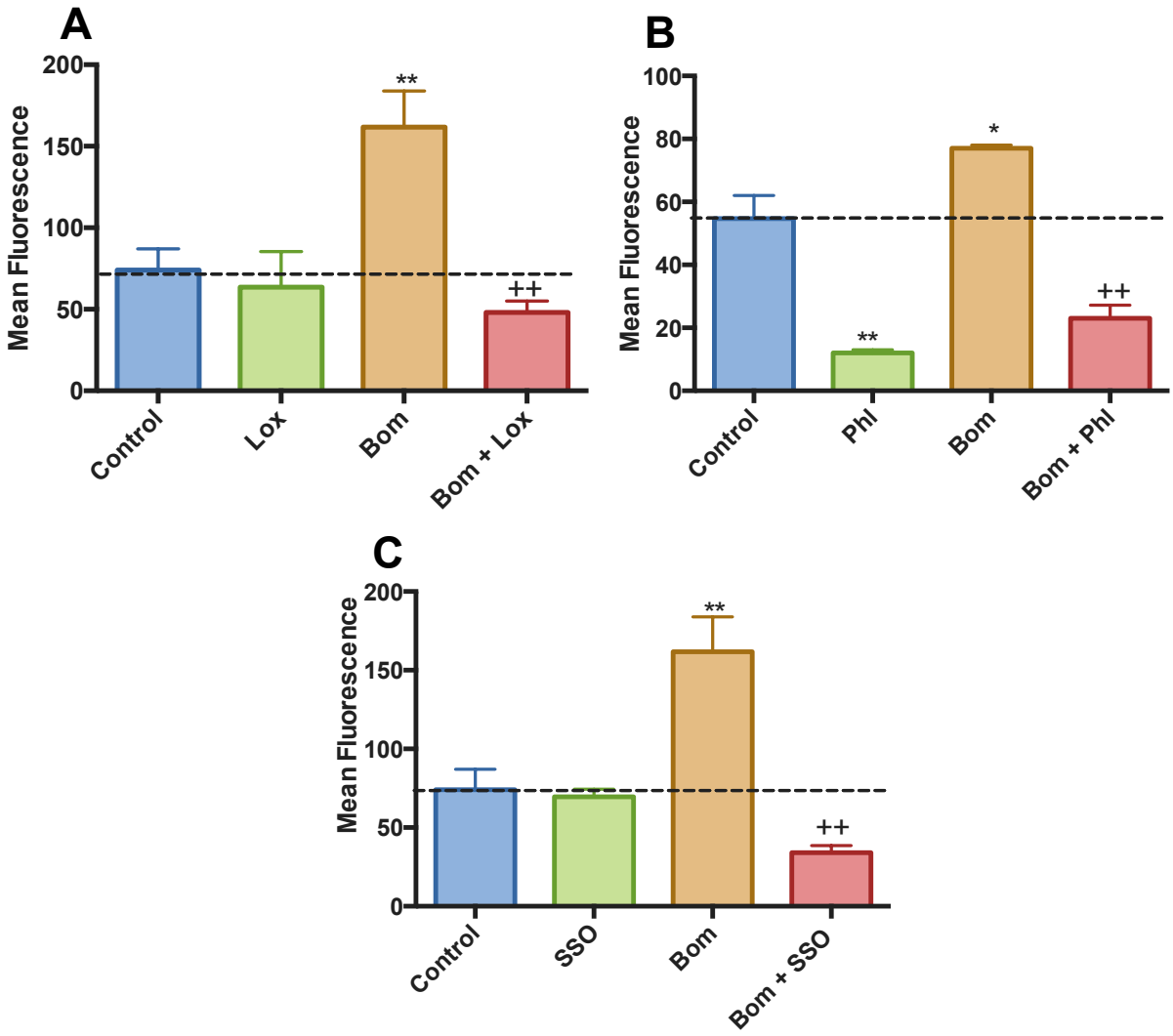
previously shown that the eGFP- population is effectively devoid of CCK expressing cells (Sykaras et al., 2012). Experiments were performed on eGFP- cell populations and the total cell population was used as a positive control. To enable comparisons, data were normalised to control mean values (non-treated cells for both populations) and then pooled. Values are represented as 'fold-increase' in mean cell fluorescence compared to non-treated values (control) (Graph 4.14).

CCK and L-phenylalanine incubation increased Bodipy-FA uptake in total cell samples and in eGFP- cell samples (Graph 4.14). Incubation of cells with 10pM CCK induced a 1.9-fold increase in Bodipy-FA uptake in both cell populations. Incubation with 10mM L-Phenylalanine caused a 1.6-fold increase for total cell population and a comparable 1.5-fold increase in eGFP cell deplete population. These data show that the effects of neither CCK nor L-phenylalanine on Bodipy-FA uptake can be deemed attributable to CCK-containing cells.



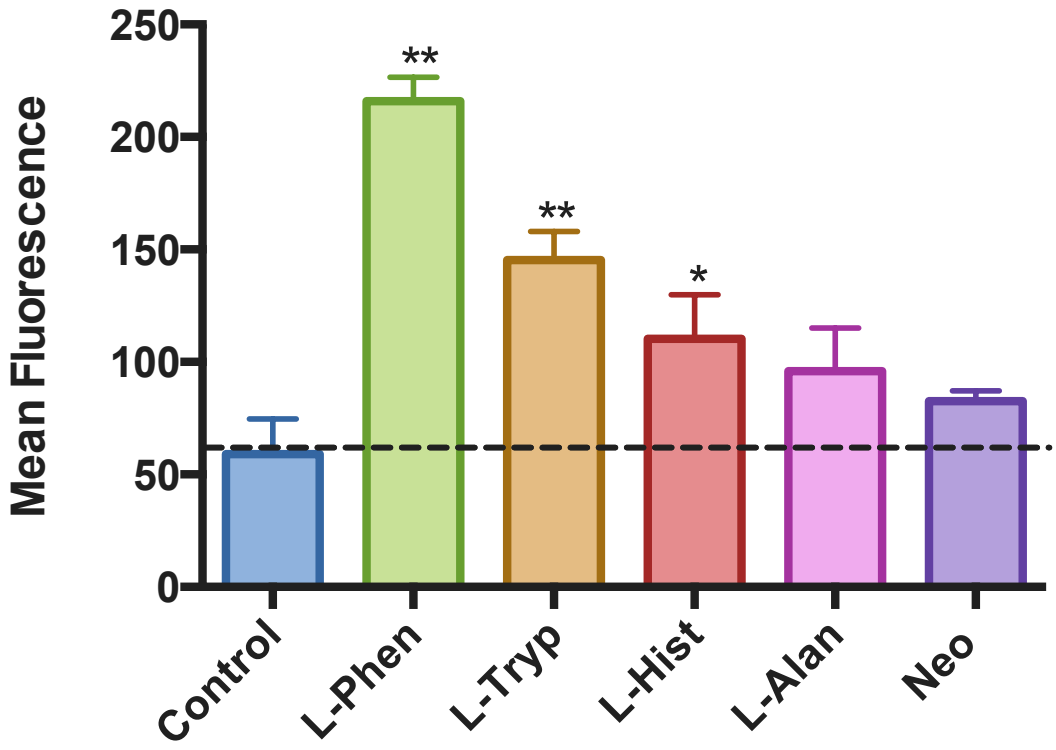
Graph 4.8. Dose response of bombesin incubation on Bodipy-FA uptake.

Intestinal cells were pre-incubated for 15mins with concentrations of bombesin ranging from 1pM to 1µM before application of Bodipy-FA for 2mins.
Significant increases in cell fluorescence were recorded for 100pM and 1nM bombesin. Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared to control. Black dash line reads across from control values.



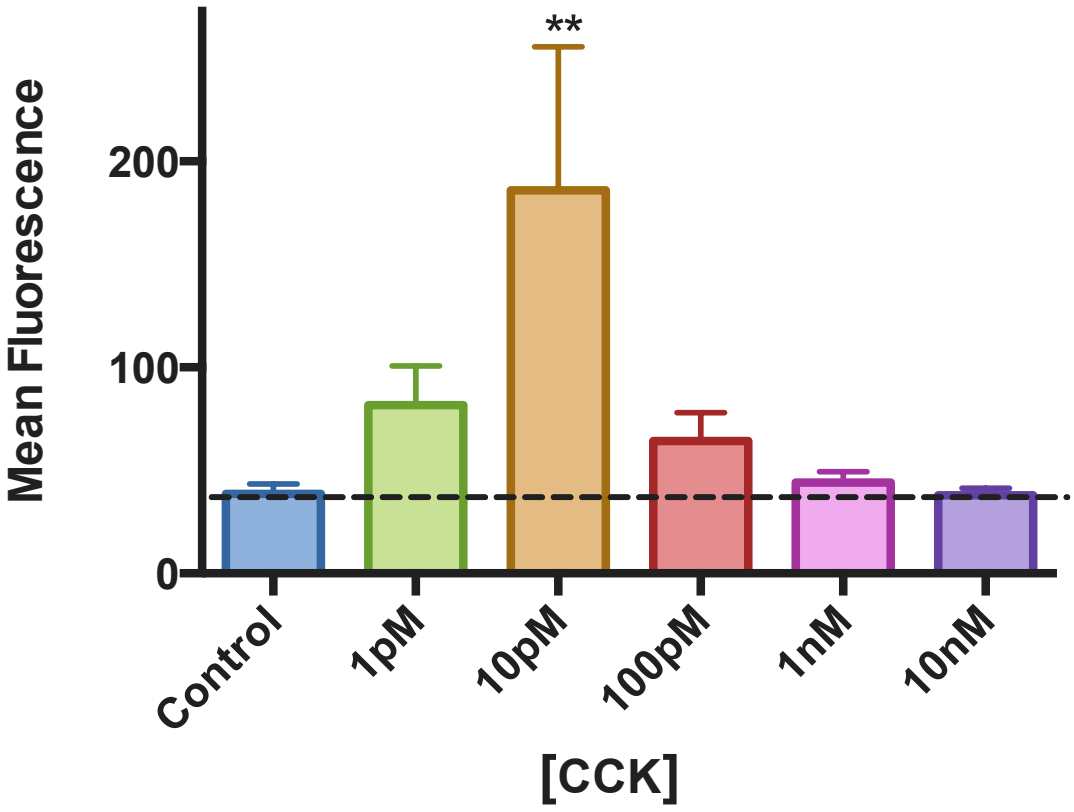
Graph 4.9. The effects of Loxiglumide, Phloretin and SSO on Bodipy-FA uptake in cells incubated with bombesin.

Bodipy-FA uptake was significantly increased by incubation with bombesin. A) Application of loxiglumide (100μM) exerted a significant inhibition upon the stimulatory affects of bombesin on Bodipy-FA uptake. Incubation with loxiglumide alone resulted in no change from control values. B) Application of phloretin (200μM) to cells significantly decreased cell fluorescence even when incubated alongside bombesin. C) Application of SSO (1μM) to intestinal cells significantly inhibited the stimulatory effects of bombesin. SSO alone resulted in no change in cell fluorescence compared to control.
Cells were incubated for 15mins with peptides, followed by incubation with Bodipy-FA for 2mins. Using FACS analysis to measure cell fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared to control; ++=p>0.01 compared to bombesin alone. Black dash line represents respective mean control value.



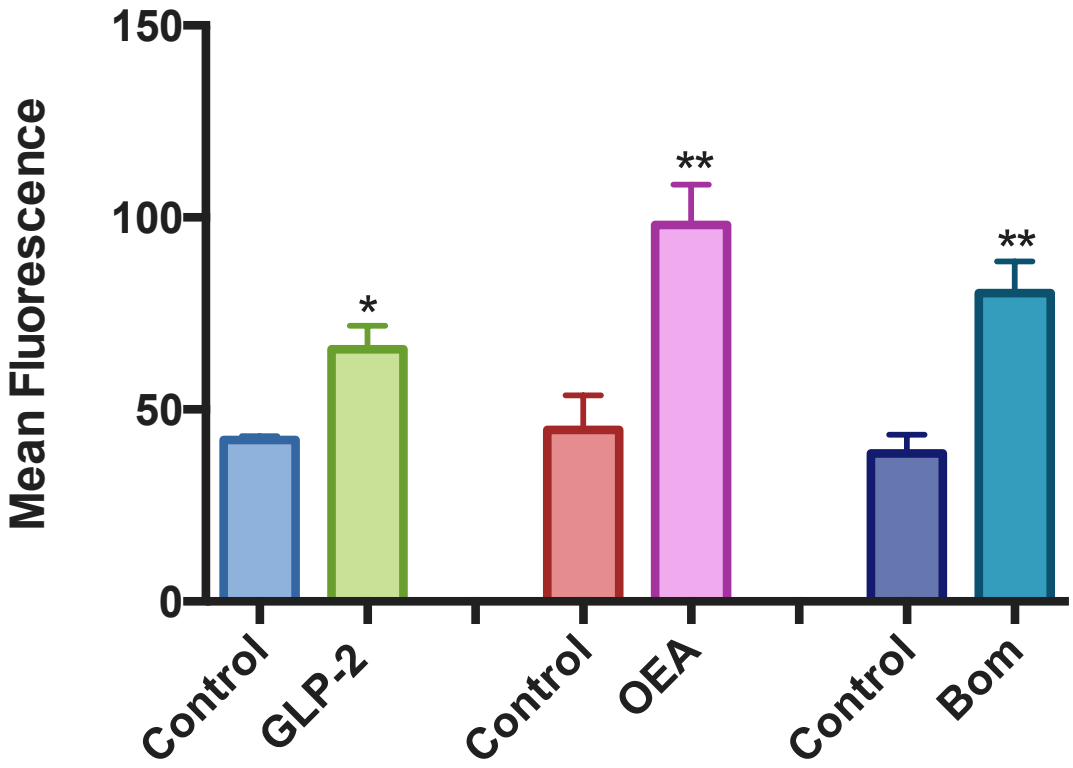
Graph 4.10. Incubation with amino acids increases Bodipy-FA uptake in intestinal cells.

