Molecular mechanisms of nutrient sensing in the human and murine colon

Lucas Baumard

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Statement of Originality

I, Lucas Baumard, confirm that the research included within this thesis is my own work or that it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below, and my contribution indicated.

All the studies were performed and analysed by myself except as stated. cDNA from the Maastricht was collected and processed by Dr Zsa Zsa Weerts and her team at the University of Maastricht. Mice were culled in the United Kingdom at Queen Mary University of London by James Gillet and Ian Goodison.

I attest that I have exercised reasonable care to ensure that the work is original and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

Signature: Lucas Baumard Date: 17/11/2021

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Table of Contents

Abbreviations

18s	18s ribosomal RNA
5-HT	5-hydroxytryptamine, serotonin
AA	Amino acid
AgRP	Agouti-related peptide
AMP	Adenosine monophosphate
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ARC	Arcuate nucleus
BMI	Body mass index
CaMKII	Calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CaSR	Calcium-sensing receptor
CCK	Cholecystokinin
C-IP3	Phosphoinositide phospholipase
CNS	Central nervous system
COX	Cyclooxygenases
DAG	Diacylglycerol
DIO	Diet-induced obesity
DPP4	Dipeptidyl peptidase-4
EC	Enterochromaffin
EEC	Enteroendocrine cells
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
FFA	Free fatty acid
FFQ	Food frequency questionnaire
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIP	Gastric inhibitory peptide
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GPCR	G-protein-coupled receptor
GPCRC6a	G protein-coupled receptor family C group 6 member A
IBS	Irritable bowel syndrome
IP3	Inositol trisphosphate
LCFA	Long-chain fatty acid
LEP-R	Leptin receptor
MCFA	Medium chain fatty acids
MCHR1	Melanin-concentrating hormone receptor 1
MUFA	Monounsaturated fatty acids
NPY	Neuropeptide Y
OEA	Endogenous oleoylethanolamide

pERK	Phospho-extracellular signal-regulated kinase
PFA	Paraformaldehyde
PI-PLC-IP3	Phosphatidylinositol-phospholipase C-inositol 1,4,5-trisphosphate
PLCβ2	Phospholipase C
POMC	Proopiomelanocortin
PTGER4	Prostaglandin E_2 receptor 4 (EP4)
PUFA	Polyunsaturated fatty acids
PVN	Paraventricular nucleus
PYY	Peptide YY
RYGB	Roux-en-Y Gastric Bypass
SCFA	Short chain fatty acid
SERT	Serotonin transporter
SFA	Saturated fatty acids
SGLT	Sodium/glucose cotransporter
SI	Small intestine
SLMVs	Synaptic-like microvesicles
SOCS3	Suppressor of cytokine signalling 3
SST	Somatostatin
STC- 1	Intestinal secretin tumour cell line: model of intestinal EECs
T1R1	Taste receptor type 1 member 1
TAG	Triacyl glycerol
TGR5	G-protein-coupled bile acid receptor
TPH1	Tryptophan hydroxylase 1
TPH1	Tryptophan hydroxylase 1
TRPM5	Transient receptor potential cation channel subfamily M member 5
VMAT	Vesicular monoamine transporter
α-MSH	α-melanocyte-stimulating hormone

Abstract

Background: Nutrient-sensing, G-protein coupled receptors (GPCRs) expressed on enteroendocrine cells (EECs) bind and are activated by specific nutrients. Receptor binding leads to cell activation and release of specific hormones/peptides, such as peptide-YY (PYY), GLP-1 and serotonin, which regulate appetite and satiety (le Roux and Bloom, 2005), (Lund et al., 2018). Research is currently lacking on the cellular mechanisms of these processes in the human and murine colon. I therefore aimed to characterise the expression and release of GPCRs, hormones, peptides.

Hypothesis: Altered nutrient receptors expression can alter cell activation and the release of hormones and peptides leading to reduced postprandial satiety.

Methods: qPCR was used to assess the relative expression of nutrient GPCRs and hormone/peptides in human colonic tissue at the genetic level, and immunohistochemistry at the protein level. Murine tissue was used in Ussing chamber experiments to determine cellular activation in response to GPCR activation and then ELISAs used to determine the change in release of hormones/ peptides from these activated cells.

Results: Expression studies revealed no difference in the mRNA expression of serotonin and PYY between BMI groups in humans. GPR40 mRNA was the only GPCR to be significantly altered, being increased in the sigmoid colon of individuals with BMI ≥25. Stimulation of murine tissue with a GPR40 agonist increased intracellular activation via ERK activation in serotonin-expressing cells, whilst a CaSR agonist decreased CaMKII activation in PYY-expressing cells. There was no observed change in cellular activation in other target GPCRs. Release of PYY or serotonin was not affected by stimulation or antagonism of GPCRs, or antagonism of the CaMKII pathway.

Conclusion: BMI does not affect the mRNA or cell expression of the examined nutrient receptors (except GPR40), hormones or expression of enterochromaffin, L cells in our cohort of human colon samples. Obesity may alter other functions such as hormone release. GPCR agonists can alter the activation state of PYY- and serotonin-expressing L and EC cells in mice, with intracellular pathway activation being agonist- and GPCR-dependent.

1 Chapter 1: Literature review

1.1 Appetite regulation

Appetite control is a homeostatic process that balances energy intake and expenditure (Perry and Wang, 2012). Food intake leads to the release of satiety signals and the cessation of eating (Druce and Bloom, 2006). Appetite regulation can begin before food enters the body; the cephalic phase is initiated by sights and smells of food as well as by environmental factors such as time of day (Schloegl et al., 2011). Post-prandially, nutrients derived from food bind to specific nutrient-sensing G-protein coupled receptors (GPCRs) on cells within the gastrointestinal tract (GIT), activating the release of orexigenic hormones such as ghrelin, or anorexigenic hormones such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) and neuropeptides such as 5-HT (Sun et al., 2018). The ileo-colonic brake is an important driver of satiety and reduction in food intake: this brake is activated when unabsorbed nutrients in the distal small intestine and colon trigger inhibition of small intestinal motility and transit and strongly reduce pancreatic enzyme secretion (Ballantyne, 2006). Although studies in animals have shown that colonic nutrient infusion can trigger these same aforementioned effects (Wen et al., 1995, Harper et al., 1979), in humans, small intestine (SI) transit was unaffected (Read et al., 1984). The hormones PYY and GLP-1 might be important drivers of the reduction in food intake mediated by the ileo-colonic brake, however this has yet to be clarified in humans.

Hedonic systems regulate appetite by controlling the reward effects of food; palatable, energy-dense foods can trigger the mesolimbic dopamine signalling-left cortex axis which is associated with reward mechanisms, including those involved in drug addiction (Freitas and Campos, 2021). The amygdala is another hub of the hedonic systems that regulates positive and negative emotions and behaviour patterns (Zhang et al., 2011). The hedonic and homeostatic systems are linked and act to control food intake and appetite together (Andermann and Lowell, 2017).

Satiation processes most likely evolved as a protective measure against overeating; this is conserved across mammals, including mice (Amin and Mercer, 2016) and dogs (Vester

Boler et al., 2012). Controlling food intake also has a role in regulating efficient digestion, by regulating ingestion (Ritter, 2004). Appetite is therefore regulated by numerous interconnected central and peripheral mechanisms, in response to nutrients and other signals (Figure 1).

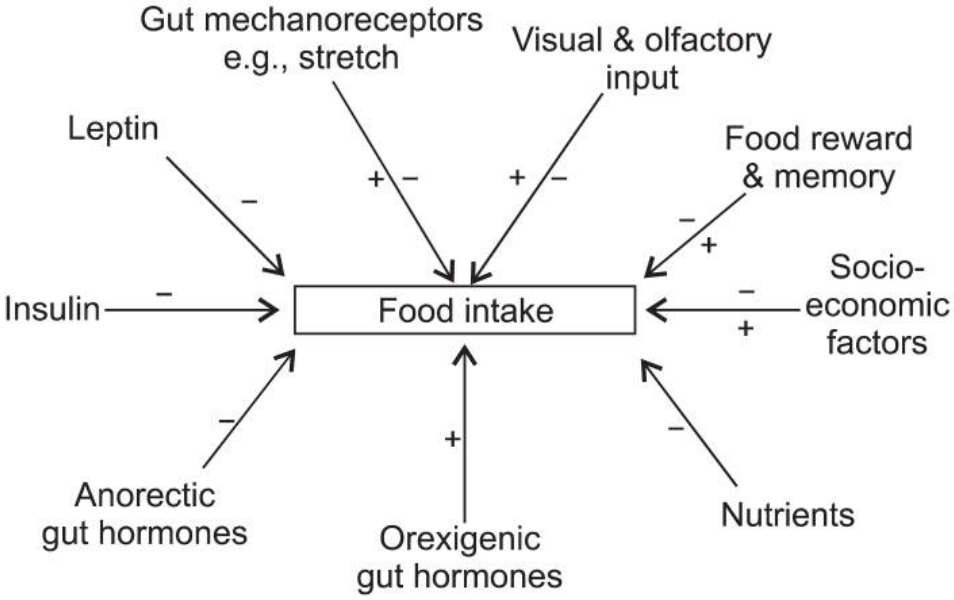


Figure 1: The major determinants of appetite control and how they modulate food intake. Several different sensory and hormonal inputs can either act to reduce (-) or increase (+) food intake. Reprinted from [4].

1.2 Peripherally mediated pathways of appetite regulation

At the level of the gut, hormones and vagal nerve stimulation act via the enteric nervous system (ENS) and central nervous system (CNS) to regulate motility, altering colonic transit of food and gastric emptying and altering satiety (De Silva and Bloom, 2012). Specialised gut epithelial cells within the gut can sense luminal stimuli and release gut peptides and other molecules -these will be discussed in further detail later in this study.

1.2.1 Appetite regulating hormones/neuropeptides

Hormones that are critical for appetite regulation can have short half-lives – being proteolyzed or cleared in the liver – indicating a limited, local effect (Page et al., 2012, Druce and Bloom, 2006). Hormones bind to mechano- and chemoreceptors in the oral cavity and gastrointestinal tract to control meal size and the onset of satiety via physiological and behavioural changes (Druce and Bloom, 2006).

Anorectic hormones, such as PYY and GLP-1, are released into circulation postprandially; the levels of these hormones are proportional to food consumption (le Roux and Bloom, 2005, Naslund et al., 1998). PYY acts on the Y2 receptor of the arcuate nucleus (ARC) to inhibit appetite and GLP-1 stimulates pancreatic insulin release and inhibits food intake (Druce and Bloom, 2006). Cholecystokinin (CCK) is also released from the GIT postprandially, and it acts on the brain stem to alter gut motility, secretions from the pancreas and gall bladder activation (Druce and Bloom, 2006). Leptin is released by adipose tissue and is a long term regulator of food intake; circulating levels are proportional to body fat (not post-prandial release), and food restriction lowers leptin levels, which can be recovered during feeding (Druce and Bloom, 2006). Much like PYY, leptin acts on neurons within the ARC, stimulating anorexigenic neurons whilst inhibiting orexigenic neurons. This leads to the inhibition of food intake (Druce and Bloom, 2006) and increased energy expenditure (Myers et al., 2010).

Some of the important hormones/ peptides involved in the satiety response are discussed below and listed by their localisation within the GIT; from stomach to descending colon.

1.2.1.1 Ghrelin

Ghrelin is an orexigenic hormone synthesised mostly in the stomach and is a potent stimulus to feeding; it is a ligand for orexigenic receptors expressed in the brain stem and hypothalamic nuclei (Druce and Bloom, 2006). Ghrelin is acylated into its active form by ghrelin-O-acyl transferase and binds to growth hormone secretagogues receptor 1a (GHSR-1a) in regions of the brain, including the arcuate nucleus (Willesen et al., 1999). The ghrelin

receptor GHSR-1a is also expressed in the hypothalamic nuclei and peripherally in almost all tissues (Howick et al., 2017).

Ghrelin and a high fat diet can both reduce the mechanosensitivity of gastric mucosal receptors, leading to increased gastric emptying and increased food intake (Page et al., 2012). Levels of the hormone are decreased after eating, particularly so by foods high in carbohydrates (Schloegl et al., 2011). In the brain, ghrelin modulates food intake as well as reward pathways. Ghrelin has been shown to increase dopamine output (Kawahara et al., 2013) and exogenous ghrelin administration has been shown to enhance the activation of central reward circuitry in response to images of "pleasurable", high-energy foods (Malik et al., 2008, Goldstone et al., 2014). Ghrelin also reduces insulin secretion, resulting in hyperglycaemia in humans (Broglio et al., 2001) and induced adiposity, increased food intake and weight gain in rodents (Tschöp et al., 2000).

1.2.1.2 Cholecystokinin

CCK is secreted by the EEC subtype I cells, expressed in the SI, and neurons in the brainstem and spinal cord (Ritter, 2004). CCK has been shown to slow gastric emptying and stimulate pancreatic secretions in humans (Fried et al., 1991), most likely caused by activity on the ventral gastric vagus nerve and gastric afferent fibres which drive relaxation of the oesophagus and inhibition of gastric contractile activity (Okano-Matsumoto et al., 2011).