Intestinal cells were incubated for 60mins with the aromatic amino acids L-Phenylalanine (10mM), L-Tryptophan (10mM) and L-Histidine (10mM), the aliphatic amino acid L-alanine (10mM) and the aminoglycoside antibiotic neomycin (100µM) before incubation with Bodipy-FA for 2mins.
Significant increases in cell fluorescence were observed for each aromatic amino acid. Both L-alanine and neomycin failed to produce a significant change in mean cell fluorescence. Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared to control.



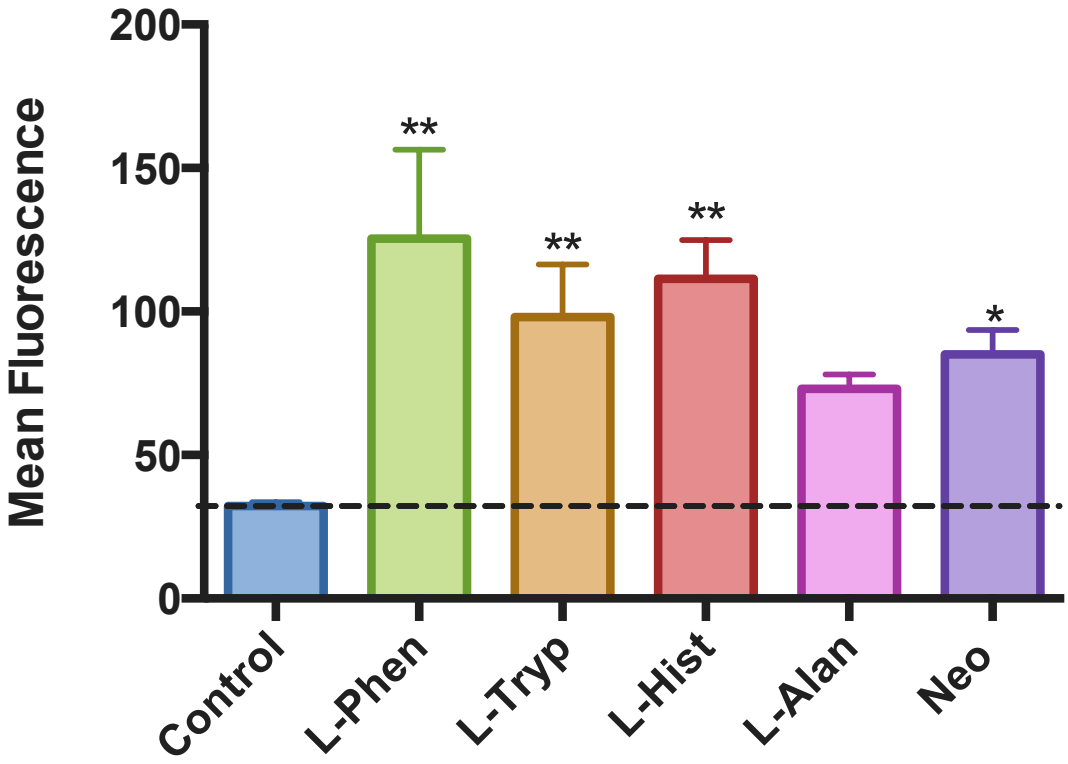
Graph 4.11. Dose Response for CCK in CCK KO cells.

Intestinal cells from CCK^{LacZ} mice were incubated for 15mins in concentrations of CCK ranging from 1pM to 10nM. This was followed by incubation with Bodipy-FA for 2mins. A significant increase in mean cell fluorescence occurred for 10pM CCK only. Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) **=p>0.01 compared to control. Black dash line represents respective mean control value.



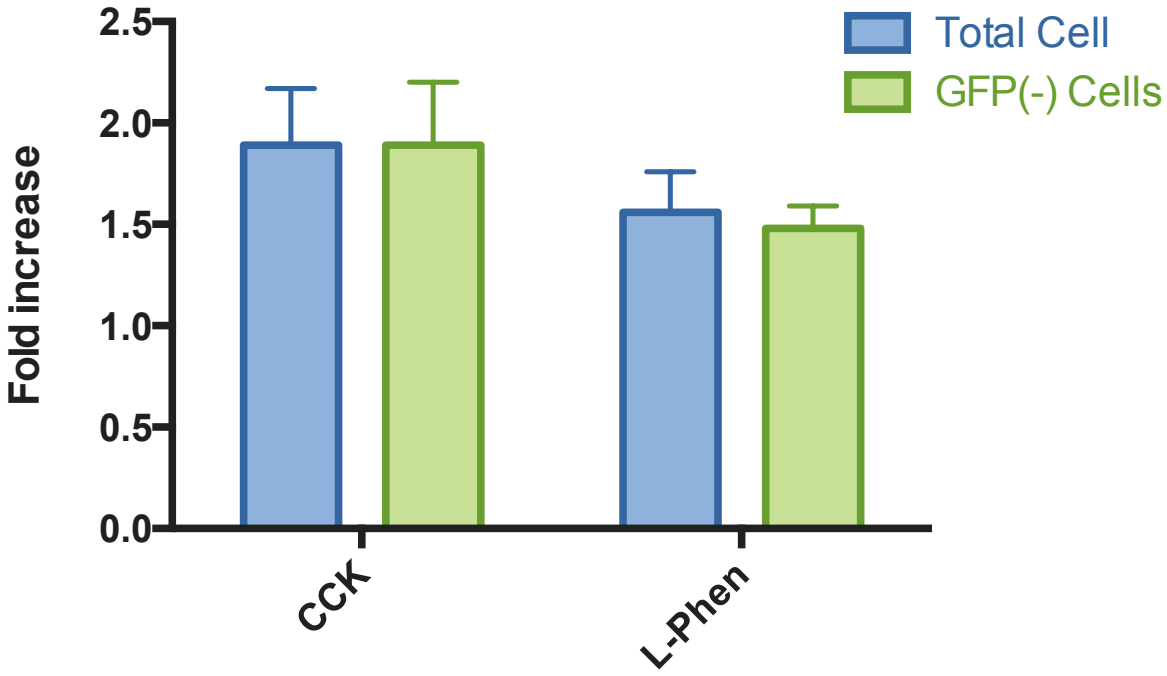
Graph 4.12. The effects of stimulatory peptides on Bodipy-FA uptake in intestinal cells from CCK KO mice.

Incubation of intestinal cells from CCK^{LacZ} mice with 10pM GLP-2, 100nM OEA and 1nM bombesin resulted in significant increase in Bodipy-FA uptake compared with control. Cells were incubated with peptides for 15mins and this was followed by incubation with Bodipy-FA for 2mins. Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) **=p>0.01 compared to control. Black dash line represents respective mean control value.



Graph 4.13. Amino acids increase Bodipy-FA uptake in intestinal cells from CCK KO cells.

Intestinal cells from CCK^{LacZ} mice were incubated for 60mins with the aromatic amino acids L-Phenylalanine 10mM, L-Tryptophan 10mM and L-Histidine 10mM, the aliphatic amino acid L-alanine 10mM and the aminoglycoside antibiotic neomycin 100µm before incubation with Bodipy-FA for 2mins. Significant increases in cell fluorescence were observed for each aromatic amino acid and neomycin. L-alanine did not produce a significant change in cell fluorescence.
Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3) *=p>0.05, **=p>0.01 compared to control.



Graph 4.14. The effects of CCK and L-Phenylalanine on whole cell population compared to a eGFP minus cell population.

The fold increase in mean cell fluorescence is represented for intestinal cells treated with 10pM CCK or 10mM L-phenylalanine. Increase is calculated against control, non-treated cells. CCK and L-Phenylalanine produced equivalent increases in Bodipy-FA uptake in total cell population compared with eGFP minus (-) cell population.
Blue bars represent mean values for whole cell population. Green bars represent mean values for eGFP- cell population. Using FACS analysis to measure fluorescence, each measurement represented 10000 events. Means represent triplicate values and error bars represent SEM (n=3).

4.3. Discussion

Gut peptides exert a multitude of effects geared to control food intake, optimise digestion and maximise nutrient absorption. As with other regulatory peptides, it is suggested that some effects of GI peptides are exerted in a paracrine manner. Paracrine signaling is proposed to exist in the intestine on several different levels (Margolskee et al., 2007, Cheeseman, 1997, Dube and Brubaker, 2007, Debnam and Sharp, 1993, Jeon et al., 2008); namely intestinal paracrine signaling pathways exist between EECs and enterocytes and between EECs themselves. This chapter investigates the capacity of CCK to modulate FA uptake by intestinal enterocytes. The underlying concept was that increased absorption of nutrients following a meal could simply be controlled by paracrine signals transmitted from nutrient sensitive EECs without recourse to higher centres.

Given that a major role of CCK is to enhance the digestion and absorption of nutrients, it would be reasonable to predict a local role within the intestinal epithelium where CCK may exert effects to modulate the absorptive properties of neighbouring cells. The paracrine role of CCK in the intestine is unknown. A local role would imply an ability to produce a rapid cellular response to alter the absorptive ability of enterocytes following the detection of dietary FA or amino acids in the intestinal lumen. In addition, the dispersed distribution of EECs also suggests that each EEC and their surrounding enterocytes may collectively represent individual functional units that communicate via paracrine signalling.

Therefore the aim of this chapter was firstly to determine whether CCK regulates FA uptake into enterocytes. To do this, a fluorescently tagged FA analogue, Bodipy-FA, was employed as an easily measurable surrogate for FA. Secondly, having observed a robust and reproducible effect of CCK on enterocyte uptake to determine the basic mechanism responsible for the observed response.

4.3.1. Development of the methodology

A Bodipy-FA method was refined and adapted (Yang et al., 2007, Gimeno et al., 2003, Nassir et al., 2007). Answers were sought to the following questions in order to validate the use of the protocol to measure dynamic changes in FA uptake in intestinal cells.

4.3.1.1. Are Bodipy-FA a reliable reporter of cellular FA uptake?

The initial aim of this study was to determine if the dynamics of Bodipy-FA uptake reflected those of FA transport. This study necessitated a method to reflect the dynamics of FA uptake; therefore it was important to determine whether Bodipy-FA analogues could be used to reflect typical cellular FA uptake kinetics. Previously, use of fluorescently tagged FAs was believed to slow the rate of FA transport across a cell membrane (Kleinfeld et al., 1997, Storch and Kleinfeld, 1986) and therefore was speculated to limit the scope to use these fluorescent analogue to establish FA uptake kinetics (Kampf and Kleinfeld, 2007, Glatz et al., 2010). However, Bodipy-FA have since been successfully used in studies of FA uptake (Gimeno et al., 2003, Yang et al., 2007, Lynes et al., 2011) therefore we sought to confirm the use of Bodipy-FA to measure FA uptake in isolated primary intestinal cells.

Based on the current knowledge of FA transport, cellular FA uptake is passive and relies on passive lipid phase diffusion or diffusional flux facilitated by membrane bound transporter proteins. Using these processes, typical FA transport for time and dose response curves have an initial linear phase, which flattens off as both diffusional and protein-facilitated FA transport reach saturation (Trotter et al., 1996, Bonen et al., 1998, Abumrad et al., 1998). The data presented in this thesis have demonstrated that incubation of dissociated intestinal cells with fluorescent Bodipy-FA described a curvilinear uptake response that was dynamic over time. Cell incubations also produced a dose-response curve over a range of concentrations of Bodipy-FA. This is reflective of the typical dynamics of cellular FA transport (Sandoval et al., 2008).

Transporter proteins enable translocation of FA into the cell. Some proteins that aid transport of FA can be considered ancillary in that they bind and anchor FA close to the plasma membrane, thus aiding FA uptake by raising the concentration gradient either side of the membrane and therefore drives FA uptake into a cell. Transporter proteins ultimately increase plasma membrane permeability to FA, particularly for LCFAs with a carbon chain length of C12 or more. This is particularly significant as the Bodipy-FA used in this experiment had a chain length of 12 carbon atoms. FA transporters have a high affinity for FA in the nM ranges so are most active in low concentration gradients (Su and Abumrad, 2009). Using this information, it is likely that in low concentrations of FA the majority of FA uptake is carrier-mediated. When increasing Bodipy-FA concentration, the extracellular concentration gradient is increased and this increases the driving force for both passive diffusion and facilitated diffusional uptake via protein transporters, which subsequently increases cellular FA uptake (Tso et al., 2004). The sensitivity to FA concentration is clearly demonstrated in the dose dependent Bodipy-FA curve. In the dose-response curve there is a steep increase in FA uptake when incubated with lower concentrations of Bodipy-FA (2.5-10μM). FA transport is saturable (Stremmel et al., 1985b); this is demonstrated in the flattening of the curve at higher concentrations Bodipy-FA. The relative increase in FA uptake for 25μM Bodipy-FA compared to 50μM is negligible. This demonstrates that both the activity of FA transporters and the diffusion capacity have been saturated.

The apparent EC_{50} value observed in the current study is within the appropriate range with affinity properties of FA transport protein FATP4 (Hall et al., 2005). Ultimately, the Bodipy-FA dose response curve displays characteristics typical of cellular FA uptake (Trotter et al., 1996, Bonen et al., 1998, Abumrad et al., 1998). A low concentration of 5μM Bodipy-FA was selected for future experiments as this was determined to be an optimal concentration with which to demonstrate dynamic changes in cellular FA uptake.

The additional parameter to be optimised was the Bodipy-FA incubation time period. Isolated intestinal cells were incubated with 5μM Bodipy-FA for incubation time periods varying from 0.5-30mins. The results obtained showed

cellular FA uptake to increase as the incubation time increased. The time-response curve achieved shows typical characteristics of cellular FA transport (Trotter et al., 1996, Bonen et al., 1998, Abumrad et al., 1998, Sandoval et al., 2008). Whether FA transport occurs by lipid phase diffusion or facilitated transport, FA transport across the plasma membrane is dependent on a concentration gradient and continues over time until equilibrium is reached. As the concentration gradient lessens over time, the driving force into the cell decreases until there is no longer net movement of FA between the intracellular and extracellular environment. There is negligible difference between the Bodipy-FA uptake recorded following 10 and 30mins incubation period, this is indicative that the cellular FA concentration has equilibrated with the extracellular FA concentration.

As a dynamic response to Bodipy-FA uptake was recorded for shorter time incubations, 2mins was select for the ability to obtain significant and dynamic results as well as ease of experimental procedure.

Taking the data from time and dose-response curves, Bodipy-FA uptake displayed properties typical to normal FA uptake. The methodology was concluded to reflect FA-transport and taken forward to monitor the effects of specific peptides and agents on cellular Bodipy-FA uptake.

4.3.1.2. Is Bodipy-FA uptake sensitive to known stimulators of FA uptake; OEA and GLP-2? Do they display a dose-response?

In the current study GLP-2 and OEA were used as positive controls because both have been demonstrated to increase FA uptake in intestinal cells. Administration of GLP-2 into hamsters caused enhanced triglyceride absorption (Hsieh et al., 2009) whereas OEA treatment enhanced Bodipy-FA uptake in isolated rat enterocytes (Yang et al., 2007).

In this study, administration of OEA and GLP-2 to isolated intestinal cells significantly increased Bodipy-FA uptake. These treatments produced a graded response that was dependent on concentration. Incubation of cells with concentrations of OEA produced a peak response at 100nM whereas incubation of GLP-2 resulted in a bi-phasic sensitivity of peptide interaction.

OEA and GLP-2 have previously been demonstrated to exert a dynamic effect upon intestinal FA uptake (Yang et al., 2007, Hsieh et al., 2009). Importantly, these studies involved *in vivo* experiments that demonstrated that the effects of these peptides upon FA uptake were reflective of the endogenous behaviours of these cells *in situ*. Yang and colleagues demonstrated that the effects of OEA upon FA uptake were attributable to increased expression of the transporter protein CD36 in enterocytes (Yang et al., 2007). Likewise the GLP-2-induced increase in FA absorption was also attributed to activity of CD36 (Hsieh et al., 2009).

OEA is an endogenously synthesised FA derivative that, in the intestine, is a ligand for two receptors; GP119 and PPAR-α (Decara et al., 2012). OEA signals to enterocytes via PPAR-α with an EC_{50} value calculated at 120nM (Fu et al., 2003). PPAR-α activation results in changes in cellular transcription activity that modulates cellular FA handling in terms of uptake, hydrolysis and metabolism (Yang et al., 2007, Rakhshandehroo et al., 2010). This includes up-regulating the expression of the FA translocase protein, CD36 (Fu et al., 2003, Yang et al., 2007, Schwartz et al., 2008). Alternatively OEA also signals through the GPCR GPR119, which has an EC_{50} of 5μM (Syed et al., 2012). GPR119 is expressed on EECs (Sykaras et al., 2012) therefore activation of GPR119 by OEA could potentially trigger the release of GI peptides that may indirectly stimulate FA uptake. GI peptides such as GLP-2 have been demonstrated to increase FA uptake through modulation of CD36 expression in enterocytes (Hsieh et al., 2009). Application of SSO, a CD36 specific inhibitor, inhibits the stimulatory effects of OEA upon Bodipy-FA uptake. Taking into account the relative EC_{50} values for PPAR-α and GPR119, and the optimal concentrations of OEA employed in the current study, it can be speculated that the effects of OEA upon FA uptake demonstrated in this thesis are most likely to be exerted through PPAR-α; to up-regulate plasma membrane expression of CD36.

OEA is synthesised in the presence of dietary fat, therefore physiological concentrations of OEA in the gut fluctuate according to feeding state and are therefore constantly varying (Lauffer et al., 2009). The OEA-induced effects on Bodipy-FA uptake reflect the requirement of the intestine to respond to these fluctuations accordingly. OEA produced a dose-response curve in which all concentrations increased Bodipy-FA uptake to a varying degree, and achieved a significant peak in uptake at a concentration of 100nM. This is marginally lower than optimal OEA concentrations recorded in previous studies, which demonstrated peak activity at 1-10μM OEA (Su et al., 2006, Lauffer et al., 2009, Galan-Rodriguez et al., 2009). These studies also demonstrated OEA to instigate its effects in a dose-dependent manner. This effect was demonstrated for the activation of GPR119 on EECs where OEA stimulated GLP-1 release from L-cells in a dose-dependent manner, with a peak in activity at 10μM and no response at 20μM OEA (Lauffer et al., 2009). OEA administration has demonstrated a dose-dependent neuroprotective role (Galan-Rodriguez et al., 2009) as well as a dose-sensitive satiety response in an *in vivo* study in male rats (Decara et al., 2012) and an inhibitory action on intestinal motility (Capasso et al., 2005). Both PPAR-α and GPR119 are sensitive to OEA concentration, and either receptor may be responsible for relaying the effects or OEA upon Bodipy-FA uptake.

GLP-2 is a bioactive peptide that is produced and secreted by enteroendocrine L-cells. GLP-2 is a product of the proglucagon gene and binds its specific receptor GLP-2R with an EC_{50} of 58pM (Brubaker and Drucker, 2002). Within the concentration range used in this current study, GLP-2 showed a biphasic response, inducing a significant increase in Bodipy-FA uptake at 10pM, 10nM and 100nM.

The dose dependency exhibited by GLP-2 is similar to that described in studies whereby the intestinal functions of GLP-2, including effects upon nutrient absorption, displayed a dose response. Kato and colleagues demonstrated such dose sensitivity in a study that administered varying concentrations of GLP-2 to rats over 14 days that resulted in increased galactose and glycine absorption that varied according to the dose of GLP-2 administered. Optimal absorption of both

amino acid and glucose occurred at a dose of 50μg/kg/day and decreased for higher and lower concentrations (Kato et al., 1999). Baldassano and colleagues also saw a dose-dependent response when looking at GLP-2 stimulated chloride secretion in guinea pig ileum. GLP-2 (100pM-100nM) had a concentration-dependent inhibitory effect upon Cl^- secretion which was reversed in the presence of the GLP-2R inhibitor, GLP-$2^{(3-33)}$ (Baldassano et al., 2009). It is documented that GLP-2R stimulation by increasing concentrations of GLP-2, causes changes in intracellular [cAMP] in an 'inverted U-shape' pattern. This has been recorded for GLP-2Rs of many different tissues and demonstrated peak stimulation at concentrations of 100pM-1nM GLP-2 with a diminished effect at higher concentrations. This is reflective of the features of GLP-2R in terms of ligand desensitisation and also of cell trafficking of the receptor (Estall et al., 2005, Estall et al., 2004). Studies on HeLa cells have also shown that GLP-2R can differentially signal through two distinct G-protein mediated pathways; the Gαs (cAMP) and Gi/Go pathways according to dose-dependent stimulation (Koehler et al., 2005). It is possible that the biphasic response for GLP-2 concentrations observed in the current study could reflect differential activation of signalling pathways or receptor desensitisation properties.

Ultimately, dose-dependent discrimination of receptor activation establishes an additional dimension of control over signalling pathways (Lauffer et al., 2009). The stimulatory response achieved through incubation of intestinal cells with OEA or GLP-2 replicate data that have previously been shown. Therefore this validated the methodology developed for the current study to determine the affect of CCK on duodenal FA uptake.

4.3.1.3 Does CCK have an effect on Bodipy-FA uptake by intestinal cells? Is this dependent on the sulphated residue of CCK? Does this display a dose-response?

The preliminary aim of the work described in this chapter was to establish whether cellular incubation with CCK modulates cellular FA uptake. The exciting finding was that CCK induced a significant increase in Bodipy-FA uptake. Incubation of enterocytes with bioactive CCK prior to exposure to fluorescent Bodipy-FA caused a reproducible and robust 2- to 3-fold increase in mean Bodipy-FA uptake. Based on a survey of the current literature this is the first report of CCK stimulating cellular FA uptake. Whilst the local effects of CCK in pancreatic acinar cells have been known for 70 years, paracrine effects within the intestinal epithelium have not previously been described. An extensive search of the current literature yielded only one report of a direct role of CCK upon enterocytes. Jeon and colleagues reported that CCK release up-regulates the expression of the ATP-binding cassette protein B1 (ABCB1) in surrounding enterocytes. The ABCB1 protein is involved in mediating the efflux of unwanted 'bitter' toxins. This depicts a protective paracrine role for CCK in the intestine, to expel unwanted bitter toxins (Jeon et al., 2008). However, a direct modulatory action for CCK on dietary nutrient uptake has not been seen before.

That CCK might modulate intestinal FA uptake has been indirectly suggested by a several studies: administration of CCK to mice raised plasma cholesterol and lipid concentrations by 6% and 13%, respectively. Feeding of olive oil to CCK-treated mice then further elevated plasma TG concentrations (Zhou et al., 2012). Furthermore, CCK KO mice display defective fat absorption, particularly for LCFAs. However, as CCK exerts many functions to co-ordinate the digestion and absorption of nutrients, this defect could therefore be reflective of alternate functions. A key contribution to this could be a reduction in bile and pancreatic juice delivery into the duodenum that would hinder the ability to breakdown dietary fats and thus restrict efficient absorption. Ultimately, CCK KO mice are

resistant to HFD induced obesity and demonstrate that CCK is important for normal fat absorption (Lo et al., 2010).

As was observed for incubation with OEA and GLP-2, CCK-induced effects on Bodipy-FA uptake showed a dose dependency. A significant peak in activity was recorded at a concentration of 10pM. This dose response is a typical representation of the binding properties of CCK-Rs. CCK-R ligand interaction and kinetics have been established predominantly through using pancreatic cells as a model. These ligand binding studies led to a speculation of a two-site model that suggested CCK-Rs have a high affinity, low capacity site and a low affinity, high capacity site (Jensen et al., 1989). This describes CCK binding with its receptor to have a slower rate of dissociation at lower concentrations, thereby prolonging the resultant effect. Such high and low affinity binding was ascribed due to the concentration-dependent effects of CCK to stimulate pancreatic amylase secretion (Jensen et al., 1989).

Since the model was proposed in 1989, specific high- and low-affinity binding sites have been neither cloned nor characterised however the data maintain the existence of alternate binding properties of CCK-Rs. Studies have even demonstrated that selective binding of CCK with its receptors at different concentrations can activate different second messenger pathways (Gonzalez et al., 1999). Whilst both receptors are coupled with the phospholipase C pathway, high-affinity CCK-R binding is associated with activation of phospholipase A2 whilst low-affinity binding is associated with activation of phospholipase D intracellular pathways. This differential activation occurs independently of each other and would elicit a significant effect on the downstream effects of ligand binding (Gonzalez et al., 1999). This has physiological significance to help cells to encode different external signals (Burdakov and Galione, 2000).

A CCK dose-dependent response has also been demonstrated in Ca^{2+} imaging studies. Large and repetitive Ca^{2+} spikes were recorded in cells exposed to pM concentrations of CCK, whereas exposure to higher nM concentrations induced a larger transient response that was associated with an immediate and rapid decline (Habara and Kanno, 1994, Burdakov et al., 2001, Jensen et al., 1989). In

addition, CCK-Rs display characteristics of desensitisation, a common property of many GPCRs. High concentrations of CCK have been demonstrated to cause receptor phosphorylation and subsequent desensitisation of CCK-R_A (Ozcelebi et al., 1996, Wank, 1998). This could represent the negligible affect of CCK at higher concentrations. The properties of the CCK-R discussed fit with the dynamic CCK-induced effects presented in the current study.

The data presented in this thesis demonstrate that the CCK-induced effect on cellular Bodipy-FA uptake is dependent on the sulphated residue of the CCK peptide. Incubation of cells with CCK-NS, which does not contain the sulphated residue, did not increase cellular FA uptake. CCK-NS preferentially binds CCK-R_B and has a 1000-fold lower affinity for CCK-R_A (Rehfeld et al., 2001). As CCK-R_B is not considered to relay any of the GI functions of CCK, CCK-NS is not considered to play a significant role within the GI tract (Rehfeld et al., 2001). The involvement of CCK-R_A in relaying the CCK-induced effects upon FA uptake was supported by application of the CCK-R_A specific antagonist loxiglumide, which abolished the stimulatory effects of CCK. This will be discussed at length shortly.
The Bodipy-FA uptake data presented are reflective of the aforementioned binding properties of CCK-NS to the CCK-Rs. Interestingly, at a concentration of 10pM CCK-NS caused an increase in Bodipy-FA uptake above control levels, although not to a significant level. This effect is probably attributable to a degree of low-affinity interaction with the CCK-R_A (Rehfeld et al., 2001). It remains possible that CCK-NS may exert an effect in the intestine whether as a tonic signal or for smaller stimulatory purposes. CCK-NS is expressed in cells of the SI and this alone suggests it may have a physiological purpose in this region (Bonetto et al., 1999).

The presented data have firstly validated the use of Bodipy-FA analogues as a method to determine dynamic changes in cellular FA uptake. The finding that CCK can modulate a cells ability to absorb FA is novel and exciting. These data have enabled the null hypothesis 'Incubation with CCK does not enhance FA uptake in enterocytes' to be rejected. The next section focuses on the mechanism through which this occurs.

4.3.2. What is the mechanism of the stimulatory effect of CCK on enterocyte FA uptake?

The data presented in this thesis have demonstrated that incubation of intestinal enterocytes with CCK can modulate cells to increase FA-uptake. This would be the logical response to maximise FA uptake into enterocytes. Taking this further, the aim was set to determine the mechanism through which this is elicited.

4.3.2.1. The effects of CCK upon cellular Bodipy-FA uptake are exerted through interaction with CCK-R_A

The preliminary aim of the experiments discussed in the ensuing section was to ascertain the mechanism by which CCK stimulated intestinal cells to increase FA uptake. To establish whether this interaction occurred through the CCK-R_A, loxiglumide was employed. Loxiglumide is a well-established and specific inhibitor of CCK-R_A that has been used extensively in many studies for over two decades (Konturek et al., 1995, Beglinger et al., 1992). Importantly, loxiglumide is 64-fold selective for CCK-R_A over CCK-R_B with an IC_{50} value of 3μM (Taniguchi et al., 1996). Therefore, at the concentration employed in the current study (100μM), this will potently inhibit CCK-R_A with negligible effects upon CCK-R_B (Taniguchi et al., 1996).