Two receptors for CCK, CCK-A and CCK-B, are expressed on vagal afferents (Ritter, 2004). 30% and 40% of vagal sensory neurons of the rat nodose and dorsal root ganglia, respectively, express CCK-A receptor mRNA (Broberger et al., 2001). 13% of collective human colonic tissue expresses CCK-B (Schmitz et al., 2001). Vagal afferents innervate a large portion of the GIT (Reimann et al., 2012). Antagonism of this receptor in two studies in rats prevented a reduction in food intake in response to intestinal infusion of lipids, fat and proteins (though not L-phenylalanine) (Yox et al., 1992, Brenner and Ritter, 1996). CCK-A receptors stimulate Y2-receptors and the release of CART (cocaine- and amphetamine-regulated transcript) neuropeptides; these act to reduce food intake and induce satiety (Reimann et al., 2012, Latorre et al., 2016). CART peptides are distributed throughout the CNS and regulate food intake, body weight and reward through CART receptors which seem

to act via GPCRs expressed centrally (Rogge et al., 2008). Mice lacking the CCK-A receptor are unable to inhibit food intake or reduce gastric emptying during exogenous CCK administration and show reduced activation of vagal afferent pathways (Latorre et al., 2016). CCK has been shown to be able to switch vagal neurons from an orexigenic to an anorexigenic state. During fasting, vagal neurons increased expression of the orexigenic-associated receptors cannabinoid receptor CB1 and melanin-concentrating hormone receptor (MCHR1); nutrient-stimulated CCK release is able to downregulate these receptors (Reimann et al., 2012). CCKA-R is also highly expressed within the gallbladder and activation by CCK induces gallbladder contraction (secretion of bile into the duodenum) (Xu et al., 2008) and aids fat emulsification and absorption (Wang et al., 2019b).

CCK might be secreted in the periphery from gastrointestinal sites abutting vagal afferent nerve fibres, and this might drive a satiation response without increasing plasma levels of CCK (Ritter, 2004). In mice and rats, CCK appears to have a dual role: at low exogenous doses of CCK, appetite was not supressed and created a mild flavour preference for an accompanying flavoured saccharide solution, whereas a higher dose suppressed appetite and created an aversion to a second flavoured solution (Perez and Sclafani, 1991). CCK has been shown to negatively regulate the orexigenic hormone ghrelin (Pizarroso et al., 2021), and this might drive much of the anorexigenic effects of the hormone.

It is posited that CCK might be the hormone that is responsible for the reduced satiety to intestinal fat; a high fat diet reduced the activity of rat neurons of the hind brain in response to CCK (Covasa et al., 2000). Rats maintained on high-fat diets treated with exogenous CCK are less sensitive to the reduction of food intake and the inhibition of gastric emptying than rats on a low fat diet (Covasa and Ritter, 1998). In addition, neurons in the nucleus of the solitary tract and the nodose ganglia had a lower activation threshold to exogenous CCK or intestinal fatty acids in high-fat-fed rats (Covasa and Ritter, 1998). CCK plasma concentrations increased after intestinal infusion of long-chain fatty acid (LCFAs) (Ritter, 2004); the suppressive effects of lipids on satiety is likely mediated by CCK. CCK release is stimulated by glucose, oleate and other fatty acids, which strongly reduce food intake, most likely via activation of nodose ganglia neurons and vagal afferents (Duca and

Lam, 2014) and by regulating intestinal motility, gastric emptying and energy homeostasis (Pizarroso et al., 2021).

The mechanism underlying the effect of CCK on food intake is poorly understood. For instance, CCK-A antagonists attenuate the reduction in food intake to peptone and protein infusions (Covasa and Ritter, 2001, Nishi et al., 2003) but not to L-phenylalanine (Yox et al., 1992). In another study in rodents though, maltose and oleate reduced food intake; only oleate elevated plasma CCK levels while unhydrolyzed casein increased CCK plasma levels but did not reduce food intake (Brenner et al., 1993). Whether obesity affects CCK secretion is also not fully clear, with separate studies showing a reduction (Deloose et al., 2016, Stewart et al., 2011), increase (French et al., 1993) or no change (Brennan et al., 2012) in postprandial CCK levels in obese subjects.

There is some evidence of crosstalk between CCK and GLP-1, as antagonism of CCK receptors decreased secretion of GLP-1 in response to oleates (Reimann et al., 2012). CCK also co-operates with the hormone leptin to peripherally regulate food intake (Ritter, 2004); nodose neurons that respond to CCK also respond to leptin, and leptin enhanced the activation of rodent vagal sensory neurons by CCK in vitro (Peters et al., 2002, Wang et al., 1997). CCK and leptin co-administration increased vagal sensitivity to CCK and increased the inhibition of gastric emptying and food intake (Peters et al., 2006). Exogenous CCK administration in the rat colon increased colonic motility and PYY release compared to control conditions, with PYY administration doing the former to a lower degree (Ko et al., 2011). This increase in colonic motility was significantly inhibited by treatment with an anti-PYY serum and was abolished by the CCK antagonist loxiglumide (Ko et al., 2011). CCK activity might therefore also be reliant on that of PYY.

1.2.1.3 Leptin

Leptin is expressed in adipose tissues and the stomach (Zhang et al., 1994). Leptin can be considered a ‘fat cell’ hormone: production is correlated with adipose tissue mass and increased BMI (Maffei et al., 1995) (Klok et al., 2007), therefore circulating levels of leptin can reflect energy storage and food intake (Wynne et al., 2005). On the other hand, exercise and increasing age decrease the release of leptin (Klok et al., 2007). In this way leptin acts as

a long-term regulator of food intake and is not a postprandial signal of food intake like CCK, PYY. Leptin has roles in reproduction (puberty) (Mantzoros et al., 1997), immune and inflammatory responses (Fantuzzi and Faggioni, 2000) and haematopoiesis, angiogenesis and bone formation (Takeda et al., 2002). Leptin is also an important regulator of energy balance: leptin deficiency caused by genetic frameshift mutations leads to rapid development of obesity, overeating and impaired satiety in individuals (Farooqi et al., 2001).

Leptin is a product of the human obese (OB) gene (Zhang et al., 1994) and acts through its receptor LEP-R/ OB-R (Schwartz et al., 1996a) which is expressed on vagal afferent neurons (Ritter, 2004) and in colonic epithelial cells (Hardwick et al., 2001). The receptor is also expressed in the anorexigenic and orexigenic neurons of the ARC (Freitas and Campos, 2021). As previously discussed, leptin and CCK have been shown to act synergistically to reduce food intake. Leptin is a signal of body energy stores to the brain, acting to decrease food intake and increase energy expenditure (Pelleymounter et al., 1995); plasma leptin can cross the blood-brain barrier and bind to LEP-R in the hypothalamus (Sahu, 2003). The ability of leptin to cross the blood-brain barrier can be regulated: starvation reduces transport whilst re-feeding increases it (Banks et al., 1996). Leptin can cause hypothalamic neurons to release orexigenic (neuropeptide Y, melanin concentrating hormone) or anorexigenic (Proopiomelanocortin - POMC, CART) neuropeptides (Schwartz et al., 1996b). Leptin is produced in the stomach in small amounts, where it might have some role in controlling meal size with other satiety peptides (Lewin and Bado, 2001). Several intestinal peptides induce gastric leptin release (Sobhani et al., 2000), including insulin (Sobhani et al., 2002).

Leptin plays an important role in the adaptation of the human body to energy deprivation via a neuroendocrine response (Klein et al., 2000). In the vagal afferent neurons of the vagus nerve, fasted rats exhibited decreased expression of the leptin receptor, which increased during feeding (Buyse et al., 2001). Dieting and fasting reduced plasma levels of leptin in humans and this change was not solely due to a change in adipose tissue (Weigle et al., 1997). A study that compared lean and obese women demonstrated that the reduced leptin production observed during fasting was blunted in obese women (Klein et al., 2000). The authors of this study suggest that the effect of obesity on glucose metabolism may be

responsible for this difference; in obese individuals there is decreased glucose production, plasma glucose concentration, and possibly glucose utilization that occurs during the first 24 h of fasting compared with lean subjects (Klein et al., 2000).

Research has showed that leptin expression is higher in serum and plasma in those with higher BMI and higher percentage of body fat (Schwartz et al., 1996a). Obese patients have many fat cells and accordingly higher leptin levels, however, leptin is no longer able to contribute to satiety and it is likely that the body is resistant to leptin; sustained high leptin levels could make cells insensitive to leptin (Remely et al., 2014). Leptin administration in obese humans only moderately affected body weight (Fogteloo et al., 2003, Heymsfield et al., 1999) whilst in rodents failed to reduce food intake (Van Heek et al., 1997). This leptin resistance could be driven by neural as well as peripheral mechanisms: altered transport into the brain or signalling within hypothalamic neurons (Wynne et al., 2005). Hypothalamic nuclei expressing LEP-R upregulate the protein, suppressor of cytokine signalling 3 (SOCS3), which is a negative regulator of leptin, therefore chronic changes in leptin expression may be responsible for resistance (Wynne et al., 2005). Knockout mice lacking neuronal SOCS3 (Mori et al., 2004) or with a heterozygous deficiency are resistant to DIO, and the latter demonstrate increased LEP-R hypothalamic signalling in response to exogenous leptin administration (Howard et al., 2004). Another study in mice showed that a phytochemical that acts as a leptin sensitizer – celastrol – reduced food intake and weight loss in DIO, hyperleptinemic mice, but not leptin/ LEP-R deficient or lean mice (Liu et al., 2015). However, when lean mice were given endogenous leptin with celastrol, they reduced food intake and body weight (Liu et al., 2015). The study authors posit that the effects of celastrol are due to increased phosphorylation of hypothalamic $STAT3^{Tyr705}$ and reduced phospho-extracellular signal-regulated kinase (pERK) phosphorylation and ER stress (Liu et al., 2015).

1.2.1.4 Somatostatin

SST is a hormone found throughout the GI tract and is released from D cells, which make up 3-5% of the EEC population in the lower tract and are at the highest frequency in the duodenum and pancreas (Buffa et al., 1978). SST is the main secretory product of D cells

and is a modulator of upper gastrointestinal secretion, sensation, and motility (Cremonini et al., 2005), released and acting locally via paracrine/ neuronal mechanisms (Engelstoft and Schwartz, 2016). Fat and protein are the major stimulants of SST release (Lucey, 1986).

The SST analogue, octreotide, has been shown to delay gastric emptying, increase fasting gastric volume, and reduce gastric volume in healthy volunteers as part of a liquid nutrient meal (Cremonini et al., 2005). A study examining the effects of intravenous SST infusion in 10 healthy subjects identified increased postprandial satiety and lower food intake (Lieverse et al., 1995). However, in obese individuals, octreotide has been shown to act in an orexigenic manner. In one study, after a fully satiating meal, octreotide reduced fullness and bloating and may therefore facilitate overeating by reducing uncomfortable sensations after a large meal (Cremonini et al., 2005). In another study, Velasquez-Mieyer *et al.* demonstrated that octreotide reduced postprandial release of GLP-1 and levels of insulin (Velasquez-Mieyer et al., 2004). However, in a previous study Velasquez-Mieyer *et al.* demonstrated that in severely obese individuals, octreotide led to significant insulin suppression, improvements in insulin sensitivity and weight loss with decreased BMI, as well as decreased leptin levels and decreased food intake and carbohydrate-cravings (Velasquez-Mieyer et al., 2003). Of interest, in the group that responded poorly to octreotide in regards to BMI loss, leptin secretion and fat mass increased and the study authors postulated that suppression of insulin secretion drove the beneficial effects of octreotide (Velasquez-Mieyer et al., 2003).

SST is a major inhibitory hormone of the digestive system, decreasing the release of all known GI hormones (including itself) and reducing exocrine functions in the GIT and pancreas (Patel, 1997, Reichlin, 1987). SST is able to modulate EEC function; 2 minutes after stimulation with SST (or octreotide) in a human cell line, EECs underwent actin filament rearrangement which translocated secretory vesicles from the cell periphery to the perinuclear region (Saras et al., 2007). Though the impact of SST on hormone secretion was not examined in this study, SST has been shown to inhibit both ghrelin and GLP-1 secretion via paracrine mechanisms (Engelstoft and Schwartz, 2016).

SST is a hormone that appears to possess orexigenic properties by reducing feelings of fullness, reducing the release of satiety hormones and encouraging overeating, but also anorexigenic properties by reducing ghrelin secretion and delaying gastric emptying. More research is needed to fully understand the role of SST in nutrient sensing in the obese and non-obese states.

1.2.1.5 PYY

PYY is a gastrointestinal hormone belonging to the pancreatic polypeptide family which includes neuropeptide Y (NPY) and takes its name from its tyrosine (Y) residues (Batterham and Bloom, 2003, Latorre et al., 2016). It is released from L cells of the distal GIT in response to intraluminal bile salts, amino acids, cholecystokinin, vasoactive intestinal peptide and GLP-1 (Zhang et al., 1993, Schloegl et al., 2011, Naslund et al., 1999b).

PYY has two forms, produced by the cleavage of the PYY peptide: PYY_{1-36}, and PYY_{3-36} – the latter is the predominant form in the colon (Karra et al., 2009). PYY is cleaved by a number of enzymes, including dipeptidyl peptidase-4 (DPP4) (Medeiros and Turner, 1994), aminopeptidase P, the metalloendopeptidase meprin β, and neutral endopeptidase 24-11 (Addison et al., 2011, Medeiros and Turner, 1994). Though there is a role for PYY_{1-36}, which is present at a higher concentration during the fasted state, PYY_{3-36} is the major circulating form after a meal (Grandt et al., 1994). PYY_{1-36} reduces food intake in rodents, but not to the same extent as PYY_{3-36} (Unniappan et al., 2006) and in obese human males, PYY_{1-36} administered intravenously was unable to increase satiety, reduce hunger or elevate levels of plasma free fatty acids (Sloth et al., 2007). L cells are the major sources of PYY (Batterham and Bloom, 2003); however, mRNA expression provides evidence for its presence in the pancreas and brain stem as well as the GIT (Broome et al., 1985) – though it is not clear whether PYY protein is transcribed by cells in these tissues. PYY expressing STC- 1 cells (murine intestinal cell line) most commonly co-express the calcium-sensing receptor (CaSR) with taste receptor type 1 member 1 (T1R1) (25%) and more rarely with the G protein-coupled receptor family C group 6 member A (GPCRC6a) (12. 8%) (Wang et al., 2019a).