Application of loxiglumide to incubating cells completely abolished the stimulatory effect of CCK on Bodipy-FA uptake. This result indicated that the stimulatory effect elicited by CCK was mediated via CCK-R_A. Significantly, incubation of intestinal cells with loxiglumide alone caused negligible change in Bodipy-FA uptake when compared to control.

The expression of CCK-R_A in the intestine has long been recognised (Lacourse et al., 1997), however this expression is believed to be confined to vagal afferents innervating the intestinal mucosa and to the smooth muscle of the SI (Sternini et

al., 1999). To date there is no report in the literature that describes CCK-R_A to be definitively expressed in the intestinal epithelium (Chandra et al., 2010).

Although expression of CCK-R_A on enterocytes remains to be confirmed, Ca^{2+} imaging studies have clearly demonstrated that CCK stimulates enterocytes. CCK induced Ca^{2+} oscillations have been demonstrated in rat and human duodenal enterocytes (Chew et al., 1998, Sjoblom et al., 2013). Furthermore, this response was abolished in the presence of CCK-R_A inhibitor devazepide and not by the CCK-R_B inhibitor YM022 (Sjoblom et al., 2003, Sjoblom et al., 2013). Moreover, further studies conducted on enterocyte clusters saw Ca^{2+} responses to spread from cell to cell, indicating a direct communication between cells of the epithelium and suggesting the existence of a functional network (Sjoblom et al., 2003, Bengtsson et al., 2009). Additionally, functional CCK-enterocytes interactions have been shown in a study by Jeon and colleagues (2008) which demonstrated that the release of CCK release stimulated by the detection of bitter toxins by the taste receptor T2R, induced an up-regulation of the ATP-binding cassette-1 (ABC1) in surrounding enterocytes. This protective mechanism was blocked by application of 10μM CCK-R_B antagonist, YM022**.** YM022 preferentially blocks CCK-R_B with a K*i* value of 68pM and has a K*i* of 63nM for CCK-R_A. The concentration employed in the work of Jeon et. al. was 10μM, which would have been expected to block both CCK-R_B and CCK-R_A receptors (Jeon et al., 2008). Collectively, the data presented in this thesis and in previous studies indicate that CCK exerts a functional role upon enterocytes and that this is relayed through the CCK-R_A.

The data presented in this thesis are similar in some respects to those reported by Hsieh et al. who showed GLP-2 to stimulate FA uptake in enterocytes (Hsieh et al., 2009) despite there being no data to confirm GLP-2R expression on enterocytes. In view of this, it may be worthwhile to compare the effects exerted upon intestinal enterocytes by GLP-2 with the effects of CCK. GLP-2 exerts a number of its intestinal effects through indirect mechanisms. An established example of this is demonstrated whereby GLP-2 conveys a potent effect to stimulate cell proliferation, however this effect is exerted indirectly through stimulating the release of insulin-like growth factor-1 (IGF-1) from intestinal

smooth muscle cells (Dube et al., 2006). Additionally, GLP-2R expression on multiple EEC subtypes suggests a mode of communicative signalling between EECs to stimulate release of additional GI peptides (Yusta et al., 2000). Although CCK-R expression has not been confirmed at the molecular level on EECs, there are data to suggest that CCK also acts as a positive modulator of EECs, with the ability to induce the release of other GI peptides. This phenomenon has been presented in studies using human subjects. Firstly, a study by Beglinger and colleagues demonstrated that administration of LCFAs raised plasma CCK and GLP-1 concentrations. Co-administration of the CCK-R_A antagonist, dexloxiglumide with LCFAs inhibited the effects upon GLP-1 concentration, implicating that this effect was mediated by CCK acting through CCK-R_A. (Beglinger et al., 2010). Equally CCK inhibits ghrelin release in a seemingly analogous way whereby intraduodenal infusion of LCFAs reduced plasma ghrelin concentrations and increased circulating PYY and CCK concentrations. The effects of LCFAs upon ghrelin and PYY release were again inhibited when co-administered with dexloxiglumide, indicating the effects were attributable to CCK acting through CCK-R_A (Degen et al., 2007). These studies demonstrate the existence of paracrine signalling pathways between EECs that involve CCK. Therefore it must be considered whether the effects of CCK could potentially be relayed indirectly through stimulation of other EECs within the cell suspension. In this way the release of additional factors may instigate the modulation of cellular FA-uptake.

The present study demonstrates that certain GI peptides are able to interact and modulate the absorptive properties of enterocytes. Two studies have previously demonstrated GI peptides to influence FA uptake. These entailed administration of GLP-2 to mice which cause an increase in TG absorption (Hsieh et al., 2009) and also a study which showed that the administration of OEA to isolated intestinal cells increased cellular FA uptake (Yang et al., 2007). However, studies have also demonstrated the ability of GI peptides to modulate carbohydrate uptake in the intestine. This is seen for GLP-1 and GIP induced up-regulation of SGLT1 expression in enterocytes, which facilitates glucose absorption in the intestine (Margolskee et al., 2007). GLP-2 also has the ability to up-regulate

SGLT1 expression and it does this through activation of the phosphoinositol-3 kinase-signalling pathway (Cheeseman, 1997, Dube and Brubaker, 2007). Glucagon is also able increase sugar uptake in enterocytes (Debnam and Sharp, 1993). These depict examples of additional paracrine mechanisms that act to enhance nutrient absorption.

The data presented in this thesis demonstrate that the CCK-induced increase in cellular Bodipy-FA uptake is exerted through interaction with CCK-R_A. It is unconfirmed whether this interaction exists directly with the enterocytes or indirectly with EECs that in turn could release factors to mediate the effects upon FA uptake. Whilst the expression of CCK-Rs within the intestinal epithelium remains unconfirmed, the data obtained from Ca^{2+} signalling studies have demonstrated that CCK is able to directly elicit a response. Future work to establish the localisation of CCK-R expression on enterocytes and EECs would help to elucidate this mode of action. This could be achieved through analysis of molecular transcripts using PCR analysis, or analysis of protein expression using western blotting or immunostaining, which would support the mode through which CCK elicits these results.

4.3.2.2. What is the mechanism of FA transport stimulated by CCK?

Phloretin is a potent and non-specific inhibitor of membrane transport proteins (Abumrad et al., 1981, Ibrahimi et al., 1996). Incubation of intestinal cells with 200μM phloretin exerted inhibition of Bodipy-FA uptake. These data indicate that the majority of cellular FA uptake at an extracellular Bodipy-FA concentration of 5μM is attributable to transporter proteins. Furthermore, the addition of OEA, GLP-2 or CCK made negligible difference to this phloretin-induced inhibition. This indicates that the increase in Bodipy-FA uptake elicited by OEA, GLP-2 and CCK is mediated through effects that are mediated by transporter proteins and not by alterations in the diffusional properties of the cells.

In control experiments in the absence of stimulation by hormone, phloretin induced a significant decrease in FA uptake. This is important as these data suggest that under control conditions the majority of FA uptake is carrier-mediated. A concentration gradient is required to drive FA into a cell; therefore limited FA uptake via diffusion could be due to a low concentration gradient. Furthermore, diffusion is more difficult for LCFA such the C12 Bodipy-FA employed, as these can necessitate the aid of transporter proteins to enable them to cross the membrane. The data therefore demonstrate that the majority of basal FA uptake in these conditions is carrier-mediated. Addition of phloretin subsequently exerts a potent inhibition upon uptake processes and the remaining Bodipy-FA uptake can be considered to represent diffusion-specific FA uptake.

There are at least three apical membrane proteins that have been identified as contributing to SI cellular FA uptake. These are CD36, FATP4 and FABPpm. CD36 acts as a FA translocase and additionally CD36 and FABPpm act as anchors for FA, which localises FA close to the plasma membrane. This helps raise the diffusion gradient to drive FA influx or to expose them to other transport proteins (Nassir et al., 2007, Lynes et al., 2011). Accumulation of FA within the cell has a feed-forward effect, particularly through the synthesis of OEA (Schwartz et al., 2008) which exerts a feed-forward effect to up-regulate expression of CD36 (Fu et al., 2003, Yang et al., 2007). Conversely, the dominant effects of FATP4 are believed to lie in it Acyl-CoA synthetase activity (Black et al., 2009).

Although phloretin has been widely used as an inhibitor of membrane transporters (Fenton et al., 2004, Potter et al., 2006, Shayakul and Hediger, 2004) including inhibition of FA transport proteins (Abumrad et al., 1981, Ibrahimi et al., 1996), it has a number of non-specific cellular effects therefore necessitated use of a more specific inhibitor of intestinal FA uptake.

The FA uptake experiments reported for OEA (Yang et al., 2007) and GLP-2 (Hsieh et al., 2009) demonstrated that the hormone-induced increase in cellular FA uptake was attributable to CD36. To test the hypothesis that CCK exerts its effect through modulating CD36 activity in intestinal cells, experiments were directed to use sulfo-N-succinimidyl oleate (SSO), which specifically targets the intestinal FA transport protein CD36.

4.3.2.3. The stimulatory effect of OEA, GLP-2 and CCK on Bodipy-FA uptake is exerted through CD36

SSO is a CD36-speicific inhibitor that binds CD36 at the FA ligand-binding site at the plasma membrane. As SSO binds the extracellular FA binding domain, this effectively inhibits both modes of CD36 facilitated FA uptake firstly as a FA channel and secondly as an extracellular FA anchor (Coort et al., 2002, Schwenk et al., 2010, Pohl et al., 2005, Lynes et al., 2011). Importantly, SSO does not permeate the plasma membrane and therefore has no effects on intracellular signalling pathways (Coort et al., 2002).

The addition of SSO to intestinal cells caused no change in Bodipy-FA uptake compared to non-treated control cell samples. These data indicate that CD36 is not involved in the basal Bodipy-FA uptake of non-treated intestinal cells. Furthermore, the addition of OEA, GLP-2 or CCK made negligible difference to SSO treated cells demonstrating that their effects in mediating an increase in cellular FA uptake are attributable to the activity of CD36. These data suggest that the 'phloretin-inhibited' transport proteins that contribute to FA uptake in non-treated cells does not involve CD36 and therefore implicates alternative membrane proteins are involved in this process. A key candidate for this is the intestinal FA transport protein FATP4 that has been demonstrated to greatly contribute to cellular LCFA uptake. The contribution of FATP4 to cellular LCFA uptake has been demonstrated experimentally where over-expression of FATP4 in HEK293 cells was observed to increase LCFA and VLCFA uptake (Stahl et al., 1999) and whereas inactivation of FATP4 in enterocytes using antisense oligonucleotides, significantly decreased cellular uptake of radio-labelled LCFA (Stahl et al., 1999). Importantly, a study conducted by Gimeno et al. (2003) used a FATP4 heterozygote mouse model that had a near 50% reduction in FATP4 protein in enterocytes. Using this mouse model, Bodipy-FA (C12) uptake experiments performed on intestinal cells demonstrated that FA uptake was reduced by 40% compared to WT (Gimeno et al., 2003). These data strongly suggest that FATP4 may be the predominant protein involved in basal intestinal FA uptake (Gimeno et al., 2003).

Although aforementioned studies depict FATP4 to mediate FA uptake in non-treated cells, it is widely documented that CD36 is essential for *in vivo* FA uptake in proximal intestine (Nassir et al., 2007, Ehehalt et al., 2008, Drover et al., 2008). Furthermore, it has been shown that the OEA-induced increase in LCFA uptake is attributable to CD36 (Yang et al., 2007) and the comparable study conducted by Hsieh et al., (2009) demonstrated that the GLP-2-induced increase of TG absorption was also attributable to up-regulation of CD36 in enterocytes. These data were validated when administration of GLP-2 exerted no effect in CD36 KO mice (Hsieh et al., 2009). Moreover, it was demonstrated that GLP-2 administration had no effect upon FATP4 expression (Hsieh et al., 2009) however changes in FATP4 expression was not measured in experiments for OEA treated cells (Yang et al., 2007). These studies clearly demonstrate that both OEA and GLP-2 administration causes significant up-regulation of CD36 expression. The data presented in this thesis reflect these findings and indicate that CCK also increases FA uptake through the actions of CD36.

It is evident that CD36 is important for intestinal FA uptake however the data suggest that CD36 is not involved in FA uptake in non-treated cells. It appears that CD36 activity is relayed following stimulation from a cellular or systemic signal. This theory is supported by the properties of CD36 that reveal that its expression is highly regulated. A large proportion of cellular CD36 expression is located intracellularly where it anticipates signals to instigate its translocation to the plasma membrane. CD36 trafficking is controlled through palmitoylation and glycosylation of the protein, which can be activated by extracellular signals such as OEA or GLP-2 stimulation (Lynes et al., 2011, Hsieh et al., 2009). This reflects the ability of a cell to respond dynamically to signals and subsequently increase FA uptake according to demand. Such requirement of activation signals for CD36 activity is demonstrated in a study conducted by Eyre and colleagues who found that transfection of CHO cells with CD36 alone did not cause any increase in cellular FA uptake (Eyre et al., 2008). This was similarly demonstrated by Hsieh and colleagues who saw negligible differences between basal plasma TG concentrations of WT and CD36 KO mice however treatment of the animals with GLP-2 significantly raised plasma TG concentrations in WT but not in CD36 KO

mice. This study went on to demonstrate that GLP-2 application increased TG absorption through an up-regulation of plasma membrane expression of CD36 in enterocytes (Hsieh et al., 2009). These studies demonstrate that CD36 requires the presence of additional factors and signals to relay its activity. These studies therefore support the data presented in this thesis where CD36 did not contribute to FA uptake in non-treated cells yet cells treated with GLP-2, OEA or CCK exhibited an increase in FA uptake that was attributable to CD36.