The PYY receptor family consists of Y receptors 1-5; PYY_{3-36} binds to all, with the highest affinity for Y_2R, but also some affinity for Y_1R and Y_5R (Karra et al., 2009). Y_2R is a 7-

transmembrane-spanning G-protein-coupled receptor (GPCR) expressed in both the CNS and vagal afferents (Schloegl et al., 2011) and is a presynaptic heteroreceptor able to regulate the release of the orexigenic peptide neuropeptide Y (NPY) (Sparrow et al., 2012). Y_2R activation reduces the release of NPY, reducing food intake, increasing energy expenditure and driving a satiety signal (Batterham and Bloom, 2003). Y_2R activation in the hypothalamus has been shown to be the main driver of PYY-mediated reduction of food intake (Latorre et al., 2016) and mice with a gene deletion in Y_2R are resistant to the anorectic effects of PYY (Batterham et al., 2002b).

Circulating levels of PYY are correlated with the caloric content and composition of foodstuffs, with plasma levels of PYY elevated postprandially (Ritter, 2004). Meals consisting solely of fat, carbohydrates or proteins alter plasma PYY levels in human subjects, with the highest levels released in response to fat, then carbohydrate, and the least, to protein-rich meals (Adrian et al., 1985). In a study where participants were given meals with different nutrient compositions, fat was shown to be the most important factor in stimulating PYY release, over protein or glucose (Batterham and Bloom, 2003). PYY was also shown to have more potent effects on food intake compared to all other anorexigenic peptides or neurotransmitters, including GLP-1, serotonin and CCK (Batterham and Bloom, 2003). Figure 2 shows that in obese, overweight individuals, levels of both PYY and GLP-1 rise sharply 30 minutes postprandially, and that a high fat, low carbohydrate diet stimulates higher levels of both hormones, compared to a high-carbohydrate/low-fat diet (Gibbons et al., 2013).

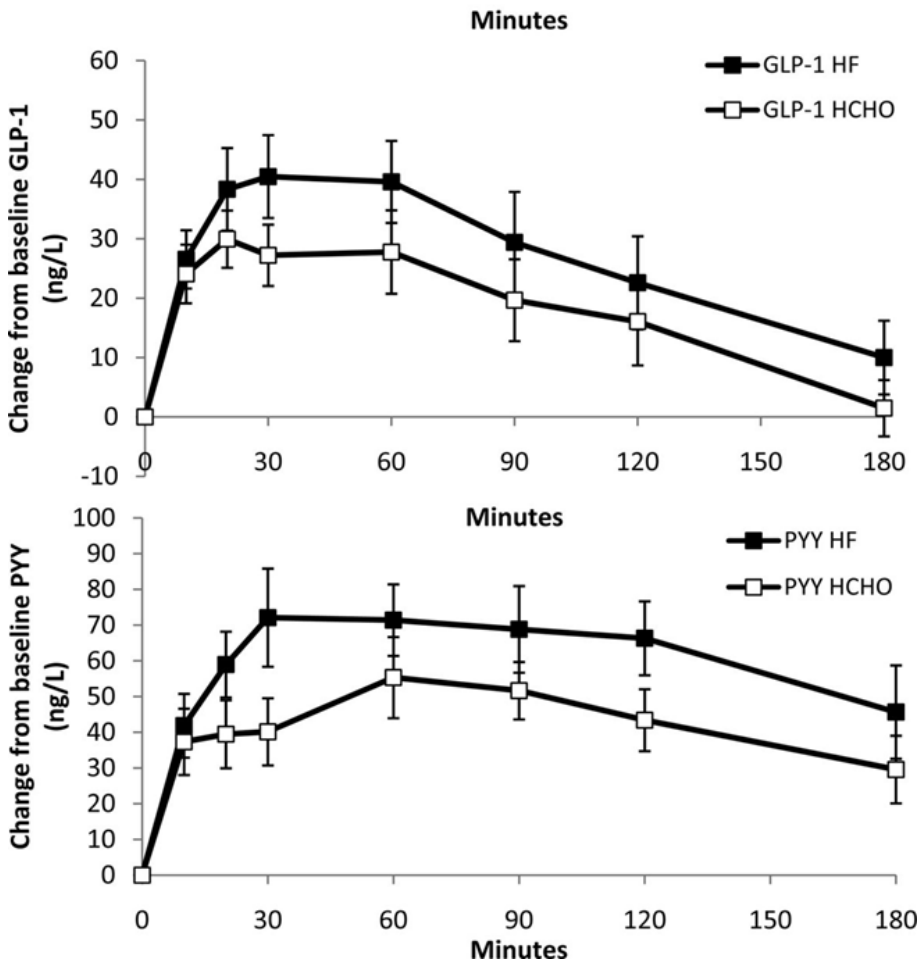


Figure 2: Postprandial plasma levels of GLP-1 (top) and PYY (bottom) after consumption of a high-fat/low-carbohydrate (HF) and high-carbohydrate/low-fat (HCHO) breakfast by obese, overweight individuals over 3 hours. Figure edited from (Gibbons et al., 2013).

PYY is able to modulate short-term food intake via CCK release (acts to rapidly stop food intake by slowing gastric secretion and activating vagal endings (Fried et al., 1991, Okano-Matsumoto et al., 2011)), and long-term food intake by the release of leptin and insulin (regulation of body energy storage) (Batterham and Bloom, 2003). There is evidence that PYY might have a role in long term body weight regulation due to its ability to modulate energy expenditure and lipid metabolism, via the synthesis and secretion of apolipoprotein A-IV (Guo et al., 2006) - a lipid-binding protein that is involved in lipid absorption and metabolism processes, glucose homeostasis (Qu et al., 2019) and suppression of food intake in the CNS (Fujimoto et al., 1993). A neuroimaging study involving exogenous PYY

administration in fasted normal weight men, showed that with increasing levels of PYY, brain regions involved in decision making and reward processing showed increased activity, with subjects given PYY vs. a placebo consuming less calories post-infusion (Schloegl et al., 2011). PYY, therefore, may be involved in behavioural modification of food intake, encouraging the consumption of low-calorie meals. In addition, PYY also has roles in vasoconstriction and the reduction in gastric acid secretion, pancreatic and intestinal secretion, and gastrointestinal motility (Batterham and Bloom, 2003), which may further promote satiety. Mice unable to produce their own PYY are hyperphagic and prone to obesity; however, if they are treated long-term with PYY they become resistant to diet-induced obesity (DIO – animal model of obesity where animals have obesity induced by a high-fat diet)) (Karra et al., 2009), pointing to a pivotal role of PYY in mediating postprandial satiety.

PYY secretion is inhibited by somatostatin (SST), an orexigenic hormone, which is an important mediator of the ileo-colonic brake (Cremonini et al., 2005). SST is stored in D cells (Buffa et al., 1978) and modulates gastrointestinal secretion, sensation and motility (Mani and Zigman, 2015) by inhibiting gastric acid secretion, parietal cells and histamine release (Latorre et al., 2016).

In obesity, lower circulating levels and postprandial release of PYY are described. A study examining plasma PYY levels in both rats and humans reported reduced levels in obese individuals during fasting and postprandially; exogenously administered PYY lowered food intake in both species (le Roux et al., 2006b). This study also demonstrated that in obese humans, double the meal calorie content was required to achieve equivalent PYY levels to normal-weight subjects, and obese individuals reported lower levels of fullness (le Roux et al., 2006b). PYY is thought to be the primary driver of the weight loss observed after gastric bypass; Korner et. al demonstrated that though fasting levels of PYY remained similar among: overweight patients with Roux-en-Y Gastric Bypass (RYGB); overweight patients with no surgery and lean individuals, post prandial levels of PYY were 2-4 times higher in the RYGB group than in the others (Korner et al., 2006). In obesity, PYY reduced feeding in rodents and humans (Karra et al., 2009); however, the lower circulating levels and postprandial release of PYY that occur in obesity (Alvarez Bartolome et al., 2002, le Roux et

al., 2006b) predispose towards the development and maintenance of obesity due to reduced satiety in these individuals (Karra et al., 2009).

Peripheral administration of PYY has been shown to reduce food intake in humans (~30%) (Batterham et al., 2002b) and reduce food intake and weight gain in rodents (Wynne et al., 2005). PYY administration has been shown to delay gastric emptying, delay pancreatic and gastric secretions and increase absorption of fluids and electrolytes from the ileum postprandially (Wynne et al., 2005). In animal studies there is some evidence for the use of continuous endogenous administration of PYY as a therapeutic option, with reduced body weight and adiposity seen in normal weight rodents (Pittner et al., 2004), DIO rodents (Adams et al., 2006) and monkeys (Koegler et al., 2005). In rodents, combining PYY administration with anorectic hormones such as GLP-1 (Neary et al., 2005), leptin (Karra et al., 2009) or a cannabinoid receptor 1 antagonist (White et al., 2008) improved weight loss and decreased feeding more than any agent alone. PYY transgenic mice are protected from DIO (Boey et al., 2008) and in rats, systemic PYY administration reduces motivation to seek high-fat food (Ghitza et al., 2007). In two human studies, administered PYY has been shown to lower food intake 90 minutes after a meal (le Roux et al., 2006b) and decrease appetite and food intake over 24 h (Batterham et al., 2002a). Lasting effects of PYY administration were not examined and it is likely that these effects are transient. PYY synergises with other hormones and peptides (GLP-1, leptin) to regulate appetite. The body's inbuilt survival mechanism to protect against weight loss during times of reduced food intake most likely counters the effectiveness of a single-agent therapy. In addition, PYY is rapidly cleaved by enzymes such as DPP4 (Medeiros and Turner, 1994): within 10 minutes of intravenous injection of the full length PYY form, the hormone was unable to activate the Y_2 receptor (Toräng et al., 2016). Table 1 shows a summary of studies looking at combining PYY with other agents and their effects on food intake.

Table 1: Comparisons of PYY combination therapies and their outcomes

Agents	Effect	Species	Route of administration, dose
PYY + exendin-4	synergistic anorectic effects (Talsania et al., 2005)	Mouse	Intraperitoneal: 3 µg PYY; 0.06 µg exendin-4
PYY + GLP-1	additive effects on feeding inhibition in rodents (Neary et al., 2005)	Rat, Mouse, human	Intraperitoneal: 100 nmol/kg PYY; 100 nmol/kg GLP-1
PYY + GLP-1 + oxyntomodulin (GOP)	increased weight loss vs. saline, improved glucose tolerance	Human	Subcutaneous: 100pmol/kg/min PYY; 4pmol/kg/min oxyntomodulin; 0.4pmol/kg/min GLP-1
PYY + cannabinoid receptor-1 antagonist	decrease food intake more when combined than any compound alone (White et al., 2008)	Mouse	Intraperitoneal: 50 µg/kg PYY; 0.3 mg/kg antagonist
PYY + amylin	increased inhibition of feeding, increased weight-reduction (Roth et al., 2007)	Rat	Intraperitoneal: 1000 µg/ kg PYY; 10 µg/kg amylin
PYY + leptin	increased inhibition of feeding (Unniappan and Kieffer, 2008)	Rat	Subcutaneous osmotic minipumps: 100 µg/kg PYY; 100 µg/kg leptin
PYY + liraglutide	similar weight-loss profile to RYGB. Reduced food intake and fat preference (Dischinger et al., 2020)	Rat	Osmotic minipump: 0.4 mg/kg/day liraglutide; 0.1 mg/kg/day PYY

PYY is therefore a very potent and important satiety hormone which has an important role in mediating post-prandial appetite.

1.2.1.6 GLP-1

GLP-1 is an anorectic hormone co-released by L cells (the primary source of GLP-1 in the intestine (Holst, 2007)) (Schloegl et al., 2011), in response to carbohydrate, fat and protein intake (Elliott et al., 1993). GLP-1 is expressed in the gut lumen and at low plasma concentrations during fasting (Amato et al., 2016). GLP-1 is produced by cleaving proglucagon (Diakogiannaki et al., 2012), (Figure 3); proglucagon can be cleaved into separate molecules in a tissue-dependent manner. For example, proglucagon is cleaved in the pancreas into a single large molecule - glucagon, whereas in the intestine cleavage produces two glucagon-like peptides (GLP-1, GLP-2), as well as the molecule glicentin, as a consequence of the activity of prohormone convertase 1 and 2 (Holst, 2007, De Silva and Bloom, 2012). GLP-1 promotes insulin release and inhibits glucagon secretion, as well as postprandial gastric acid secretion and gastric emptying, whilst encouraging gastric distention: these generate feelings of fullness (Pizarroso et al., 2021).

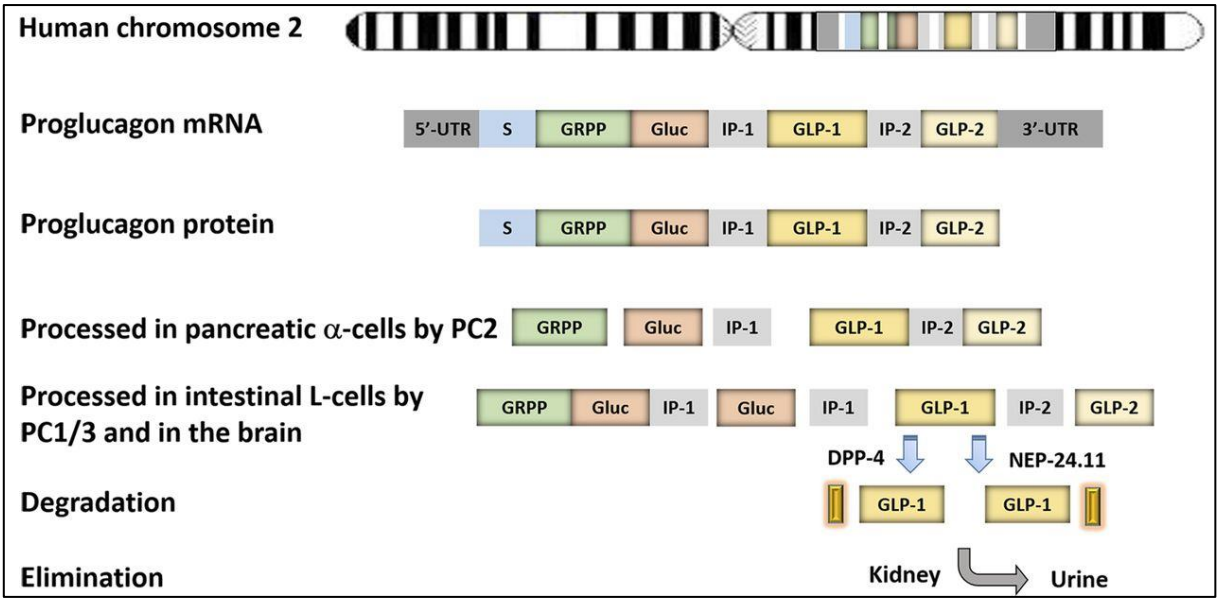


Figure 3: Gene structure, expression, processing, degradation, and elimination of proglucagon. In L cells of the intestine and the brain, proglucagon is processed by PC1/3 into IP-1/2, GLP-1, and GLP-2. In pancreatic α cells, proglucagon protein is processed by PC2 into GRPP, Gluc, IP-1, and a major proglucagon fragment. GLP-1 is degraded by DPP-4/ NEP24.11 and eliminated via the kidney in urine. Figure reprinted from (Graaf et al., 2016).

GRPP: glicentin-related polypeptide

PC1-3: prohormone convertase 1-3

Gluc: glucagon

IP-1/2: intervening peptide-1/2

NEP-24.11: neutral endopeptidase 24.11

DPP-4: dipeptidyl peptidase IV

Research in dogs (Sugiyama et al., 1994) and the colonic neuroendocrine tumour cell line GLUTag (Gribble et al., 2003) shows an important role of the luminal sodium/glucose cotransporter 1 (SGLT-1) in mediating the effects of GLP-1 (Amato et al., 2016). GLP-1 release after stimulation with glucose in an organoid culture was shown to be SGLT-dependent (Goldspink et al., 2020); oral glucose administration is a strong promoter of GLP-1 release (Wu et al., 2011). Interestingly, although GLP-1 has been shown to be released in response to sweet stimuli, artificial sweeteners do not cause GLP-1 release (Duca and Lam, 2014). Indeed, artificial sweeteners have been shown to alter host microbiome, glucose homeostasis, and are associated with increased caloric consumption, food intake and

weight gain in animals and humans (Pearlman et al., 2017) and it is likely that these effects are at least partially driven by this decreased GLP-1 release. Lipids also strongly stimulate GLP-1 secretion, potentially via the action of the long-chain fatty acid receptor GPR120, which has been shown to stimulate GLP-1 release in response to fatty acids (Holst, 2007). GLP-1 secretion is regulated by paracrine signalling of SST (Holst, 2007). Human colonic cultures have been shown to release GLP-1 in response to agonist stimulation of the nutrient receptors, G-protein-coupled bile acid receptor (TGR5) and GPR40 (Habib et al., 2013). GLP-1-expressing STC-1 cells have been shown to express the nutrient receptors CaSR, GPRC6a and TAS1R1, with CaSR being the most commonly co-expressed (Wang et al., 2019a). GLP-1 is rapidly degraded by DPP4 (Figure 3); the enzyme cleaves GLP-1 into an inactive form which nevertheless competes for binding sites on the GLP-1 receptor (Amato et al., 2016). It has been shown that of the GLP-1 that enters the circulation, less than 25% remains in an active, uncleaved form, with only 10-15% able to reach the brain and pancreas (Amato et al., 2016). GLP-1 has a half-life in plasma of around 2 minutes (Pizarroso et al., 2021). This raises questions as to the intrinsic activity of GLP-1 within areas of the brain like the hypothalamus.

GLP-1 inhibits gastric emptying and glucagon secretion, acting with PYY to reduce gastric acid secretion (Schloegl et al., 2011) and absorption of nutrients in the intestine (Naslund et al., 1999a). Glucagon acts to inhibit upper gastrointestinal functions such as small intestinal motility, under the ileal brake mechanism, most likely via vagal pathways (Holst, 2007). It also one of the incretin hormones (gastric inhibitory peptide (GIP) being the other) secreted after nutrient intake (Nauck and Meier, 2018), regulating insulin release and inhibiting glucagon secretion from the pancreas in response to food ingestion - though this regulation is impaired in obesity, where hyperglycaemia is a common feature (Madsbad, 2014). GLP-1 significantly increases insulin secretion, to such an extent that along with GIP they can fully abolish the entire insulin response (Holst, 2007). A meta-analysis of human studies has shown that energy intake was reduced by around 12% after GLP-1 infusion, with hormone levels and gastric emptying significantly reduced compared to a saline infusion (Verdich et al., 2001). Liraglutide, a GLP-1 analogue, has been shown to increase feelings of fullness and decrease desire for sweet, salty, savoury and fatty foods, with subsequent increases in

postprandial PYY levels (which is reduced in obesity) and reductions in body fat (Kadouh et al., 2020). Interestingly, the same study showed diminished post-prandial plasma GLP-1 levels, which the authors attributed to reduced gastric emptying and the effects of weight loss (Kadouh et al., 2020).

GLP-2 has roles in regulating intestinal capacity, modulating gastric transit and mucosal cell growth and is of interest in therapies for conditions of compromised intestinal function (Thulesen, 2004). There is some evidence that GLP-2 has roles in regulating lipid absorption and energy uptake as well as glycaemic control (Amato et al., 2016); however, for this project I will be focusing on GLP-1, for which research is more conclusive on its role in nutrient sensing.

The receptor for GLP-1, GLP-1R, is expressed in pancreatic islets, the brain, heart and kidney and throughout the GIT, including intestinal L cells (WEI and MOJSOV, 1996). In the rat brain, GLP-1R is expressed in regions that regulate food intake such as the hypothalamus and arcuate nucleus in particular (Göke et al., 1995). GLP-1R is expressed on the surface of beta cells and activates these cells in response to GLP-1, to facilitate glucose-dependent mitochondrial ATP production as well as β-cell proliferation and differentiation (Tsuboi et al., 2003). This has led to interest in GLP-1 as a therapeutic option in autoimmune diabetes, with some evidence of lasting improvements in glucose homeostasis and β-cell maintenance in rodents (Tourrel et al., 2002).

GLP-1 secretion in obesity is unclear, with conflicting studies showing reduced (Muscelli et al., 2008, Ranganath et al., 1996), increased (Fukase et al., 1995) or no difference (Knop et al., 2012, Vilsboll et al., 2003) in postprandial GLP-1 levels in obese vs. lean participants. Muscelli *et al.* demonstrated that obese individuals given oral glucose had significantly reduced plasma GLP-1 levels (Muscelli et al., 2008) and Ranganath *et al.* also showed the same response to an oral carbohydrate - but not oral fat – meal in patients (n=6) (Ranganath et al., 1996). Knop *et al.* did not demonstrate a significant change in plasma GLP-1 levels to oral glucose in obese patients, but they did in obese patients with Type 2 Diabetes, and they observed elevated fasting plasma glucagon levels (Knop et al., 2012). Vilsbøll *et al.* also did not observe any increase in plasma GLP-1 after challenges with high fat and high protein

food, and they also observed elevated fasting plasma glucagon levels (Vilsboll et al., 2003). GLP-1 secretion postprandially was increased (10-20 times in plasma) in obese patients after gastric bypass, which was attributed to altered transit of nutrients through the gut and improved glycaemic control and energy restriction (Dirksen et al., 2012). Increased DPP-4 levels are detected in obese type 2 diabetics, with activity unchanged between obese and non-obese T2D patients (Sarkar et al., 2019), however, it is not clear whether this drives changes in GLP-1 activity and food intake. GLP-1 release is not constant throughout the day in humans: release peaks around 6 pm and is lowest at 10 am, and then again at 11 pm (Figure 4A) (Muñoz et al., 2015). However, this cycle is lost in obesity, with a higher (than normal weight) peak at 6 pm but only minor dips at 10 am and 11 pm (Figure 4C) (Muñoz et al., 2015). This is a factor that should be considered when assessing biopsy samples from individuals, who will be overnight fasting and operated upon around 10 am each day. I may therefore see lower GLP-1 release/ expression from cells due to this diurnal pattern.

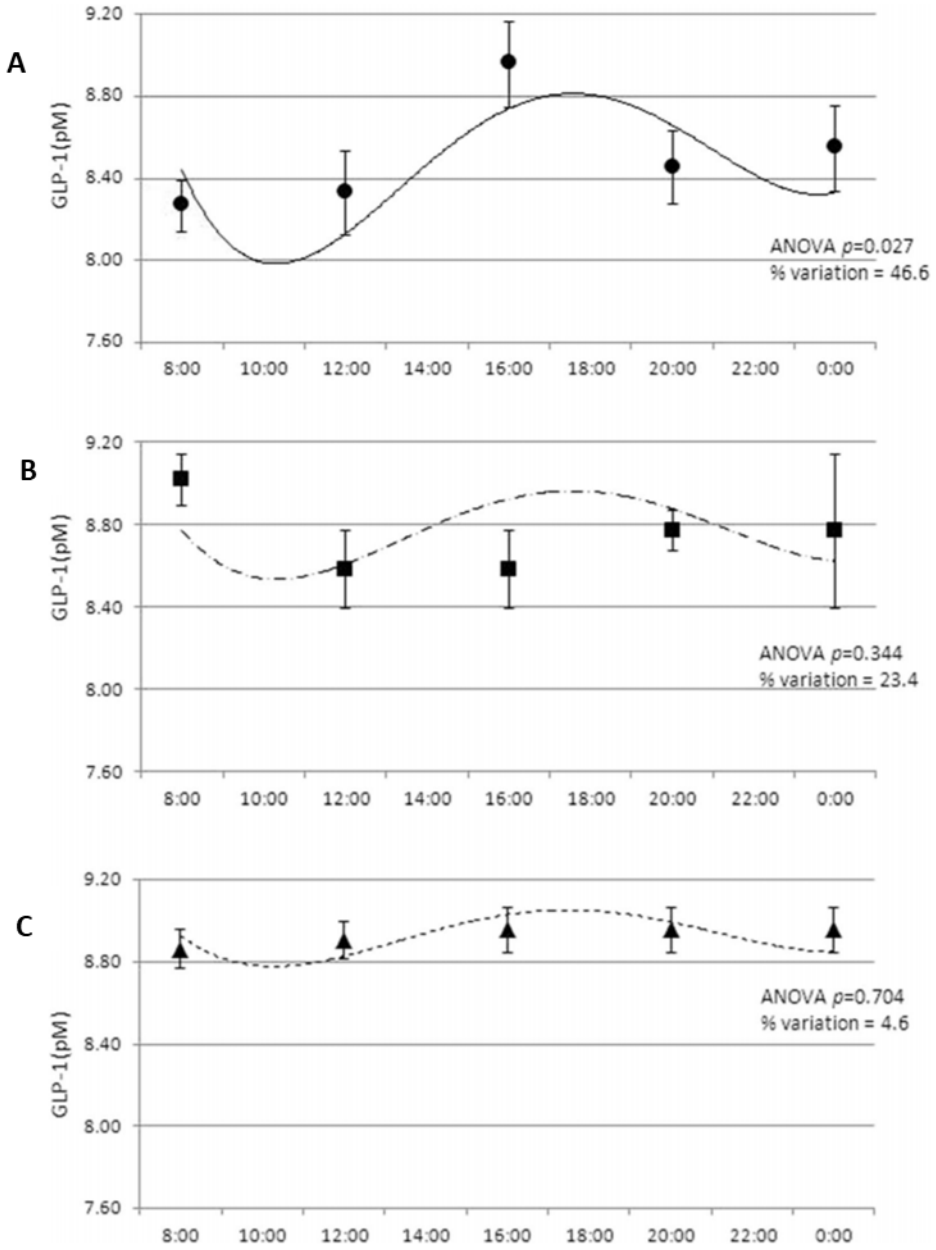


Figure 4: Raw group-average data (mean ±SEM) and fitted cosinor curves in normal weight (A), overweight (B), and obese groups (C) of plasma GLP-1 levels over a 16-hour time course. Blood samples were taken after an overnight fast and then every 4 hours after, with patients given an identical diet throughout the day. Reprinted from (Muñoz et al., 2015).