The properties of CD36 described above, highlight the degree of control a cell has to manipulate its activity according to physiological demands and stimuli (Schwenk et al., 2010). CD36 activity is not only subject to short-term signals but also exposure to long-term signals such as prolonged dietary changes. This has been demonstrated in high- and low-fat diet studies that observed fluctuations of CD36 expression that significantly increased or decreased accordingly to diet manipulation (Sukhotnik et al., 2001, Sukhotnik et al., 2003). Collectively, these studies clearly demonstrate that CD36 activity is dynamic.

The data presented in this thesis indicate that OEA, GLP-2 and CCK increase cellular FA uptake through modulating the activity of CD36. Therefore the null hypothesis 'carrier-mediated transport is not implicated in CCK-induced enterocyte FA transport' can be rejected.

Summary

The data presented in this section demonstrate that CCK exerts an effect upon intestinal cells to increase Bodipy-FA uptake is relayed through interaction with the CCK receptor: CCK-R_A. Furthermore, this section has demonstrated that OEA, GLP-2 and CCK increase Bodipy-FA uptake through a facilitated transporter mechanism. This was identified to be specifically mediated through activity of the fatty acid translocase protein; CD36. The perceived mechanistic pathway is outlined in Figure 4.1. Subsequent experiments were carried out to test the hypothesis; 'ligand-stimulated release of CCK from CCK-cells does not induce FA uptake'.

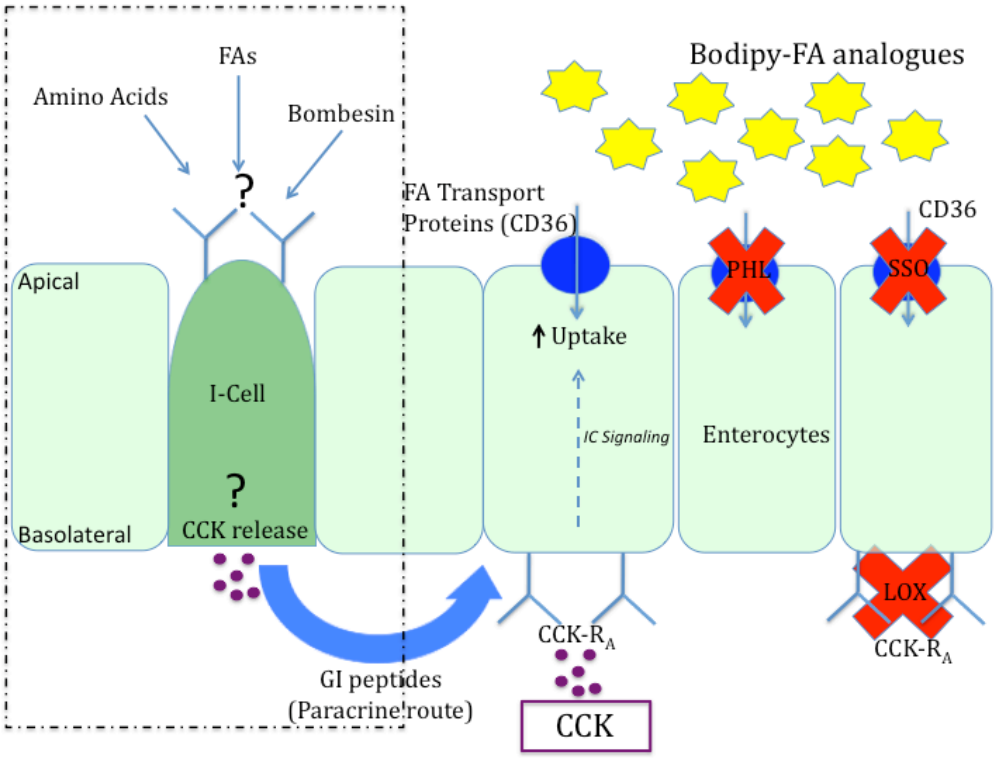


Figure 4.1. The perceived mechanistic pathway of paracrine signaling by CCK to up-regulate FA-uptake in intestinal cells.

The data has demonstrated that CCK can modulate cellular uptake of Bodipy-FA through interaction with CCK-R$_A$. Phloretin (PHL) depicted the effect to be mediated by transporter proteins and application of sulfo-N-succinimidyl oleate (SSO) pinpointed this to be exerted by the fatty acid translocase protein; CD36.
Subsequent experiments were designed with the aim to relate these effects the activity of I-cells. The dashed black box depicts this hypothesis. It is predicted that stimulation of I-cells by amino acids, fatty acids (FA) or bombesin can induce CCK secretion. Release of CCK could then exert its paracrine role upon cells of the intestinal epithelium to increase FA uptake.

4.3.3. Stimulation of I-cells to induce the effects of CCK on Bodipy-FA uptake

After the novel and exciting discovery of the effects of CCK upon intestinal FA absorption, a major question remained: are these effects directly mediated by I-cells? Therefore, the study was taken further with the aim to target I-cells by stimulating them to release CCK and subsequently relay effects upon cellular FA uptake.

4.3.3.1. Bombesin stimulates an increase in Bodipy-FA in a dose-responsive manner. Does this involve CCK?

Bombesin is a tetrapeptide that was originally isolated from frog skin. Bombesin has been used experimentally for decades as a model of inducing the release of CCK (Banks, 1980, Chen et al., 2006, Wang et al., 2011, Cuber et al., 1989). In mammals these exist as bombesin-like peptides (BLP). Two analogous BLPs are gastrin-releasing peptide (GRP) and neuromedin B (NMB).

Application of bombesin to intestinal cells elicited a dose-dependent increase in Bodipy-FA uptake. Additionally incubation of intestinal cells with bombesin alongside the inhibitors phloretin and SSO displayed the same pattern of inhibition as observed for OEA, GLP-2 and CCK. In this way, application of phloretin decreased Bodipy-FA uptake and application of SSO resulted in no change in uptake compared to control values. Furthermore incubation of cells with loxiglumide alongside bombesin inhibited the stimulatory effects elicited by the tetrapeptide. The data acquired in these experiments indicate that bombesin mediates an effect upon intestinal cells to increase FA-uptake. Application of SSO demonstrated that the membrane protein CD36 mediates this effect. Furthermore, loxiglumide inhibited the stimulatory effects of bombesin upon Bodipy-FA uptake. This suggests CCK to be involved in this signalling pathway. Therefore, these data suggest that bombesin stimulates cellular FA uptake through stimulating the release of CCK from I-cells.

Bombesin-induced CCK release has been widely accepted for over four decades. This was initially shown through work on cell-line models such as STC-1 cells where bombesin stimulates Ca^{2+} oscillations through Ca^{2+} release from intracellular stores (Chen et al., 2006, Wang et al., 2011) and the subsequent release of CCK, via stimulation of the G-Protein Gαq (Hira et al., 2009, Takahashi et al., 2000). It is also important to note that GRP and NMB receptors are expressed by STC-1 cells which suggests expression on EECs (Snow et al., 1994).

The data achieved using cell line models are mimicked in mammalian systems where administration of BLPs causes a rise in plasma CCK concentrations (Banks, 1980, Cuber et al., 1989, Konturek et al., 1976, Erspamer et al., 1974). This is coupled with an onslaught of effects within the body that mirror the functions of CCK; bombesin induces a potent satiety effect (Gibbs et al., 1997b, Gibbs et al., 1997a, Lee et al., 1994) and also modulates many aspects of GI functions, including delayed gastric emptying (Scarpignato and Bertaccini, 1981), gall bladder contraction (Erspamer et al., 1974) and the release of pancreatic secretions (Taylor et al., 1979, McDonald et al., 1983, Ghatei et al., 1982). In mammals, GRP and NMB receptors are expressed in the GI tract and in the brain, which are implicated to relay the effects of bombesin listed above (Ladenheim et al., 1992, Minamino et al., 1983, Stein and Woods, 1982). Collectively, these data suggest that bombesin could induce its effects indirectly through stimulating the release of CCK. This is reflective of the data presented in the current study where the stimulatory and dose-responsive effects of bombesin, reflect the effects achieved following cell incubation with CCK.

Interestingly, a study has previously shown bombesin to exert effects upon the absorptive properties of intestinal cells. Rats with surgery-induced short bowel syndrome were administered bombesin for 10 days, which caused the intestinal absorptive capacity to increase when compared to control rats. However, it is possible that these data are reflective of the trophic effects of bombesin, which can stimulate intestinal growth and enhance absorption over time (Uluutku et al., 2004).

The effects elicited by bombesin upon cellular Bodipy-FA uptake showed a dose-dependency. Bombesin elicited a significant increase in enterocyte FA uptake at concentrations of 100pM and 1nM. This is reflective of previous studies that have demonstrated many of the effects of bombesin to be elicited in a dose-dependent fashion. Two such studies were demonstrated using Swiss 3T3-cells. These studies monitored the effect of bombesin upon cellular Ca^{2+} mobilisation and subsequent formation of diacyl-glycerol (Takuwa et al., 1987) and secondly in a DNA synthesis study observing the mitogenic effects of bombesin (Rozengurt and Sinnett-Smith, 1983). In both of these studies the effects of bombesin were optimal at concentrations of 100pM and 1nM and then declined a higher concentration of 10nM. These data mirror the effects of bombesin observed in the current study.

To further probe the involvement of CCK to elicit this response, the experiment was repeated using cells isolated from a CCK KO model. Intestinal cells from CCK^{LacZ} mice were incubated with bombesin and interestingly also saw a significant increase in Bodipy-FA uptake. Intestinal cells from CCK^{LacZ} mice produced a comparable profile of Bodipy-FA uptake upon administration of OEA, GLP-2, CCK and bombesin as that seen for wild-type (eGFP-CCK) mice. This indicates that the cellular machinery required to elicit these effects is still present in CCK^{LacZ} mice and therefore was not through CCK. Whilst these results were puzzling, it is highly probable that, given the nature of the enteroendocrine system, bombesin may in fact stimulate the release of a combination of GI peptides, which could collectively elicit the effects upon FA uptake. There are many studies, which suggest that bombesin can stimulate EECs throughout the GI tract and induce the release of multiple regulatory peptides. This has been shown firstly in cell line models where bombesin induces Ca^{2+} oscillations and hormone release; GIP release in GIP/Ins cells and (Li and Wice, 2005) and GLP-1 release from GLUTag cells (Reimann et al., 2006) through interaction with G-protein signalling pathways. Bombesin has actually been shown to stimulate the release of numerous GI peptides as well as CCK in *in vivo* and *in vitro* models; this includes multiple products of the preproglucagon gene, insulin, neurotensin,

gastrin, somatostatin and secretin (Glad et al., 1996, Schaffer et al., 1997, Taylor and Fuller, 1994, Sukhotnik et al., 2007a, DuVal et al., 1981).

GI peptides have numerous overlapping functions. By stimulating the release of multiple peptides, bombesin may exert its extensive effects by acting through any combination of GI peptides. Over the years some of the actions of bombesin, originally believed to be attributable to CCK, have been unravelled to disprove this theory. This explains a few additional roles of bombesin including effects on raising blood glucose levels (Plamondon and Merali, 1993), a trophic effect within the pancreas and intestine (Liehr et al., 1990) and a protective role in many GI disorders (Sukhotnik et al., 2007a, Jensen et al., 2008) which are not a reflection of the principle actions of CCK.

Reviewing the literature, it appears that a number of the effects of bombesin which were originally believed to be relayed indirectly through the actions of CCK, were not the case. Firstly, a study by Liehr and colleagues found that bombesin-mediated pancreatic enzyme secretion was not mediated through CCK as previously thought but is in fact caused through direct interaction with acinar cells (Liehr et al., 1993). This was enforced by studies that showed bombesin could directly induce Ca^{2+} signalling in pancreatic acinar cells (Lee et al., 2003, Schulz et al., 1999). This was replicated in studies that have looked into the satiety effect of bombesin that also appeared to be independent of CCK; demonstrated through both a vagotomy model and through administration of CCK inhibitors alongside bombesin (Smith et al., 1981a, Smith et al., 1981b). Again, this could be explained by the expression of GRP and NMB receptors within the brain, which indicates a direct interaction of BLPs with brain regions to relay its satiety effects (Ladenheim et al., 1992, Minamino et al., 1983, Stein and Woods, 1982).

With respect to the current data, whilst it is evident that bombesin can influence the activity of multiple EECs, it is also possible that bombesin could inflict the observed effects through a direct interaction with the absorptive enterocytes. Bombesin receptors are expressed along the intestinal epithelium throughout the

length of the GI tract (Narayan et al., 1991, Ferris et al., 1997, Kachur et al., 1982, Seybold et al., 1990). The high signal acquired during ligand binding studies suggests bombesin receptors are not confined to the sparse EECs but are more likely expressed in enterocytes and other cell types within the epithelium. Direct interaction of bombesin with enterocytes is believed to be the mode in which BLPs exert direct effects on enterocyte growth (Carroll et al., 2002) and turnover (Sukhotnik et al., 2007b, Sukhotnik et al., 2007a). Therefore it is possible that bombesin could be exerting a direct effect on enterocytes to influence their absorptive properties (Riviere et al., 1990).

In addition to the potential of a direct cellular interaction, bombesin has also been shown to influence the expression of brush-border enzyme gene expression, many of which have the capacity to influence the activity of FA transporter proteins. Importantly, bombesin was shown to increase intestinal alkaline phosphatase (iAP) levels (Hodin et al., 1994) which dephosphorylates CD36 and subsequently increases its activity (Lynes et al., 2011). CD36 is a key transporter of LCFA in the intestine and therefore iAP regulation poses a significant potential in bombesin induced FA uptake. However it is difficult to perceive how this may occur in isolated cells as opposed to an *in vivo* model.

Whilst it remains possible that bombesin is stimulating the release of CCK from EECs in the cell suspension it is clear that the results are not exclusively relayed through this mechanism. Therefore, the results of these experiments and the multiple effectors involved in bombesin's activity do not allow the conclusion that bombesin stimulates CCK release from I-cells.