Though post-prandial GLP-1 levels are not as responsive to food intake, its ability to influence food intake does not seem to be altered. Naslund *et al.* demonstrated that obese men given intravenous GLP-1 with a meal had significantly reduced hunger ratings, reduced energy intake (~21%), delayed gastric emptying, decreased plasma glucose levels and reduced plasma concentrations of PYY (Naslund et al., 1999a). In another study by Naslund *et al.*, obese subjects were given subcutaneous GLP-1 pre-prandially, and compared to a placebo, had reduced calorie intake (15%) and lost 0.5 kg over a 5-day period (Naslund et al., 2004). Exogenous GLP-1 administration could therefore be an interesting strategy for obesity treatment. There are several therapies involving the use of GLP-1 receptor agonists (albiglutide, liraglutide, lixisenatide) to control hyperglycaemia in T2D, that have shown varying weight loss vs. placebo, alongside reduced blood glucose levels (Aroda et al., 2012), with semaglutide and liraglutide currently approved for weight loss (Muscelli et al., 2008).

1.2.1.7 Serotonin

Serotonin (also known as 5-HT) is a monoamine involved in sleep onset, pain sensitivity, blood pressure regulation and mood control [3]. It has also been shown to have a role in regulating the intestinal barrier and mucus secretion (Lund et al., 2018). Carbohydrate-rich food, especially glucose, induces insulin release, which increases serotonin levels in the brain (Wurtman and Wurtman, 1995, Sankoda et al., 2019) and decreases blood glucose levels (Yamada et al., 1989). 'Carbohydrate craving' is a mechanism by which serotonin release is thought to have addictive effects; those who overeat carbohydrate heavy foods such as snack food or 'junk' food, may take advantage of the food-induced, large release of serotonin to improve their mood (Wurtman and Wurtman, 1995).

Serotonin is synthesised from tryptophan by the enzyme tryptophan hydroxylase 1 (TPH1) in enterochromaffin (EC) cells of the GIT, in a rate limiting process (France et al., 2016, Watanabe et al., 2010) and acts as a peripheral hormone here. Tryptophan is available from various food sources: including proteins and carbohydrates (Acevedo-Triana et al., 2017). TPH2 also synthesises serotonin, but the gene is only expressed in neuronal cells, where altered expression is associated with severe effects on mental health (Buttenschøn et

al., 2013). Interestingly, TPH1 requires molecular oxygen to function, and rats breathing 100% pure oxygen showed increased synthesis of serotonin (Cooper et al., 2003). This might have noteworthy implications for the role of exercise, pollution and even climate and geography on serotonin availability and therefore satiety regulation. Serotonin availability is reduced by reuptake; serotonin nerve terminals have high-affinity serotonin uptake sites that reuptake serotonin via plasma membrane transporters such as the serotonin transporter (SERT) (Cooper et al., 2003).

A study by Lund *et al.* examining GPCR expression of serotonin-positive FACS-purified murine cells of the colon and SI, demonstrated that SI ECs do not express GPR119, GPBAR1 or CaSR, and express at low levels GPR43, whereas in the colon GPBAR1 and GPR43 were highly expressed, with GPR119 and CaSR lowly expressed (Lund et al., 2018). When Lund *et al.* examined hormone receptor expression, they found that colonic EC cells most highly expressed somatostatin receptors 1, 2, 3, GLP-1R (GLP-1R 760x enriched) and PYY Y1R, with the SI differing only in GLP-1R enrichment level (220x) and PYY receptor sub-type (Y6) (Lund et al., 2018). The study authors further demonstrated GLP-1R staining on serotonin positive enteroendocrine cells (colonic epithelial cells – EECs) in the colon (54%) and that stimulation of tissue with a GLP-1R agonist induced serotonin release in the colon (Lund et al., 2018). This could lead to a super-additive effect of GLP-1 and serotonin release on satiation and suppression of gastric emptying. The authors postulated that in the small intestine, because ECs are devoid of GPR119, GPBAR1 or CaSR, they rely on neighbouring GLP-1-containing EECs to indirectly sense nutrients and release serotonin, whereas in the colon ECs are able to sense nutrients (GPR43: SCFAs, GPBAR1: bile acids among others) directly (Lund et al., 2018). This study provides interesting evidence of transcript expression of GPCRS and transcript/ protein expression of hormone receptors as well as differing roles of SI and colonic ECs.

Serotonin has been shown to be released from serotonin-containing vesicles on the membranes of cells in a mechanosensitive manner by the action of Piezo2 mechanosensitive ion channels expressed on EECs, including ECs (Alcaino et al., 2018). Mechanical stimulation of a subset of ECs led to a Piezo2-dependent intracellular Ca^{2+} increase and release of serotonin (Alcaino et al., 2018). Antagonists at the serotonin 5-HT_3 receptor have been

shown to abolish glucose-/ mannitol-induced (duodenal infusion) inhibition of gastric emptying and the authors attributed this to glucose-induced release of serotonin, activating 5-HT_3 receptors on afferent nerve endings (Raybould et al., 2003). It is also likely that CaSR activation dependent serotonin release drives gastric emptying, with the ability of CaSR agonists to reduce the glycaemic (glucose lowering) response to an oral glucose tolerance test being almost completely blocked by administration of a 5-HT_3 antagonist (Muramatsu et al., 2014). Gastrointestinal motility may act as a mechanistic trigger of serotonin release as an alternative trigger to canonical, nutrient-stimulated, GPCR-activated serotonin release from cells.

There are 14 (excluding various splice variants) serotonin receptor subtypes which are distributed in a tissue-dependent manner throughout the CNS, peripheral nervous system, GIT, cardiovascular system and blood (Stiedl et al., 2015, Hoyer et al., 2002). 13 of these receptors are GPCRs whilst 5-HT_3R is a ligand-gated ion channel (Hoyer et al., 2002). These receptors are broadly classified into 7 groups, and Table 2 lists serotonin receptor sub-types involved in appetite regulation centrally, peripherally and their demonstrated functions.

Serotonin receptors are coupled to the $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_s$ signalling pathways (McCorvy and Roth, 2015). The 5-HT_1 sub-family signals through $G\alpha_i$, inhibiting adenylyl cyclase and decreasing cAMP levels (Hurley et al., 1998) (Lin et al., 2002). The 5-HT_2 sub-family have been shown to be coupled with $G\alpha_{q/11}$ increasing intracellular calcium levels via inositol triphosphate and diacylglycerol (McCorvy and Roth, 2015). There is however evidence that the 5-HT_{2A} and 5-HT_{2C} subtypes can inhibit cAMP via $G\alpha_i$ (Lucaites et al., 1996, Garnovskaya et al., 1995). Lastly, the 5-HT_4, 5-HT_6, 5-HT_7 subfamilies are coupled to $G\alpha_s$ (McCorvy and Roth, 2015). 5-HT_4 has been shown to couple with adenyl cyclase to increase cAMP levels in the porcine hippocampus (Bockaert et al., 1990), with some evidence for increased calcium levels human atrial myocytes (Ouadid et al., 1992). 5-HT_6 and 5-HT_7 activation increases cAMP levels (McCorvy and Roth, 2015).

Table 2: 5-HT receptor types, location and functions.

Receptor	Location(s)	Function(s)
5-HT_{1A}	Myenteric plexus	• Neuroendocrine regulation of adrenocorticotrophic hormone (regulates cortisol release) (Jørgensen et al., 2001) • Decrease in blood pressure and heart rate and increased locomotor responses (Wilkinson, 1991) • Modulating anxiety-related behaviours (Hoyer et al., 2002)
5-HT_{2A}	Periphery, CNS, cortex	• Mediate contractile responses in vascular smooth muscle (bronchus, uterus, urinary tract) (Hoyer et al., 2002) • Platelet aggregation and increased capillary permeability (Hoyer et al., 2002) • Implicated in schizophrenia, depression, and Tourette's (Meltzer and Roth, 2013)
5-HT_{2B}	Rat: fundus, gut, heart, kidney, lung and brain Mouse: intestine, heart, kidney and brain	• Fundic smooth muscle contraction (Hoyer et al., 2002) • Centrally, receptor activation implicated in hyperphagia in rats (Kennett et al., 1997)
5-HT_{2C}	CNS	• Central serotonin$_{2C}$ receptor activation induced hypoactivity, hypophagia in rats (Vickers et al., 2001) • Receptor antagonism increased food intake and weight gain in rats (Bonhaus et al., 1997) • serotonin$_{2C}$ receptor KO mice: increased food intake and obesity (Bickerdike et al., 1999)
5-HT_3 family	Central (hippocampus, solitary tract), peripheral (pre-/postganglionic autonomic, sensory nervous system) neurons	• Regulate intestinal motility and secretion (De Ponti and Tonini, 2001)

5-HT_4 family	Brain, vascular smooth muscle	• Triggers acetylcholine release (guinea pig ileum), contracts the oesophagus and colon (Hoyer et al., 2002) • Mediating secretory responses to serotonin in intestinal mucosa • Colonic peristalsis (Kadowaki et al., 2002) • Receptor knock-out mice exhibit stress-induced feeding disorders (Compan et al., 2004)
5-HT5 family	Cerebellum (Pasqualetti et al., 1998)	• Possible role in psychiatric disorders (Thomas, 2006) and memory (Gonzalez et al., 2013)
5-HT_6 family 5-HT_7 family	Thalamus, hypothalamus, hippocampus, as well as the peripheral tissues	• 5-HT_6 regulates feeding: antisense oligonucleotide treatment reduced both food consumption and body weight (Bourson et al., 1995) • Antipsychotic functions (Meltzer and Roth, 2013) • 5-HT_7 is an antidepressant target (Abbas et al., 2009)

Gut-derived serotonin is unable to cross the blood-brain barrier; TPH1-deficient mice have little serotonin in the blood and GIT, however, levels of serotonin in the brain are normal (Watanabe et al., 2010). Gut-derived serotonin that leaves the GIT is mostly sequestered within platelets to aid in blood clotting (Young et al., 2018). Non-sequestered plasma serotonin (~2% (Spohn and Mawe, 2017)) has been shown to inhibit osteoclast function (Yadav et al., 2010), increase hepatic glycogen synthesis and cholesterol content and stimulate gallbladder bile excretion, which acts to increase lipid metabolism and decrease plasma triglyceride levels in rodents (Watanabe et al., 2010). Watanabe *et al.* showed a concentration-dependent effect of intraperitoneal serotonin injection; with a 1 mg dose, serotonin increased plasma glucose and insulin concentrations whereas at 0.03 mg serotonin slightly decreased plasma insulin, without hypoglycaemia (Watanabe et al., 2010). Serotonin is released in response to carbohydrate rich foods (foods generally associated with an obese diet) (Wurtman and Wurtman, 1995) and plasma levels of serotonin in obese patients have been shown to be higher than in non-obese humans post intraduodenal glucose infusion (Young et al., 2018). Mice fed a high fat diet, however, had the same levels

of serotonin synthesis as normal diet mice (France et al., 2016), this might be an important species difference. If serotonin release is increased in obesity, this might drive pathologies related to high blood sugar and poor insulin control. It also might provide evidence of serotonin playing both a protective and harmful role in obesity, with diet causing a switch in phenotype.

Colonic mRNA expression of TPH1 was also shown to be unchanged in obese vs. non-obese humans (Young et al., 2018). The same study showed that the amount of serotonin in EC cells was unchanged with obesity and posited that the increased numbers of EC cells was responsible for the increased overall amount of serotonin, and that this drove the obese state (Young et al., 2018). The mechanical sensations of stretching in the gut wall that occur during ingestion and digestion have been shown to stimulate serotonin release from ECs via paracrine and autocrine release of ATP (Reimann et al., 2012). This can provide an important signal of fullness and help regulate food intake.

Gut-derived serotonin also has an important role in regulating energy reserves during fasting; in rodents serotonin acts on 5-HT_{2B} receptors to promote hepatic gluconeogenesis and lipolysis in white adipose tissues, and 5-HT_3 receptors to inhibit adaptive thermogenesis in brown adipose tissue (Crane et al., 2015, Oh et al., 2015). The peripheral and central serotonin systems are independent and separate, and for the purpose of this project I will be focusing on gut (TPH1)-derived serotonin as an important mediator in nutrient sensing in obesity.

Genetic deletion or pharmacological blockade of TPH1 protects mice from high fat diet-induced obesity, insulin resistance and fatty liver disease (Young et al., 2018). Serotonin receptor agonists have been shown to reduce weight gain in mice and reduce caloric intake in lean and obese humans (Halford et al., 2007). Our group has previously shown that expression of TPH1 and the numbers of ECs were increased in an obese mouse group compared to a normal weight group (though this increase did not reach statistical significance) (Peiris et al., 2018). Furthermore, expression levels of colonic TPH1 in obese mice decreased to match those in lean mice after RYGB surgery (Peiris et al., 2018). Young *et*

al. showed that plasma serotonin levels increased in obese vs. non-obese patients after intraduodenal glucose infusion, but that the colonic mRNA expression of TPH1 was unchanged (Young et al., 2018).

Serotonergic drugs agonists have been shown to facilitate weight loss by accelerating satiety onset, increasing metabolic rate and inhibiting carbohydrate cravings (Wurtman and Wurtman, 1995). Inhibiting serotonin synthesis in the brain induced weight gain in rats (Namkung et al., 2015). Serotonin release also activates small intestinal and colonic vagal afferent nerves (Lund et al., 2018). There is also evidence that ECs in the colon have basal extensions containing secretory vesicles which are thought to communicate directly with primary afferent neurons (Gunawardene et al., 2011). Of these mucosal projections of primary afferent neurons, extrinsic nerves are thought to transmit feelings of nausea and discomfort to the CNS, whilst intrinsic neurons of the colon are involved in peristalsis and secretion (Gershon and Tack, 2007). It is therefore likely that serotonin can activate these afferent neurons in a paracrine manner and is the mediator responsible for these effects.

1.2.2 Central mechanisms regulating food intake

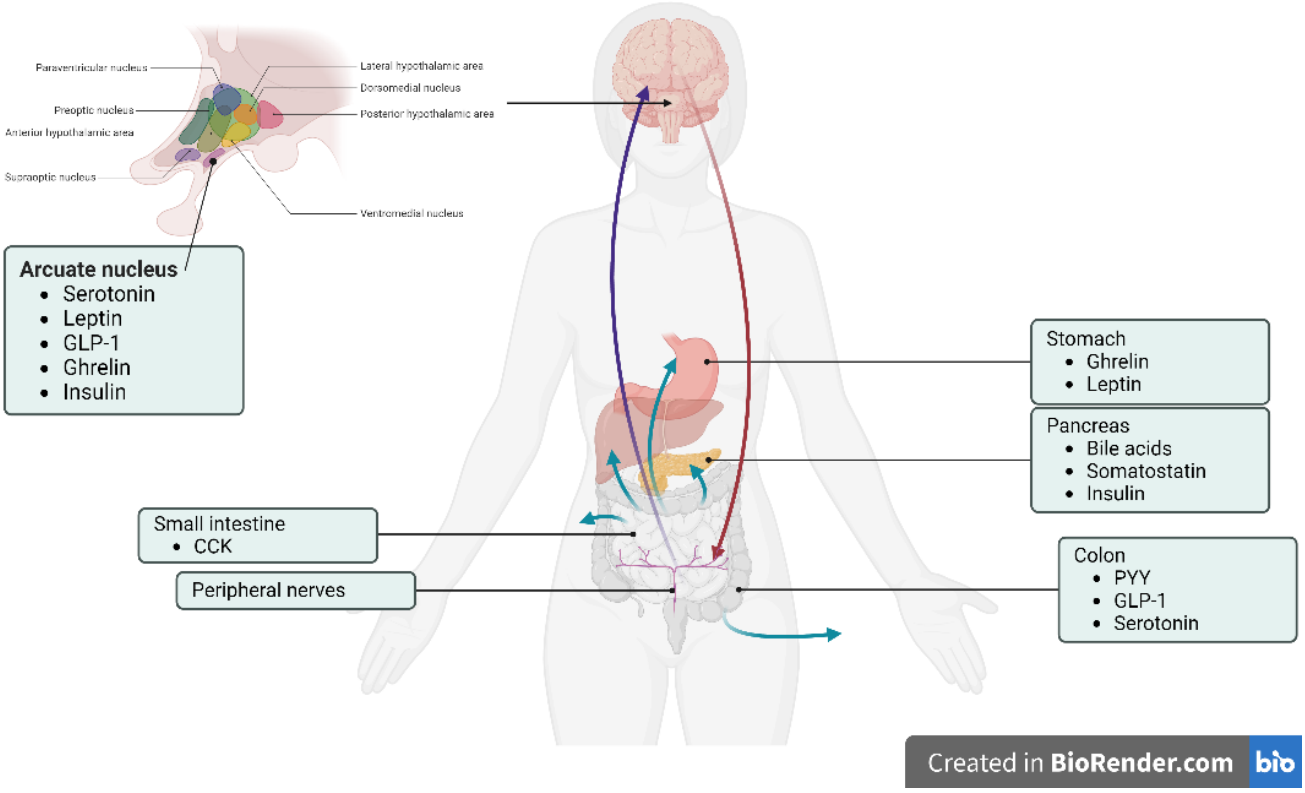


Figure 5: A summary of the peripheral and central signallers of satiety. Hormones like PYY, GLP-1 and CCK are released from the GIT and act on local cells, tissues and peripheral nerves (blue arrows). Peripheral signals (hormones, nerve signals) can drive satiety/ hunger pathways in the brain (purple arrow) by inducing e.g., NPY/ POMC/ CART peptides. Central signals (hormonal, taste, olfactory, visual) can drive satiety/ hunger pathways in the periphery (red arrow) by inducing hormone release, muscle activity etc. Created with BioRender.com

Hormones and neuropeptides released peripherally are able to travel to and act on the hypothalamus; specifically the hypothalamic arcuate nucleus (ARC) region (Perry and Wang, 2012) (

Figure **5**). The hypothalamus is the main region of the CNS that is involved in appetite regulation and is the major homeostatic centre of the brain (Schloegl et al., 2011). The ARC

is situated at the base of the hypothalamus and is not fully separated from the rest of the circulation by the blood-brain barrier, allowing access of circulating factors to its neurons (Murphy and Bloom, 2004). The ARC transfers peripheral hormone signals on energy/ nutrient availability to other brain regions (Lieu et al., 2020). Within the ARC there are two distinct neuronal populations; orexigenic, which co-expresses agouti-related peptide (AgRP) and NPY, and anorexigenic, which release POMC and CART peptide (Druce and Bloom, 2006). POMC is a precursor molecule that is cleaved into various molecules including α-melanocyte-stimulating hormone (α-MSH) (Murphy and Bloom, 2004). α-MSH is an anorectic peptide that is an agonist for the MCR3 and MCR4 receptors (Ellacott and Cone, 2004). AgRP is the natural MC3 and MC4 antagonist and has been shown to increase food intake in mice deficient in the MC4 receptor (Rossi et al., 1998). Mice lacking MC3R are not obese on a normal diet, but do have increased adiposity; however, mice that lack MC3 are obese (Butler et al., 2000). NPY is an orexigenic molecule, much like AgRP, that instead stimulates feeding through the Y1 and Y5 receptors (Gehlert, 1999). The AgRP-expressing population of neurons has been shown to drive rapid responses to feeding (minutes/ hours), whereas the POMC-expressing population has been shown to alter long-term feeding (hours/ days) (Aponte et al., 2011, Zhan et al., 2013). NPY- and AgRP-expressing neurons within the ARC act in an orexigenic manner; they strongly stimulate feeding via the activation of the paraventricular nucleus (PVN) NPY receptors and inhibition of the melanocortin system via antagonism of MCR3/4 (Wynne et al., 2005). However, NPY/AgRP-knockout mice show no obvious changes in food intake or body weight, therefore these pathways are unlikely to be involved in regulating energy homeostasis (Microbiology, 2004). The hormones leptin, serotonin and GLP-1 have been shown to activate, and insulin inhibit, POMC neuronal activity

Figure 5; these same hormones and insulin have also been shown to inhibit NPY neuronal activity (Lieu et al., 2020). These neurons are therefore able to alter their activity in response to hormonal changes that might originate in the GIT. Receptors for hormones released from the gut (including PYY) and for insulin are also expressed on neurons within the hypothalamic ARC and when stimulated, change the activity of these neurons and the release of neuropeptides, and therefore feeding behaviour and energy expenditure (Haynes et al., 2020, Small and Bloom, 2004). These neurons have also been shown to alter their

activity in response to nutrients and hormones in circulation such as glucose, ghrelin, and GLP-1 (Haynes et al., 2020). PYY acts on the hypothalamus, where it causes changes in the neurons and neuropeptides involved in appetite (Batterham et al., 2002b, Karra et al., 2009). The ARC is a site with high levels of expression of the PYY receptor and is a key site of action of PYY (Batterham and Bloom, 2003). Circulating PYY levels in human participants were shown to increase before nutrients could have reached L cells in the colon; therefore, a neuronal or hormonal mechanism is likely behind its initial release (Karra et al., 2009). The anorectic effects of intra-arcuate injection of PYY were abrogated by blocking its receptor (Y_2R) (Abbott et al., 2005). PYY modulates the activity of the NPY (orexigenic) and POMC (anorexigenic) neurons in the arcuate nucleus of the hypothalamus (Batterham and Bloom, 2003). PYY administration in mice increased c-fos expression (a marker of neuronal activation) within the ARC, increased vagal nerve activity and altered hypothalamic neuropeptide release (Karra et al., 2009). The proglucagon gene is also expressed in the CNS; cells immunoreactive for GLP-1, glucagon and glicentin are present in the human brain stem (Holst, 2007). In animal studies, GLP-1 has been shown to activate afferent sensory neurons, the hypothalamic nuclei that regulate glucose metabolism, as well as stimulating insulin release from the pancreas (Balkan and Li, 2000, Nakabayashi et al., 1996). Indeed, proglucagon expression has been found to be associated with oxyntomodulin-processing neurons, which have been shown to release oxyntomodulin, GLP-1 and -2 (all products of proglucagon processing), which reduce food intake (Holst, 2007). Mice injected intraperitoneally with GLP-1 had reduced food intake and reduced signal intensity of the PVN and ventromedial nucleus regions of the hypothalamus (Schloegl et al., 2011). However, because of the short half-life of GLP-1 in circulation, it is currently not known whether gut-released GLP-1 is able to reach and activate receptors in the hypothalamus and brain stem (Latorre et al., 2016).

The vagus nerve is the main component of the parasympathetic nervous system which regulates digestive functions (among others) by connecting the brain and GIT (Breit et al., 2018). The vagus projects into the nucleus of the solitary tract in the brain which is able to communicate with the hypothalamus (Wynne et al., 2005). CCK has been shown to activate vagal fibres in both a paracrine and neurocrine manner; CCK-A receptor-knockout rats are

hyperphagic, diabetic and obese (Bi et al., 2001). This study showed that peripheral CCK signalling was abrogated and NPY expression was increased in these rats and there was a significant (doubling) increase of meal size compared to wild-type animals (Bi et al., 2001). Vagal nerves are also involved in monitoring stomach and gastric distension, which are strong drivers of food intake and satiety, with an inverse relationship between the two (Oesch et al., 2006). Indeed, an implantable vagus nerve stimulator has been approved in humans for weight control, and studies have shown that stimulating the vagus nerve artificially can induce weight loss through reducing food intake and altering energy metabolism, though CCK and PYY release were unchanged (Ikramuddin et al., 2014, Val-Laillet et al., 2010). The mechanism of action behind this effect is, however, poorly understood.

Afferent nerves innervate the colonic mucosal epithelium and connect to the CNS, and have been shown to terminate approximal to EECs (Berthoud et al., 2004). Tension receptors respond to muscular contraction and distension, and mucosal receptors to sensitizing or inhibiting chemical mediators released from cells, including EECs (Berthoud et al., 2004). Sensory information from these nerves do not transmit sensations of pain to the CNS, but instead signals that regulate homeostasis (Blackshaw and Gebhart, 2002). These afferent endings have been shown to express receptors for PYY (Y2), serotonin (5-HT_3) and GLP-1 in the mouse, and when GPCR agonists lauric acid and TUG891 were co-administered, there was evidence of synergistic activation of vagal afferent responses (Peiris et al., 2021). In human colonic mucosa, co-administration of these agonists increased PYY and GLP-1 release by 2-4 times more than either alone (Peiris et al., 2021). In addition, PYY and GLP-1 administration indicated that receptors for both co-exist on the same afferent endings, with the induction of powerful responses in the fibres, indicating a high level of synergism between EEC and afferent ending activity (Peiris et al., 2021). Antagonists for serotonin, PYY and GLP-1 receptors on these fibres reduced the response to their relevant hormones (Peiris et al., 2021), indicating an important role of these fibres in modulating satiety. Afferent nerves and fibres are therefore able to rapidly signal to the CNS the colonic hormonal environment before these hormones enter the circulation.

Other hypothalamic nuclei and neuropeptides also have roles in regulating appetite and body weight. The brain stem has a role in controlling food intake via reward and feedback mechanisms; opioids, serotonin and endocannabinoids are all involved in such mechanisms, with endocannabinoid antagonists shown in trials to reduce appetite and body weight (Druce and Bloom, 2006). Monoamines such as serotonin can be released from the gut in response to food ingestion (Wynne et al., 2005, Druce and Bloom, 2006). Endocannabinoids may be involved in regulating food intake in the hypothalamus; defects in normal leptin signalling are associated with high levels of endocannabinoids in the hypothalamus of obese mice and rats (Wynne et al., 2005). Rimonabant is an antagonist for the endocannabinoid receptor, cannabinoid-1, and has been found to reduce appetite and body weight by around 5% in 4 studies in obese humans (Curioni and André, 2006).

Enteric nerves in the GIT can also signal energy intake and regulate food intake. Tension receptors within the GIT are able to signal gastric distension to the CNS to generate sensations of satiety and fullness (Page and Li, 2018). The GLP-1 receptor is expressed on mucosal afferent endings (Bucinskaite et al., 2009) and GLP-1 has been shown to increase afferent activity to drive a reduction in food intake and insulin release (Hayes et al., 2011).

Also of note is the recorded sexual dimorphism in metabolism; there are gender differences in metabolic responses to exercise, energy utilisation and storage (Lieu et al., 2020). For example, in the ARC, male mice have fewer POMC neuronal fibres and increased expression of the NPY gene, whilst female mice are more sensitive to the anorectic effects of centrally injected leptin (Lieu et al., 2020). In humans, intranasal administration of insulin reduced body fat in men, but not women (Hallschmid et al., 2004).