4.3.3.2. Amino acids stimulate an increase in Bodipy-FA uptake. Does this involve signalling through CCK?

I-cells release CCK upon detection of nutrients in the lumen. The most potent substrates of which are FA with a chain length of ≥C12 (McLaughlin et al., 1999, Feltrin et al., 2004) and amino acids (Meyer et al., 1976, Liou et al., 2011a, Liddle, 1995, Feltrin et al., 2004). In the present study, attempts were made to stimulate

I-cells to release CCK by incubating them with C12 dodecanoic FA prior to measuring Bodipy-FA uptake, but these experiments proved unsuccessful because by virtue of adding C12, the prevailing FA gradient driving FA flux was altered making comparison of control with FA treated unfeasible. For this reason, amino acids were used as a nutrient ligand working on the premise that amino acids acting through the Ca^{2+} sensing receptor (CaSR) trigger release of CCK from CCK expressing cells (Liou et al., 2011c, Wang et al., 2011).

Several studies have shown that amino acids can stimulate CCK release in both STC-1 cells and primary I–cells (Hira et al., 2008, Liou et al., 2011c, Wang et al., 2011, Daly et al., 2013). Amino acid signalling is sensitive to its structure; aromatic amino acids have been shown to be potent stimulators of CCK release, and also display different degrees of potency. Furthermore this response appears to be selective for amino acids with L-isoform orientation (Wang et al., 2011, Daly et al., 2013, Liou et al., 2011c).

Duodenal cells were incubated with a range of aromatic L-amino acids and observed to significantly stimulate Bodipy-FA uptake. Within this study, the largest increase in Bodipy-FA uptake was recorded for L-phenylalanine and L-tryptophan. These data are reflected in the profile of CCK release for both L-phenylalanine and L-tryptophan, in which L-phenylalanine was the most potent of the amino acids tested in each study (Wang et al., 2011, Liou et al., 2011c, Daly et al., 2013). In the current study, L-histidine was included as an additional aromatic amino acid and also induced a significant increase in Bodipy-FA uptake although to a lesser extent. This demonstrates variability in signalling by different L-isoform aromatic amino acids.

Interaction of the L-amino acids; L-phenylalanine and L-tryptophan with I-cells is elicited, at least in part, through the CaSR (Liou et al., 2011c, Wang et al., 2011, Hira et al., 2008). In both the study by Liou and colleagues and that described by Wang and colleagues, experiments were performed upon primary CCK-cells isolated from an eGFP-CCK mouse model. Wang et al. observed Ca^{2+} fluxes in CCK-cells upon application of L-phenylalanine and L-tryptophan, and the CaSR

inhibitor Calhex231 blocked these effects. Non-aromatic amino acids failed to induce a response (Wang et al., 2011). Liou et al. also demonstrated Ca^{2+} fluxes in CCK-cells in the presence of L-phenylalanine, which was not reproduced by D-phenylalanine. The effects of L-phenylalanine were abolished in CaSR KO mice (Liou et al., 2011c). In these instances, amino acids act as an allosteric modulator of CaSR, in the presence of extracellular Ca^{2+}, to drive Ca^{2+} influx and signalling within the cell, resulting in release of hormone (Conigrave et al., 2000, Mangel et al., 1995, Hira et al., 2008).

Aromatic amino acids appear to bind sites on the CaSR that are different to the binding sites for calcimimetics (Mun et al., 2004, Zhang et al., 2002a, Zhang et al., 2002b). The study conducted by Liou and colleagues demonstrated that although calcimimetics and extracellular Ca^{2+} alone were able to stimulate CCK-cells, neither significantly stimulated CCK release. This suggests that L-phenylalanine specifically directs intracellular signalling in a way to potentiate CCK release (Liou et al., 2011c). This mirrors the data of the current study whereby incubation with neomycin, a potent agonist of the CaSR (Ye et al., 1996), did not significantly increase cellular Bodipy-FA uptake. These details are interesting as they show that ligand binding to CaSR must occur at distinct sites that activate specific signalling pathways to potentiate hormone secretion. This develops the idea that CaSR acts as a primary sensor for dietary aromatic amino acids in the intestine and mediates hormone secretion in response to this (Liou et al., 2011c). This was confirmed upon exposure of L-phenylalanine to intestinal cells isolated from CaSR knockout mice where the signalling response in I-cells was abolished (Liou et al., 2011c).

In addition to aromatic amino acid signalling through CaSR, some amino acids have also been reported to stimulate CCK release through interaction with the taste receptor T1R1/T1R3 (Daly et al., 2013). L-leucine and L-glycine stimulate CCK release through the T1R1/T1R3 receptors and importantly L-phenylalanine is able to signal through both CaSR and T1R1/T1R3 (Daly et al., 2013). The combined involvement of at least two receptors known to contribute to L-phenylalanine signalling might be expected to amplify the resultant response

compared to L-tryptophan that signals through CaSR and not T1R1/R3 (Nelson et al., 2002, Daly et al., 2013).

The principle receptor by which L-histidine signals through has not been elucidated. L-histidine has been shown to exhibit a very low degree of signalling through T1R1/T1R3 (Nelson et al., 2002) and its aromatic structure may also imply interaction with CaSR. Indeed this may alternatively involve interaction through an unconfirmed receptor, such as GPRC6A (Oya et al., 2013) or GPR93 (Choi et al., 2007b, Choi et al., 2007a). It would be useful to confirm the effects of L-histidine and also L-phenylalanine and L-tryptophan in the place of a CaSR antagonist to confirm the contribution of CaSR in eliciting the effects on Bodipy-FA uptake.

L-alanine was added to incubating cells as a negative control, as previous studies have shown L-alanine does not stimulate I-cells or stimulate CCK release (Wang et al., 2011). Interestingly, L-alanine also exhibited a small stimulatory effect upon Bodipy-FA uptake although not to statistically significant value. This result could be explained by possible stimulation of other EEC subtypes by L-alanine through alternative receptor mechanisms or by directly interacting with enterocytes to affect uptake. Notably, the study by Wang and colleagues did not report the effects of L-alanine on other GI hormones, or mention a direct effect upon enterocytes therefore these possibilities cannot be ruled out (Wang et al., 2011).

Taken together the results of the current study and the published literature suggested that the aromatic L-amino acids employed might indirectly increase Bodipy-FA uptake through the actions of CCK. To confirm this theory the experiment was repeated on intestinal cells isolated from CCK KO mice, thus eliminating endogenous CCK from the cell suspension. Unexpectedly, these experiments reproduced the pattern of Bodipy-FA uptake as seen for eGFP-CCK mice. This surprising result showed that CCK was not responsible for mediating the amino acid induced increase in Bodipy-FA uptake. This could be the result of amino acid-stimulated release of additional GI peptides that may mediate this

effect, or through a direct interaction of amino acids with the absorptive enterocytes.

CaSR is highly enriched in CCK-cells (Wang et al., 2011) and not expressed in absorptive enterocytes (Liou et al., 2011c) importantly however, CaSR is also highly expressed by other EEC subtypes. CaSR expression has been described in L-cells, K-cells and G-cells (Buchan et al., 2001, Ray et al., 1997, Reimann et al., 2004, Mace et al., 2012). Additionally, Mace et al., (2012) saw L-amino acids stimulated GIP, GLP-1 and PYY secretion from rat small intestine, effects that were elicited through CaSR with a dependence upon extracellular Ca^{2+} (Mace et al., 2012). This is important to consider as our previous data suggested that other GI peptides might have the capacity to stimulate FA uptake. It is likely that other GI peptides may be involved in these responses. To clarify the release of CCK or other peptides from the cell suspension it would be informative to measure amino acid induced peptide secretion by radioimmunoassay. However, in practice, the complexities to measure secretion of mixed peptide types and concentrations, particularly in such a small cell populations are too vast.

The suggestion that amino acid signalling may be exerted through multiple EEC subtypes is supported by expression analysis of T1R3, a receptor shown to elicit amino acid-induced hormone release, that showed only 50% co-localisation with CCK-positive cells (Daly et al., 2013). This indicates significant expression of T1R3 on other cell types. Additionally, amino acid stimulation of GPRC6A, a family C group 6 subtype A GPCR has been shown to elicit GLP-1 secretion from GLUtag cells (Oya et al., 2013) thus implicating a role for this receptor on EECs. This indicates that amino acid interaction with T1R1-T1R3 or GPRC6A may elicit hormone release from other EEC subtypes.

Whilst it is clear that amino acids are potent stimulators of EECs, it is important to consider that amino acids may be able to exert a direct action on enterocytes. Significantly, amino acid receptors such as the protein-hydrosylate activated GPCR, GPR93, are ubiquitously expressed in the intestinal epithelium (Sai et al., 1996, Choi et al., 2007a). It is possible that interaction through these receptors

could activate cell signalling pathways and influence permeability to FA directly however this mode of action is speculative.

Ultimately, the amino acid signalling network is extensive and incredibly complex. Multiple amino acid receptors exist in the lingual epithelium, which is probably reflected in the intestine (Yasumatsu et al., 2012). Amino acid sensing and transportation can also be carried out by one or more specific amino acid transporters, with substantial overlap of substrates of amino acid subtypes (Boudry et al., 2010). Aromatic acids alone have the ability to signal through various receptors of which may collectively relay some of the effects upon Bodipy-FA uptake.

The data presented in this thesis show that incubation of intestinal cells in amino acids causes an increase in Bodipy-FA uptake. This suggests that the intestine can respond to the presence of one nutrient type and induce the uptake capacity for a different nutrient type. Therefore the null hypothesis 'ligand-stimulated release of CCK from CCK-cells does not induce FA uptake' can be partially rejected. The fact that CCK release was not demonstrated from CCK-cells means the role of CCK to elicit these 'ligand-stimulated' effects cannot be confirmed. Furthermore previous data in this thesis have demonstrated that GI peptides, including CCK, can increase Bodipy-FA uptake in a similar pattern. It was postulated that bombesin and then amino acids were also able to increase FA uptake, exerting their effects indirectly through the release of CCK. However, use of intestinal cells from CCK KO mice showed that this was not, in its entirety, the case.

4.3.3.3. Can an eGFP-ve cell population be used as a representation for the effects of endogenous CCK on Bodipy-FA uptake?

Based on the results of the experiments discussed above in section 4.3. it became evident that some nutrient ligands might be triggering release of other GI hormones that in turn stimulate FA uptake. Data from the experiments utilising CCK^{LacZ} mice demonstrated that CCK alone was not responsible. Therefore, a

series of experiments were undertaken with the aim of determining whether CCK-expressing cells in the duodenum were responsible for sensing nutrients and releasing hormones that via a paracrine action, stimulated FA uptake.

Fluorescent activated cell sorting (FACS) was employed to deplete duodenal cells of CCK-expressing cells. Dissociated duodenal cells replete with CCK expressing cells were compared to CCK-cell depleted samples. These cell samples were incubated with either L-phenylalanine or CCK as a positive control to enable a direct comparison for I-cell activity within the preparation. Interestingly, incubation of CCK cell depleted samples and whole cell populations with CCK or L-phenylalanine achieved comparable results. Incubation with CCK was included as a positive control and confirmed cell viability and responsiveness was similar for both populations. Incubation of CCK-cell depleted samples cells with L-phenylalanine maintained a 1.5-fold increase in Bodipy-FA uptake that mirrored values obtained for total cell population and therefore indicated CCK-containing cells were not required for L-phenylalanine to induce an increase in FA uptake.

There are three possible explanations for these data. Firstly, cells containing CCK in the GI tract are not essential to elicit the observed response upon Bodipy-FA uptake. However, the data presented in this thesis have demonstrated that CCK can elicit a significant effect upon FA uptake in intestinal cells. If intestinal CCK-cells were not involved in this response then the alternative explanation would suggest that CCK derived from a different source in the body might elicit this effect. Alternative areas of CCK production exist in the brain and pancreas (Dockray, 1976, Shimizu et al., 1998). If this were the case, delivery of CCK to enterocytes would occur through the circulation or via neural transmission. However, since the experiments were performed *in vitro* any influences from systemic hormones or neural inputs were not present.

Alternatively, L-phenylalanine may interact directly with enterocytes and modulate absorptive properties. L-phenylalanine may interact with amino acid receptors such as GPRC6A that are expressed on enterocytes and activate intracellular signalling pathways (Oya et al., 2012, Oya et al., 2013). A number of amino acids have also been demonstrated to act intracellularly, to influence cell signalling pathways following their cellular uptake (Wu, 2009). In the case of

phenylalanine this includes affects upon GTP cyclohydrolase-I expression and activity which is involved in the hydrolysis of guanosine triphosphate (GTP) and has subsequent downstream affects on nitric oxide synthesis (Shi et al., 2004). However, although nitric oxide has been shown to increase glucose transport in muscle cells (Fryer et al., 2000) there is nothing in the literature to suggest this may affect cellular FA uptake. Furthermore experiments using porcine intestinal cells showed that oxidation of phenylalanine within enterocytes is negligible (Chen et al., 2007) and therefore does not result in the generation of multiple by-products that may influence aspects of cell signalling.

Thirdly, the presence of non-eGFP EECs within the intestinal cell preparation retains the possibility that L-phenylalanine may stimulate these cells to release peptides. Amino acids are the natural ligand for many EEC types (discussed in section 4.3.3.2.) and the induced release of alternate GI peptides may elicit an effect upon intestinal cells to modulate FA uptake.

Unfortunately the logistics of this experiment were very difficult. To limit the time cells were subject to FACS sorting, only a small number of cells could be obtained. This made the experiment difficult to perform and required that data from a number of experiments be pooled and normalised to represent a fold-increase in Bodipy-FA uptake compared to the control of each experiment. Due to time constraints no further experiments were performed to explore the validity of the three possible explanations, but it was felt that further experiments were necessary before firm conclusions could be drawn.

To be able to draw any conclusions as to the involvement of CCK-cells in mediating the hormonal effects to modulate intestinal FA absorption it is necessary to identify a more accurate way to exclusively implicate I-cells. EECs have a multitude of overlapping functions and stimulatory profiles. The experimental use of nutrients as a ligand to exclusively stimulate CCK-cells is evidently difficult due to the potential effects upon other EEC types. A more specific stimulant of CCK-cells, such as a GPR40 agonist may be a more astute direction to focus this aim.