1.3 Obesity

1.3.1 Clinical manifestations and prevalence

Obesity is defined by the World Health Organisation as “abnormal or excessive fat accumulation that presents a risk to health”(World Health Organisation, 2014). Body mass index (BMI) is used to classify whether a person is underweight, healthy, overweight or

obese, by dividing a person's weight by the square of their height (World Health Organisation, 2014). A person with a BMI of 25-29.9 (kg/m^2) is considered overweight, with 30-39.9 obese and with a BMI of over 40 severely/ morbidly obese. Generally, a BMI of 18.5 – 24.9 is considered to reflect a healthy weight, with anything under 18.5 classified as underweight (Public Health England). BMI is used alongside other physiological factors to predict the risk for developing diseases related to excess weight, i.e., metabolic, immune and cardiovascular disorders. Waist size is often used as an additional indicator of excess fat; waist sizes above 94 cm and 80 cm (men, women respectively) increase risk of obesity-related health problems and diseases (Public Health England). Alongside effects on patient morbidity and mortality, obesity also has significant costs: health and economic impacts of obesity cost the UK £27 billion in 2014 (Public Health England, 2019b). Annual UK government spending on treating obesity and diabetes in 2017 was higher than the amount spent on the combined budgets of the police, fire services and judicial system (England, 2020).

Obesity is one of the most significant health issues facing the Western world and it is a growing problem; since 1993 the prevalence has increased by 11%, affecting 26% of adults in the UK, with another 36% being overweight (Public Health England, 2019a, England, 2020). The UK has an obesity epidemic second in Europe only to Hungary, and ranked 6th worldwide (OECD, 2019). This is a global trend, and it is postulated that by 2022 there will be more obese children and adolescents worldwide than underweight (Abarca-Gómez et al., 2017). Furthermore, the proportion of obese adults who fall into the very high risk category (those with high/very high waist circumference and BMI) is also increasing (England, 2019). Obese adults are more susceptible to a wide range of comorbidities, including type 2 diabetes (Nguyen et al., 2008), metabolic disorders and abnormalities such as dyslipidaemia (Grundy and Barnett, 1990), cardiovascular disease (Klein et al., 2004) including hypertension (Wilson et al., 2002), stroke (Rexrode et al., 1997) and a variety of cancers (Calle et al., 2003). In Europe it has been demonstrated that every 5 points past a BMI of >25 leads to a 39% increase in the risk of mortality, with heart disease, stroke and respiratory disease being the main co-morbidities (Di Angelantonio et al., 2016). It is

estimated that around 30,000 deaths per year are due to obesity, with obesity reducing life expectancy by around 9 years (England, 2020).

1.3.2 Pathophysiology

There are several systemic changes associated with obesity that predict the progression of a number of diseases and metabolic dysregulation. Increased body fat in obese individuals is mostly due to the increase in the size of fat cells, though numbers of fat cells can also be increased (Salans et al., 1973). Increased adipose cell number and/ or increased size is referred to as hyperplastic obesity, with increased cell size alone referred to as hypertrophic obesity (Salans et al., 1973). The distribution of this additional, excess fat, can influence the type of disease that may arise; excess abdominal adipose tissue and triglyceride content in liver and skeletal muscle, and fat infiltration of heart tissue, are associated with hepatic and skeletal muscle insulin resistance, and impaired ventricle function and coronary heart disease, respectively (Klein et al., 2004, Alpert, 2001).

The association between obesity and disease is highest for hypertension and diabetes, at 51% and 27% prevalence, respectively (Nguyen et al., 2008). Obesity caused by excessive calorie intake can adversely affect glucose regulation, promote insulin resistance and glucose intolerance due to overproduction of glucose (Grundy and Barnett, 1990). Obesity is associated with Type 2 diabetes, i.e., diabetes mellitus. Obesity has also been negatively linked with a poor outcome of infection with the SARS-CoV-2 virus and the virus-induced disease, COVID-19; there are increased mortality and morbidity rates in obese individuals, driven by negative clinical lung manifestations (Kang et al., 2020).

1.3.2.1 Dysregulation of central appetite control mechanisms

It is thought that under conditions of energy/ caloric abundance, the hypothalamus becomes resistant to nutritional and adiposity signals (leptin, fatty acids and insulin) and that this can lead to obesity and insulin resistance (Lam et al., 2005). This is likely due to an attempt by the body to increase energy storage from this increased availability of energy sources. There is also evidence that this increased energy availability - even increased availability of nutrients which normally stimulate satiety – also causes the dysregulation of normal satiety mechanisms. A study by Morgan *et al.* demonstrated that rats on a normal

diet, given centrally administered oleic acid, a LCFA, had a reduced food intake and decreased hypothalamic NPY expression (Morgan et al., 2004). However, when rats were overfed, these anorectic effects were lost and hypothalamic NPY expression was not decreased; the authors postulated that this would contribute to the rapid weight gain and insulin resistance observed (Morgan et al., 2004). Calcium-permeable (depending on RNA editing, receptor subunit composition) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) in the nucleus accumbens mediate cue-triggered motivation for food, and their activity and expression has been shown to be induced in rats by sugary, fatty junk-foods (in a sex dependent-manner) (Alonso-Caraballo et al., 2021). Interestingly, these AMPARs also drive cue-triggered motivation for cocaine (Alonso-Caraballo et al., 2021) - adding to the evidence that junk food acts in a drug-like manner. Another study that looked at the impact of a high sugar diet in diet-induced obese mice found not only that mice preferentially ate high sucrose foodstuffs, but also had changes to "numerous gastrointestinal genes involved in macronutrient sensing, appetite regulation, nutrient metabolism and intestinal microflora" (O'Brien et al., 2021), suggesting an important role of this nutrient – and particularly its abuse - in obesity.

1.3.2.2 Dysregulation of peripheral appetite control mechanisms

Dysbiosis refers to the replacement of benign/ beneficial intestinal microbiota (microbiota is a collective term for the resident micro-organisms) with pathogenic microbiota, and is a common effect of obesity or its related comorbidities (for instance the treatment of underlying conditions with antibiotics) (Pizarroso et al., 2021). Turnbaugh *et al.* proposed that the obese gut microbiome has an increased capacity to harvest energy from the host diet (Turnbaugh et al., 2006). In addition, using wild-type and leptin-deficient (ob/ob) mice, they demonstrated that the microbiome of the obese gut could produce a higher level of monosaccharides and short chain fatty acids (SCFAs) (Turnbaugh et al., 2006). The authors postulated that this would give these obese mice extra energy from indigestible food components, compared with lean animals. Bäckhed *et al.* suggested that the gut microbiota (murine) can control fatty acid oxidation in the host via suppression of the adenosine monophosphate (AMP)-activated protein kinases and induce fat storage via suppression of fasting-induced adipose factor (Backhed et al., 2007). A study examining the

effect of a probiotic (*S. cerevisiae* containing fruit juice) in diet-induced obese rats showed reduced bodyweight, fat mass and improved hepatic markers (Konda et al., 2020). The study authors concluded that an increase in the intestinal population of the *S. cerevisiae* yeast was responsible (Konda et al., 2020). The microbiome and diet can therefore alter the energy balance of the host. It has been demonstrated that humans display a higher concentration of SCFAs in the faeces of obese, compared with lean individuals (Schwiertz et al., 2010) so it is possible that these effects seen in rodents also exist in humans. In this study however, the diet or food intake of individuals was not recorded or quantified in detail (only: "All participants subsisted primarily on a western diet") – this is a weakness to the study (Schwiertz et al., 2010).

Obesity can be driven by defects in normal appetite and energy homeostasis. Mutations or outright deletions in the POMC gene that lead to a change in POMC cleavage products, have been shown to cause early onset obesity and adrenal insufficiency (Krude et al., 1998), and hyperphagia and obesity, respectively (Yang and Harmon, 2003). As previously discussed, mutations in the MC4 receptor are also linked with obesity as well as changes to basal energy expenditure and hyperinsulinemia (Farooqi et al., 2003).

1.3.3 Obesity and diet

Obesity is thought to be driven by changes in the human diet over time, particularly following the industrial and subsequent agricultural revolutions. Increased consumption of calorie-dense foods, soft drinks and increased 'snacking', alongside decreased physical activity can drive the obese state (Druce and Bloom, 2006). A report found that in the UK over a quarter of adults, and a fifth of children, eat food from food outlets at least once per week, and that these meals were associated with "higher energy intake; higher levels of fat, saturated fats, sugar, and salt, and lower levels of micronutrients" (Science, 2007). In addition to an increased calorie load through intake, people in the UK are 20% less active than in the 1960s, and this is predicted to reach 35% by 2030. This decreased activity starts in the young, with only 22% of children aged 5-15 meeting the UK government's guidelines on physical activity (England, 2016). These environmental changes exacerbate any genetic predisposition to fat storage; during times of food plenty, survival was often reliant on the

ability of the human body to store fat to aid survival during times of scarcity (Druce and Bloom, 2006). However, in the Western world and where junk food is normalised and the fact that “unhealthy” foods are significantly cheaper than “healthy foods” - there is no scarcity; only plenty.

1.3.4 Treatment of obesity

Currently, effective treatments of obesity are limited. Behaviourally focused strategies to reduce food intake and promote exercise are poorly maintained and have limited effectiveness – varying greatly from person to person (Curioni and Lourenco, 2005).

1.3.4.1 Lipase inhibitors

Pharmacological treatments include orlistat; the only drug approved in the UK results in a 3% reduction in body weight loss vs. dieting alone (Drew et al., 2007). Orlistat is a gastric and pancreatic lipase inhibitor which reduces dietary fat absorption from the intestine (Drew et al., 2007) by competing with the active site of gastric and pancreatic lipases, therefore reducing triglyceride hydrolysis and subsequent absorption of these products (Guerciolini, 1997). There is some evidence that orlistat is able to improve glycaemic control and reduce the risk of T2D in obese/ overweight individuals, as well as improve blood pressure and cholesterol levels (Drew et al., 2007). The side effects of orlistat are malabsorption (of vitamin D and calcium) (Filippatos et al., 2008) and changes to bowel movements (increased urgency, oily/ fatty faeces) (Uk).

Cetilistat is a pancreatic lipase currently approved in Japan for the treatment of obesity (George et al., 2014). The drug acts to reduce the conversion of triglycerides into free fatty acids, increasing triglyceride excretion into urine (George et al., 2014). In phase II trials cetilistat induced significant weight loss and was well tolerated in 442 obese patients in a 12-week study (Dourish et al., 2008). The drug is not currently approved in the UK.

1.3.4.2 CNS-targeted drugs

Other treatments include drugs that target the CNS. These include drugs targeting hormone receptor pathways such as the 5-HT_6 receptor agonist loracserin, and the GLP-1 agonist liraglutide -these drugs have been shown to reduce mean weight compared to a

placebo by 3.06 and 5.5 kg respectively in a review of 50 reports and 43,443 subjects (Coulter et al., 2018). Loracserin has been shown to increase satiety and reduce food-cravings and impulsivity (Roth et al., 1998), however in 2020, the FDA requested that the drug be removed from the market because of potentially increased cancer risk (Sharretts et al., 2020). Liraglutide was initially approved to treat T2D but was shown to be associated with weight loss, with the mechanism most likely due to an increased slowing of gastric emptying (Halawi et al., 2017).

Semaglutide is a GLP-1 analog which has recently (2020) been shown to increase weight loss, lower glucose levels and cardiovascular symptoms in a phase 3 trial of diabetic patients (Gabery et al., 2020). Semaglutide was more effective (2-4%) than other GLP-1 analogs in reducing body weight (Overgaard et al., 2019). Weight loss was shown to be independent of energy expenditure, instead modulating food preference and intake (Gabery et al., 2020). The weight loss is thought to be mediated by the activity of GLP-1R receptors in the brain, specifically on neurons involved in meal termination in the lateral parabrachial nucleus (Gabery et al., 2020).

Bupropion is a drug that targets POMC neurons, blocking dopamine and noradrenaline reuptake and possibly stimulating dopamine and noradrenaline release - to decrease appetite and increase energy expenditure - and has been shown to reduce mean weight compared to a placebo by 6.15 kg (Coulter et al., 2018). Topiramate was initially approved for the treatment of epilepsy but demonstrated weight loss properties in epilepsy trials (Ben-Menachem et al., 2003), with studies in rats demonstrated it acted to enhance thermogenesis (Richard et al., 2002). Topiramate has been shown to reduce mean weight compared to a placebo by 7.45 kg (Coulter et al., 2018).

1.3.4.3 Bariatric Surgery

At present, bariatric surgery is the most efficacious treatment for obesity. The most common bariatric surgery is gastric bypass, specifically RYGB. This procedure creates a pouch from a small part of the upper stomach and attaches this directly to the jejunum (Figure 6) (Gulliford et al., 2017). This causes several changes to the gastrointestinal mechanical and satiety mechanisms. Food is now able to bypass most of the stomach as

well as the duodenum and upper jejunum (Dirksen et al., 2012), with undigested food shunted directly into the distal small intestine, a site usually naïve to such a nutrient stimulus. Nutrients cause the release of hormones from these cells and the stimulation of the vagal afferents, which act to reduce gastric emptying and food and energy intake (Peiris et al., 2018). GLP-1 and PYY have been shown to be significantly increased after bypass surgery (le Roux et al., 2006a, Peiris et al., 2018). The increase in these hormones is likely to underlie the significant weight loss seen in obese patients - usually around 35-40%, that is maintained for at least 15 years (Brolin, 2002). Gut-brain communication via vagal-spinal sensory pathways is also altered; vagal afferent fibres can signal the increased extension of the small intestine caused by an undigested food bolus and alter food intake, therefore acting as vagal tension sensors (Hao et al., 2014). The sensory structures, intra-ganglionic laminar endings, are expressed in the myenteric plexus – the tissue layer between the circular and longitudinal smooth muscle layers – and are likely to sense the increased tension and stretch in the bypass region (Hao et al., 2014). This vagal activation is postulated to activate central sensory pathways; in gastric bypass patients, increased sensations of distension were associated with smaller meals (Hao et al., 2014), and in rats, increased neural activation in satiety-associated brain areas was observed compared to unoperated rats (Berthoud et al., 2011). Although effective, gastric bypass is associated with both acute and long-term complications. Acute complications are similar to those that follow most abdominal surgeries, including risk of infection, haemorrhages and rhabdomyolysis (Pories, 2008). Long term complications include nutritional deficiencies (often leading to malnutrition), hypoglycaemia, hernias and psychological disorders (Pories, 2008).