4.3.4. Overview: EECs and FA uptake

It is clear that GI peptides have a definite role in the control of intestinal FA uptake. As stated by Ducroc et al. "All of these hormones are likely to exert paracrine effects via receptors on basolateral membranes" (Ducroc et al., 2005). However, attempting to define the precise paracrine role of specific GI peptides is incredibly complex. Knockout studies for individual EEC subtypes have continually failed to produce a notable phenotype, which adds to the uncertainty of specific hormonal mechanisms. However, a lack of total EEC population, induced through knockout of Ngn3 gene, resulted in a 50% death rate within 2 weeks of birth, coupled with retarded growth and very little body fat (Mellitzer et al., 2010, Mellitzer and Gradwohl, 2011). The problem was pin-pointed to defective lipid absorption, where reduced lipid concentrations were seen inside enterocytes coupled with a global reduction in chylomicrons, total plasma cholesterol and TGs (Mellitzer et al., 2010). This is also important clinically in so far as a patient with Ngn3 disruption exhibited extreme nutrient malabsorption attributable to an almost total absence of EECs (Cortina et al., 2007). Therefore, whilst the extent of paracrine signalling for specific GI peptides remains unclear, it is obvious they impart fundamental significance concerning FA uptake.

The methodology employed in this chapter has demonstrated that Bodipy-FA analogues can be used to demonstrate cellular FA uptake. However, it is important to remember it is difficult to demonstrate intestinal FA uptake using isolated cells in suspension. An important property of intestinal epithelial transport is cellular polarity, which is central in the movement of nutrients across a cell. This polarity may also reflect expression of transporters and receptors in defined portions of the cell and this distinction is lost when cells are suspended. Additionally, epithelial cell-to-cell contacts are important to maintain epithelial polarity and for cell communication and may also influence uptake kinetics or transporter protein location which needs consideration when interpreting the data presented in this thesis (Pohl et al., 2005). However the data obtained using this protocol was reproducible and was determined the best model to demonstrate short-term FA uptake in intestinal cells.

4.3.5. Conclusion

This chapter has demonstrated for the first time that CCK can increase FA uptake in small intestinal cells. It has been further demonstrated that this is elicited through signalling through CCK-R_A and an increase in activity of the FA translocase CD36. Incubation of cells with the CCK-cell ligands; bombesin and L-amino acids achieved a similar result yet data acquired using cells from CCK KO demonstrated this effect was not exclusive to CCK. It can be postulated that a cocktail of GI peptides are able to modulate intestinal cells with the aim to enhance FA absorption according to stimuli and demands. The implications between these data and the findings presented in chapter 3 will be interpreted in the summary discussion.

Chapter Five

Summary Discussion

5.0. Summary of the findings

Enteroendocrine cells are key modulators within the GI tract, relaying signals reporting the presence and type of ingested nutrients to peripheral and central tissues, with the primary aim to optimise digestion and acquisition of nutrients, as well as controlling further food intake. The integrated response of EECs is achieved by the controlled release of GI hormones. The fact that the EEC population constitutes only ~1% of the total intestinal epithelial cell population and are difficult to visually discern from other types of intestinal cells, has previously hampered the ability to study EECs. The development of transgenic animals with genetically labelled EEC populations, such as the eGFP-CCK mouse model used in the current work, has opened the door to EEC research by enabling identification and study of these previously elusive cells.

In this thesis two different views of EECs have been presented that detail novel characteristics of EECs. Firstly, data were presented that demonstrated a multi-hormone complement in CCK-cells of mouse duodenum. These data are contrary to the long-standing 'one-cell one-hormone' classification system that was promoted in 1970 by Creutzfelt. Secondly, a paracrine role for CCK was discovered whereby intestinal cells that were pre-treated with CCK showed an increase in FA uptake. This was an exciting discovery because it suggests that *in vivo* CCK can modulate the absorptive ability of intestinal cells. In addition, these data suggest that certain functional attributes of the SI are partly, if not wholly, 'closed circuit', meaning that an increase in permeability to a solute need not rely on higher centres or in fact other tissues.

These data have been discussed within the relevant chapters and this summary discussion chapter will explore the broader implications of the findings and suggest future directions for this research.

5.1. Characterisation of a Multi-Hormonal Complement Expressed in CCK-cells of Mouse Duodenum

It was established that duodenal eGFP-cells of eGFP-CCK mice represented CCK-cells. Extensive analysis using immunostaining techniques established that the hormonal content of CCK-cells was not exclusive to CCK. CCK-cells were observed to contain an array of anorectic hormones and interestingly, half of duodenal CCK-cells contained the orexigenic hormone ghrelin. CCK-cells that co-expressed other peptides were observed in both the crypt and villi regions supporting peptide co-expression as a feature of EECs throughout their lifespan. Co-expression data were validated using dual staining for CCK alongside key gut peptides and detailed peptide co-expression to exist within EECs at a spectrum of intensities. Furthermore, the majority of intracellular peptide labelling within co-expressing cells was located to separate vesicles although this requires validation, which could be achieved through immuno-gold labelling and electron microscopy.

Collectively, these data refute the long-standing hypothesis that EECs have the ability to transcribe protein from only one peptide precursor and substantiate the newly founded property that EECs possess the molecular machinery to express multiple GI peptides. Co-expression of multiple peptides within EECs has been supported by studies that have conducted protein and mRNA analysis of isolated EEC types (Egerod et al., 2012, Habib et al., 2012). Additionally, transgenic models of targeted cell ablation controlled by specific GI peptide precursors also affected the expression of other GI peptides (Lopez et al., 1995, Rindi et al., 1999, Egerod et al., 2012). These studies have demonstrated that multiple peptide precursors are active within individual cell types. However, the co-expression of ghrelin in mature CCK-cells is a novel finding.

Intracellular co-localisation of peptide labelling depicted the majority of co-expressed peptide was contained within separate secretory vesicles. This suggests a cell may be able to control differential release of peptide however co-release of peptides has not yet been demonstrated. The

potential physiological advantages for EEC peptide co-expression were discussed in section 3.4.2.5. These advantages encompassed the multitude of overlapping roles of GI peptides and thus suggested that peptide co-release could potentiate a desired response. There are examples of such combined peptide responses evident in the literature. Firstly, both GIP and GLP-1 act to enhance glucose stimulated insulin secretion. However, activity of both peptides potentiates the response to achieve the full incretin effect (Kim and Egan, 2008). Similarly, a study by Sandberg and colleagues demonstrated that low doses of CCK-33 could potentiate GIP induced insulin secretion (Sandberg et al., 1988b). Furthermore, secretin and GIP have been separately demonstrated to enhance the pancreatic roles of CCK to stimulate release of bicarbonate juice and amylase enzymes (Bold et al., 1995, Kim et al., 1999). Whilst the related effects of these anorectic hormones are relatively straightforward to interpret, peptide co-expression is more difficult to determine in terms of CCK and ghrelin due to the classically opposing roles of these peptides. However, because of the additional contrasting roles attributed to octanoylated 'active' ghrelin and non-octanoylated ghrelin (DAG) it is important to establish which form of ghrelin is expressed within CCK-cells before these data can be fully interpreted. This could be achieved through probing cells with an antiserum specific for octanoylated 'active' ghrelin, or alternatively for expression of the enzyme GOAT, which is critical for the production of active ghrelin.

The spectrum of intensities of the co-localised peptides within stained cells was intriguing and highlighted the heterogeneous nature of EECs. These properties suggest an EEC may be able to modulate peptide expression according to incoming signals. This is supported by the known plasticity of the enteroendocrine system that is facilitated by a short life cycle (3-7days) of intestinal epithelial cells. Such modulation of EEC characteristics may be established in differentiating cells to influence final cell phenotype or an alternative hypothesis is that a cell may be able to alter its characteristics and peptide expression during its lifespan by altering the transcriptional

activity of different peptide precursors according to signals and demands (Egerod et al., 2012).

Studies have demonstrated that prolonged changes in environmental signals influence terminal differentiation of EEC subtypes. Such systemic modulation of EEC characteristics have been demonstrated in models of intestinal pathology (Rubin et al., 2000, McDermott et al., 2006, O'Hara et al., 2004) and also following long-term dietary changes (Murphy and Bloom, 2006, Moran-Ramos et al., 2012) which can alter EEC expression, distribution and activity (described in section 1.2.4). Furthermore, alterations in nutrient exposure to portions of the GI tract, such as that which occurs following bariatric surgery, has been repeatedly shown to transform the circulatory profile of gut peptides (Ockander et al., 2003, Mingrone et al., 2012, Schauer et al., 2012). Such changes in circulating peptide concentrations inflict the resultant benefits of bariatric surgery including weight loss and a sustained reduction in appetite in addition to multiple other effects. Importantly, the resultant effects of bariatric surgery also include sensitive alterations in incretin hormone concentrations, which cause improved glucose tolerance in patients. The effect of bariatric surgery upon incretin hormone activity is so significant that has been shown to reverse incidence of diabetes within 6 days post-operatively, even prior to significant weight loss (Mingrone et al., 2012, Schauer et al., 2012). Significantly, the onset of these effects is reflective of the timescale for EEC re-generation in which the entire EEC population is renewed within a week (Gordon et al., 1992). Integration of signals can influence EEC differentiation and subsequently alter properties and characteristics of the EEC population; this is commonly known as EEC reprogramming.

The above examples of EEC reprogramming reflect the plasticity of the enteroendocrine system. It is in this way that the GI tract has the ability to adapt according to environmental and systemic signals to maintain optimised nutrient handling, a capability that without doubt conferred an evolutionary advantage on species that possessed it. Most significant alterations in EEC distribution and peptide expression are probably initiated during cell development, however it has also been speculated that

an EEC may be able to respond to signals during its lifecycle to adapt accordingly. The peptide co-expression data presented in this thesis indicate mature EECs possess the molecular machinery to transcribe multiple peptides within its lifespan. Therefore it is postulated that an EEC may be able to integrate signals and infer acute changes in peptide expression by altering the transcriptional activity of different peptide precursors. This would enable a more rapid response to alter peptide expression within mature EECs. It is then also speculated that the cell may be able to control the differential release of alternate peptides however this is yet to be determined and is confounded by the difficulty to maintain EECs in culture. Systemic modulation of mature EECs could explain the immediate alterations in plasma concentrations in GLP-1 and PYY observed within 2 days of bariatric surgery (Papamargaritis et al., 2012). Review of the literature has revealed only one study that has reported acute changes in cellular environment to induce an alteration in a cells transcriptional activity. Lee and colleagues demonstrated that induced hypoxia in Caco-2 cells increased transcription of lactase enzyme within cells, increasing enzyme mRNA levels as rapidly as one hour following treatment (Lee et al., 2002b). This is a direct example that intestinal cells can adapt transcriptional activity in response to extracellular environmental signals. This property would be physiologically advantageous to cope with temporary fluctuations in nutrient delivery within the system such as temporary fasting or nutrient overload.

The aforementioned studies give examples of EEC reprogramming in response to environmental and systemic changes. However, whilst these studies report resultant alterations in circulating profiles of GI peptides, these measurements are not reflective of hormone expression within a single cell or even at the local tissue level. With this in mind, it would be interesting to determine alterations in the peptide complement within EECs following manipulation of diet and in particular examine models of a typical high-fat 'Western' diets or regimes to mimic well established fad diets such as a high protein and fat, low carbohydrate diet, typified by the

'Atkins' diet plan. These experiments would provide insight to understand the adaptability of the enteroendocrine system and would provide scientific evidence to the application of dietary changes.

Additionally, due to the impressive and currently obscure effects observed following bariatric surgery, a very exciting aim for the future would be to develop a viable mouse model of bariatric surgery to determine the postoperative changes in the hormonal complement of EECs. It is predicted that there is a complete shift in EEC expression and hormonal content, whether this changes the complement and distribution of heterogeneous EECs remains to be determined. It would also be interesting to establish whether significant alterations are achieved only from generation of new EECs or if a mature EEC has the capacity to significantly alter peptide expression. This information would elucidate the extent of plasticity of the enteroendocrine system and would ultimately enhance the scope to modulate it therapeutically.

5.2. Modulation of Enterocyte Fatty Acid Uptake by Gut Hormones

EECs release hormones including CCK in response to nutrients (Meyer et al., 1976, Liddle, 1995, McLaughlin et al., 1999, McLaughlin et al., 1998, Liou et al., 2011c, Daly et al., 2013). Following secretion, CCK signals via receptors on nearby vagal afferent neurones or alternatively by entering the circulation via the capillaries within the villi for transportation to receptors on local and distant target tissues. CCK exerts a spectrum of effects within the body with the ultimate end-point to optimise the digestion and absorption of nutrients. With this in mind, a paracrine role of CCK was investigated to determine whether CCK might exert a local effect to modulate the absorptive properties of neighbouring enterocytes.

The current study utilised Bodipy-FA analogues to determine dynamic changes in FA uptake. Using this methodology it was confirmed that pre-incubation with CCK increased FA uptake in intestinal cells. Incubation of

intestinal cells with OEA and GLP-2 increased cellular FA uptake, re-iterative of previously established data (Yang et al., 2007, Hsieh et al., 2009). Experimental attempts to implicate CCK-cells in this process involved application of the CCK-cell ligands bombesin and L-amino acids which imposed an increase on FA uptake reflective of that achieved following incubation with CCK. At a glance these data indicated that the cellular release of CCK was eliciting these effects, however it is maintained that CCK release was not determined or confirmed in any experiment. Unfortunately due to the surprising lack of specificity of the chosen experimental ligands, it was ultimately difficult to determine the direct contribution of CCK-cells to elicit these effects. This is reiterated in experiments that were performed using cells from CCK KO mice that demonstrated the effects of bombesin or L-amino acids were not exclusive to CCK. A more specific CCK-cell ligand such as a GPR40 agonist may have proved to be more selective than the nutrient-based ligands employed. However, as GPR40 is also expressed on other EEC subtypes (Edfalk et al., 2008) this would still not eliminate the potential involvement of other GI peptides to elicit these effects upon cellular FA uptake. As such, the methodology described in Chapter 4 to strip SI eGFP-cells from an intestinal cell population isolated from eGFP-CCK mice remains the best representative to clarify the role of CCK-cells to influence FA uptake. However, unexpectedly, the data acquired from these experiments suggested that the stimulatory-effects induced through incubation with L-phenylalanine did not involve CCK-cells. This suggests a possible direct effect of nutrient ligands upon absorptive cells irrespective of GI peptide activity. To test this possibility, it would be useful to establish whether enterocytes are able to respond to extracellular nutrient presence. An initial approach to demonstrate this could be achieved through Ca^{2+} signalling studies. However, the significant effects observed upon incubation of intestinal cells with CCK as well as with OEA and GLP-2, all hormones known to be present in EECs, maintain the premise that EECs contribute to these effects and release peptides that can act to increase cellular FA uptake.

GI peptides have a multitude of overlapping and on occasion reciprocal effects. For example, CCK and GLP-2 have the ability to modulate cellular FA uptake, whereas GLP-1 inhibits FA absorption (Mellitzer and Gradwohl, 2011, Hsieh et al., 2009). What the net emergent effects of co-secretion of several hormones may be is open to conjecture and highlights the need for integrative studies rather than reductionist approaches.

The multi-hormone complement of duodenal EECs established in chapter 3 indicates that an EEC can contain multiple hormones. Determination of peptide co-secretion from a cell following its stimulation would be a landmark, as detail of hormone co-expression alone does not prove that peptides are co-released. It would be incredible to establish whether EECs are able to co-release a cocktail of peptides and to subsequently determine the relative consequences of their co-release.

With this in mind, future experiments to establish the combined effect of GI peptides upon cellular FA uptake would appear a better representation of the physiological environment. The co-release of a combination of peptides could explain the potentiated increase in cellular Bodipy-FA uptake that was observed following pre-treatment with L-amino acids compared to incubation with CCK alone. The initial approach to investigate this theory would entail the co-application of peptides that have been established as co-expressed in EECs (Chapter 3). It would be particularly interesting to determine the absorptive effects following incubation of cells with CCK alongside DAG and/or ghrelin. This may also elucidate evidence of an antagonistic role for DAG/ghrelin that was speculated and discussed in chapter 3.4.2.6 and may help to explain co-expression of these peptides within duodenal EECs. The scope to design such experiments is extensive given the potential range of peptide combinations as well as the dose-sensitivity for each peptide that may fluctuate when co-administered. It is also possible that peptide effects may exist at a variety of specific targets within cells. This may extend towards properties of the apical or basal membrane to affect flux across the cell. However, determining the differential effects of membrane properties is not within the realms of this experimental design. It would also be interesting to probe whether the

effects of peptides upon cells isolated from different regions of the SI differ according to regions that the relative peptides are most highly expressed. An example of this would postulate that the effects of GLP-2 might be heightened upon ileal cells compared with duodenal cells.

The findings that OEA, GLP-2 and CCK and probably additional GI peptides can increase nutrient absorption present a novel approach to treat a number of nutrient malabsorption pathologies. Enhancing the understanding of this system could establish clinical significance with implications to treat cases of short bowel syndrome, lipid malabsorption illnesses and may have influence upon obesity treatments (Yang et al., 2007).

Collectively, these data have demonstrated that CCK has the ability to double the absorptive ability of intestinal cells yet it is not the only GI peptide with this capacity. These data support the notion that GI peptides may exert a paracrine effect upon neighbouring cells to modulate absorptive properties and enhance nutrient uptake without requirement of signals from higher centres. This reflects properties of the GI tract to exist as a closed circuit in which the enteroendocrine system is able to co-ordinate signals to optimise uptake of nutrients with a rapid onset of effects.

5.3. Interpretation of the two data sets:

The multi-hormone complement demonstrated in duodenal EECs (chapter 3) indicates that nutrient-induced stimulation of EECs may result in the co-release of multiple peptides. The data presented in chapter 4 demonstrate that CCK and other GI peptides can exert an effect on intestinal cells to increase FA uptake. These two data sets go hand in hand to explain some of the activities of EECs. Firstly; EECs can contain more than one hormone and secondly; more than one hormone acts upon intestinal cells to modulate absorptive functions.

The data presented in this thesis and in recent publications (Habib et al., 2012, Egerod et al., 2012) collectively challenge the classical 'one cell-one hormone' property of individual EEC subtypes. It has been suggested that EEC classification could potentially be determined by the dominant hormone that is expressed within a cell (Helander and Fandriks, 2012). However, what appears to exist can simply be defined as intestinal 'hormone-cells' that possess specificity for nutrient stimulation and perhaps a defined complement of hormones that reflect location in the GI tract, as well as a multifaceted environmental and systemic signals. These signals may acutely or tonically alter the spectrum of hormone co-expression. This can be interpreted as a heightened degree of efficiency within the enteroendocrine system and through release of multiple peptides that collectively act to control nutrient handling and, as such, to optimise digestion and absorption of nutrients.

It is obvious from global ablation studies that EECs are important for well-being. In humans, absence of EECs presents clinically with extreme nutrient malabsorption (Cortina et al., 2007) depicting a vital role to control nutrient handling within the body. However, the individual contribution of each peptide expressed in EECs at present is hard to assess because of the dearth of literature addressing individual roles. Additional functional studies are required to confirm the characteristics of EEC activity and the effects that a combined release and activity of GI peptides may have.

The work presented in this thesis has involved defining the hormonal content of EECs in fixed tissue sections and monitoring cellular FA uptake following application of peptides and compounds to intestinal cells in solution. What would be useful is to connect the two data sets by performing functional experiments upon isolated EECs. An attractive future goal would be to monitor CCK-cell stimulation by nutrients. This could be achieved in the first instance by measuring intracellular Ca^{2+} signalling following exposure to different ligands or likewise through measuring the hormones released within a cell suspension. The availability of the eGFP-

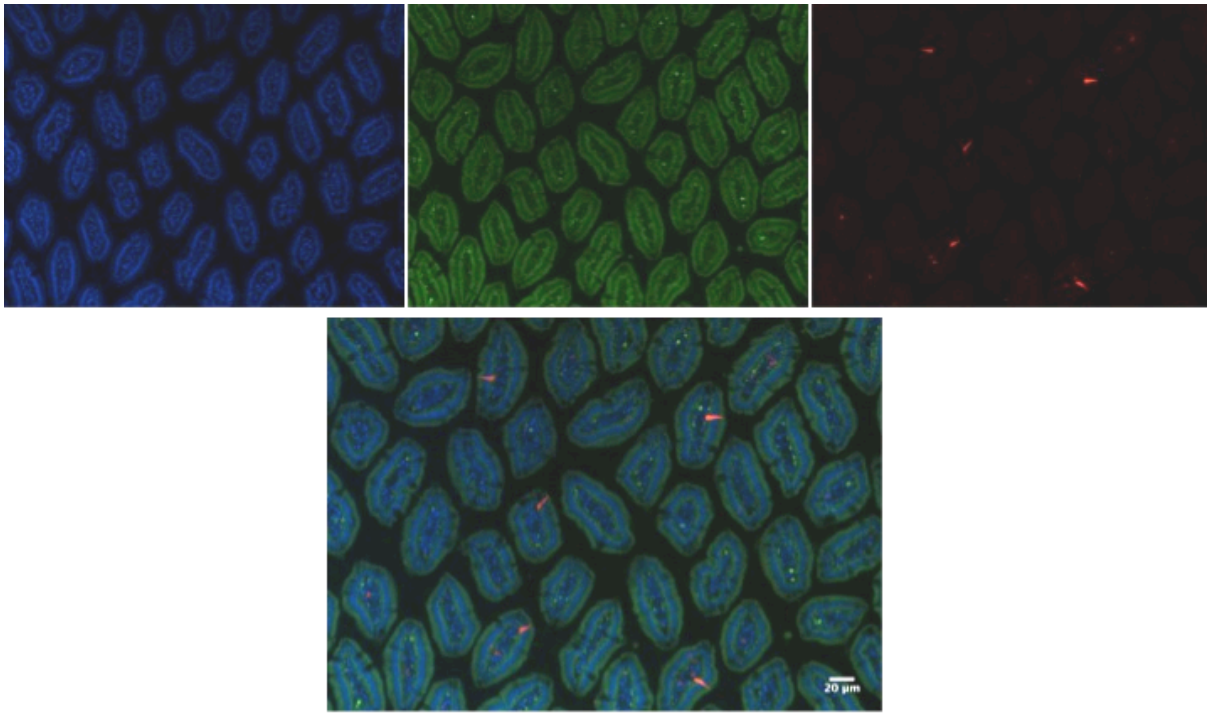
CCK mouse model is an exciting model for future investigations as it enables identification and isolation of hormonal cells. However problems with cell viability of small intestinal cell preparations continue to hinder progress.

5.4. Concluding Remarks

The current work demonstrates that EECs of the duodenum express a spectrum of bioactive peptides. Furthermore, a paracrine action of CCK-signalling is implicated to increase the absorptive ability of neighbouring enterocytes. These data suggest that CCK-cells have the ability to integrate nutrient signals and secrete a cocktail of hormones in response. These findings imply an increased complexity to the enteroendocrine system whereby GI peptides may work together to potentiate a desired response without requirement of signals from higher centres.

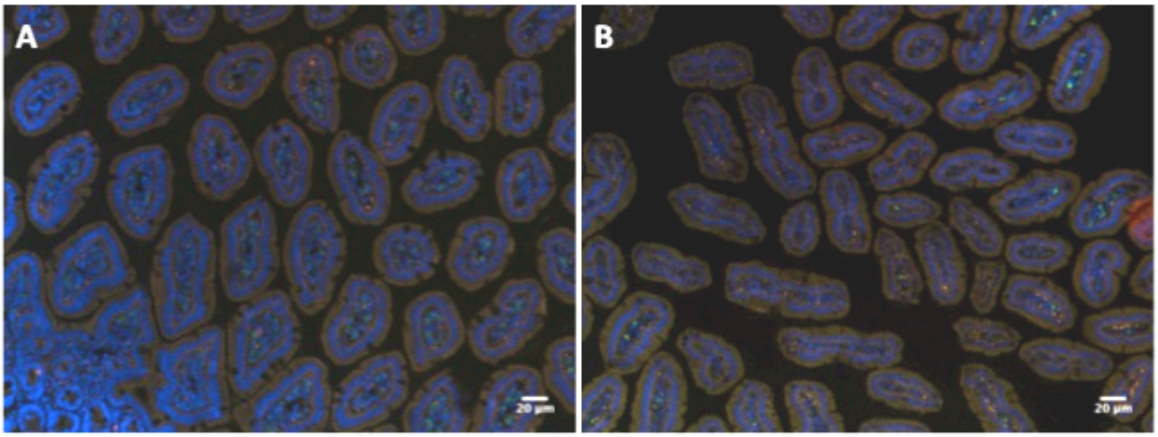
There is still a long way to go in terms of characterising EECs. However, with the scope of new technologies and expanding knowledge, along with a collaboration of data between laboratories conducting EEC research, the task of characterising EECs is becoming increasingly possible.

6.0. Appendices



Appendix 3.1. Anti-eGFP immunostaining of paraffin embedded tissue sections from eGFP-CCK mice.

Sections (4µm) were immunostained with an anti-GFP coupled with an Alexafluor594 secondary antiserum. Confirmation that endogenous eGFP disappear after processing. Blue fluorescence represents nuclei staining with Hoechsst 33452, and green fluorescence represents endogenous eGFP whilst red represents labelling with eGFP-antiserum. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.



Appendix 3.2. Control images for secondary antisera.

Duodenal sections (4µm) that have undergone the immunostaining procedure without application of primary antisera. This acts as a control for the secondary antisera as it provides detail of specific labelling.
A) Anti-mouse Alexafluor488 and Anti-rabbit Alexafluor594. B) Anti-mouse Alexafluor488 and Anti-guinea-pig Alexafluor594. Blue fluorescence represents nuclei staining with Hoechsst 33452. Red represents Alexafluor594 and green represents anti-GFP labelling with Alexfluor488. Both images show a small degree of background auto-fluorescence. There is no specific fluorescence labelling within the epithelial cell layer. Images were taken on a snapshot widefield microscope, 20X objective. Scale bar represents 20µm.

6.1 Future Directions

Listed here is the foremost selection of experiments to be conducted following on from the data presented in this thesis:

Characterisation of a Multi-Hormonal Complement Expressed in CCK-cells of Mouse Duodenum

• Employ immuno-gold labelling and electron microscopy to confirm intracellular peptide localisation to separate vesicles within co-expressing cells.

• Map the distribution of cells expressing GOAT enzyme and determine its expression within CCK-cells.

• Determine a shift in the hormonal complement of EECs from different regions of the GI tract.

• Determine changes in the hormonal complement of EEC in models of diet manipulation.

• Determine changes in hormonal complement of EECs in a mouse model of bariatric surgery.

• Perform functional studies to determine differential or simultaneous release of peptides

Modulation of enterocyte fatty acid uptake by gut hormones.

• Incubation of intestinal cells with a GPR40 agonist to implicate CCK-cell activity to modulate cellular FA uptake.

• Perform complementary studies using Bodipy-FA of different chain lengths.

• Determine the effects of other key GI peptides upon cellular FA uptake.

• Determine effects of individual GI peptides upon cellular FA uptake in cells from different portions of the SI.

• Determine the cellular effects following pre-treatment of cells with two or more GI peptides upon FA uptake.

• Perform live cell imaging of Ca^{2+} fluxes in isolated eGFP-cells and enterocytes upon exposure to nutrients.

• Perform PCR analysis of eGFP+ and eGFP- cells to determine the expression of receptors in enterocytes and EECs; CCK-Rs, Bombesin receptors, GLP-2R.

7.0. References

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