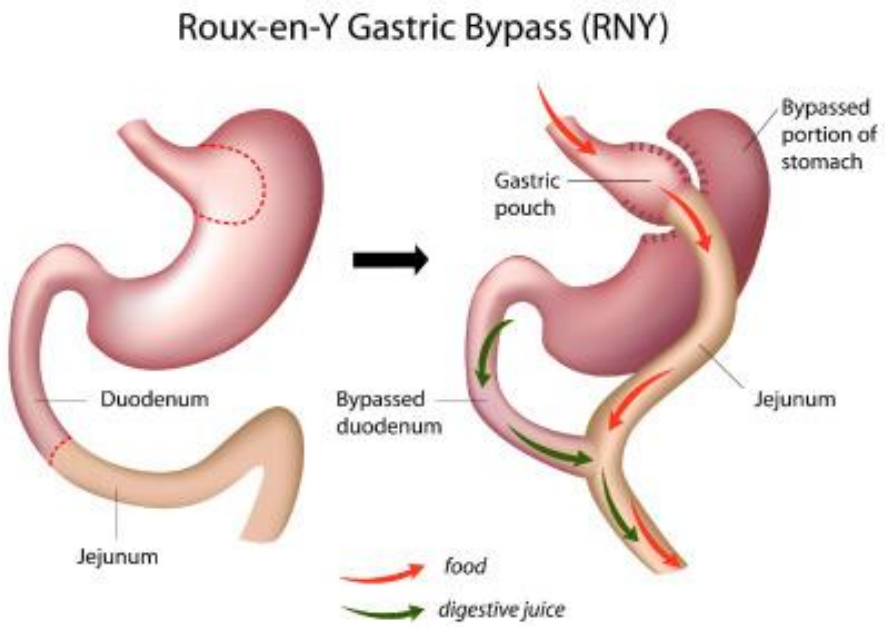


Figure 6: Roux-en-Y bypass surgery procedure; the majority of the stomach and upper intestine is bypassed and instead the upper stomach connects to the small intestine. The rest of the stomach remains connected to the digestive system to allow products of the stomach such as acids, enzymes to enter the intestine. Reprinted from (Hospital, 2020).

A better understanding of nutrient sensing in the gastrointestinal tract could help drive new therapies and treatment options. However, current understanding of nutrient sensing in the normal, as well as the obese gut, in humans remains limited.

1.4 Mechanisms of nutrient sensing in the colon

Understanding how sensing of dietary nutrients and subsequent hormone release leads to satiety may offer important insights into promoting appetite reduction. However, I first needed to understand how nutrient sensing functions in the normal and obese colon. The colon is not the main site of nutrient absorption; most nutrients have already been absorbed in the stomach and small intestine (Cheng et al., 2010). The primary biological function of the colon is to remove water from the luminal contents (Velagapudi et al., 2010) and compact waste (Cheng et al., 2010). However, not only does the colon hold the highest number and diversity of microbiota, but it is the main site for the production of serotonin (Gershon and Tack, 2007) and bacterial by-products that have a role in nutrient sensing and obesity, but also has the highest density of L cells (Gunawardene et al., 2011). The colon receives peptides and amino acids that have been broken down from larger proteins by digestive enzymes in the upper GIT. These are absorbed by transport systems on intestinal epithelial cells, to induce downstream signalling (Rasoamanana et al., 2012). In the colon, both the volume of colonic content (gas, faeces) as well as the microbiota can influence sensations of digestion and fullness (Livovsky and Azpiroz, 2021).

Figure 7 shows the relative mRNA expression of various nutrient receptors in different sections of the murine GIT: the colon expresses receptors for a variety of different nutrients, with the expression of GPR120, GPR43 and CaSR being highest in the colon (Symonds et al., 2015). Various hormones are released throughout the GIT, including the colon, with diverse roles in regulating digestion, metabolism and appetite (Svendsen et al., 2015). These hormones can act to inhibit or promote appetite and food intake, regulate blood sugar levels and affect gut physiology (Svendsen et al., 2015). The fact that most gut mediators (such as GLP-1) are broken down rapidly by proteases means that they are at the highest concentration locally (Rasoamanana et al., 2012), underlining the role of the colon in driving postprandial signals. Colonic infusions of fatty acids have been shown to increase PYY and GLP-1 secretion – however, the mechanisms are not fully clear. One study showed that colonic infusion of isolated rat colon with the SCFA acetate and butyrate, increased secretion of GLP-1 and PYY and increased intracellular cAMP levels; however, this was independent of GPR43/41 (Christiansen et al., 2018). Another study showed that increased

levels of PYY and GLP-1 to propionate stimulation in colonic crypt cultures, were abrogated in GPR43 knockout mice (Psichas et al., 2015).

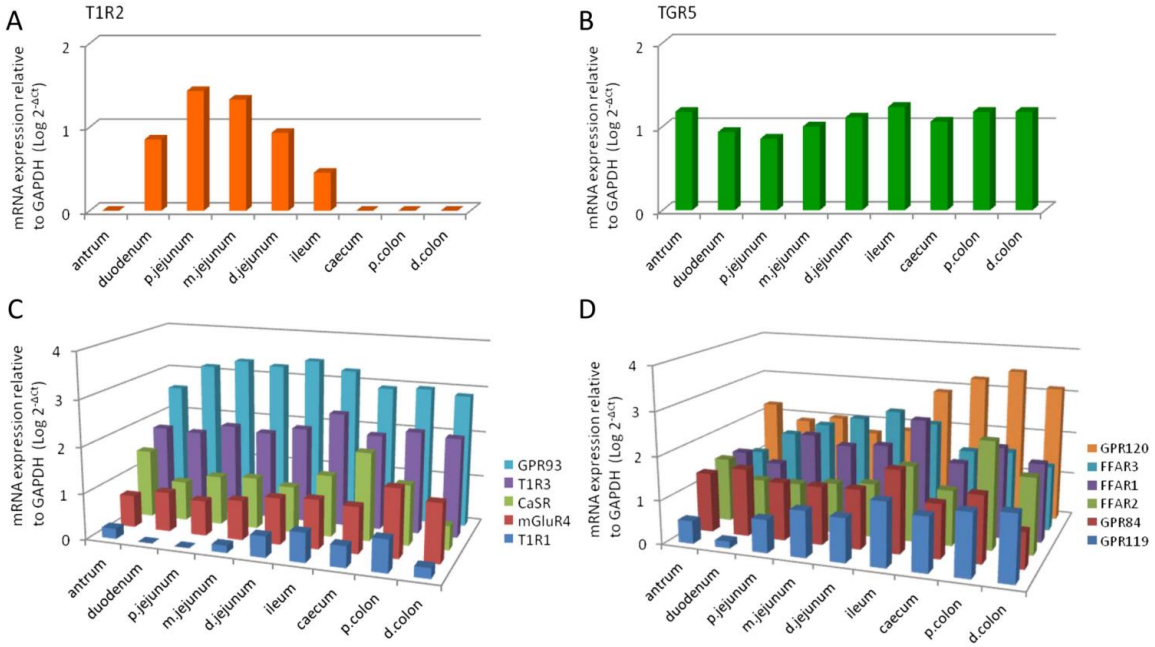


Figure 7: mRNA expression of genes (human) encoding nutrient receptors (relative to GAPDH) for (A) carbohydrates (TAS1R2); (B) bile acids (G-protein-coupled bile acid receptor); (C) amino acids and fatty acids (D) within the gastro-intestinal tract. FFAR1 = GPR40, FFAR2 = GPR43, FFAR3 = GPR41. Reprinted from (Symonds et al., 2015)

As discussed, hormones in the colon can be released from a variety of colonocytes (epithelial cells of the colon) in response to specific nutrients in food, hunger or insulin status. Cells of particular interest are EECs: intestinal epithelial cells with demonstrated taste transduction machinery such as chemoreceptors on their apical surfaces (Bertrand and Bertrand, 2010, Rasoamanana et al., 2012).

1.5 Enteroendocrine Cells

EECs are derived from pluripotent stem cells located in intestinal crypts (Gunawardene et al., 2011) and differentiate in response to the transcription factors neurogenin 3, Pax4, Pax6 (Schonhoff et al., 2004) and pancreatic-duodenal homeobox 1 gene (Yamada et al.,

2001). EEC subtypes vary in morphology and shape (Figure 8) but generally respond to nutrients (Gunawardene et al., 2011), acting as sensors of luminal content (Pizarroso et al., 2021). EECs feature apical cytoplasmic processes, with microvilli extending towards the luminal surface, secretory vesicles and basal processes (in the large intestine) that extend towards adjacent epithelial cells (Gunawardene et al., 2011, Sjolund et al., 1983). The apical processes allow them to make direct contact with the luminal contents, including nutrients (Feher, 2017). EECs respond to luminal contents, chemicals, toxins and micro-organisms (Latorre et al., 2016). In addition to nutrient sensing, EECs can act to co-ordinate defence responses against harmful substances via emesis, diarrhoea and food aversion (Latorre et al., 2016).

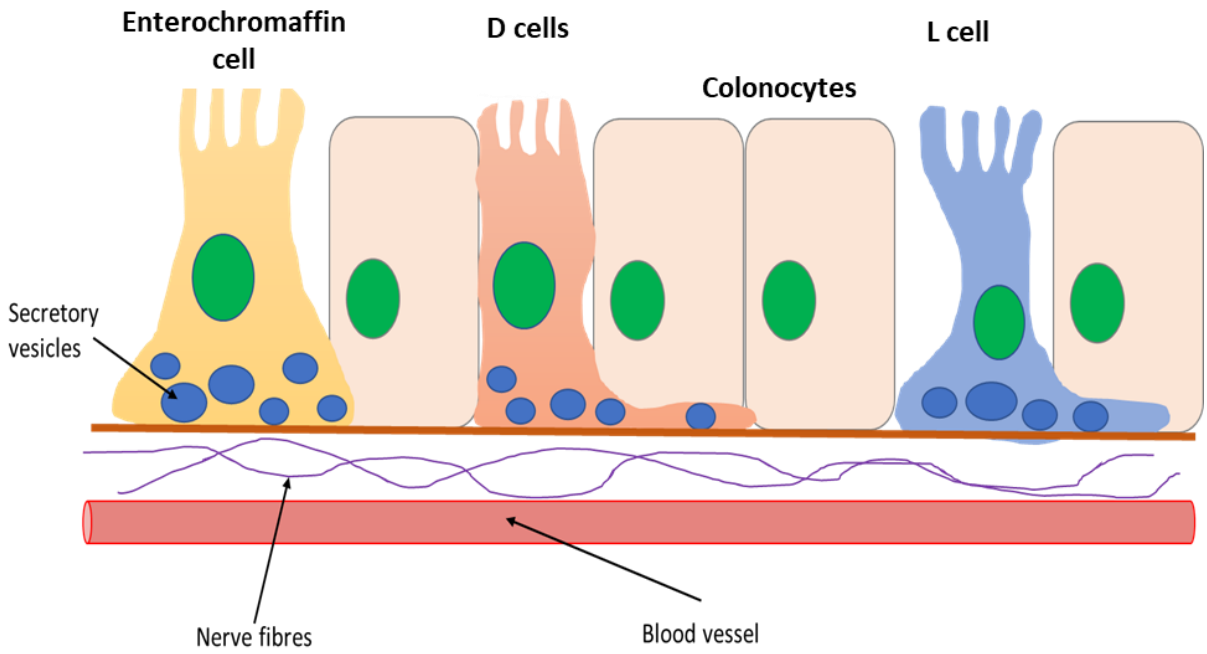


Figure 8: General morphology of various enteroendocrine cells (enterochromaffin (EC), D, L cells) within the colonic tissue.

EECs make up 1% of the intestinal epithelial cell population and are less common in the colon than in other GIT tissue (Sternini et al., 2008) (being lowest within the colon before rising again towards the rectum (Gunawardene et al., 2011)). Despite comprising only 1% of epithelial cells in the GIT, EECs form the largest endocrine system in the body (Reimann et al., 2012). EEC subtypes in the colon express various nutrient receptors specific to certain

nutrients, and once activated induce the release of hormones and mediators (Figure 9). These subtypes are not evenly distributed throughout the GIT (Gunawardene et al., 2011) (neither are nutrient receptors - Figure 7), perhaps reflecting different roles and functions for different parts of the colon. Though EECs are regularly classified by the expression of a specific hormone or GPCR, single-cell RNA-seq experiments demonstrate heterogeneity in expression (Haber et al., 2017), with EECs able to simultaneously co-express 20 varieties of gut peptides (Duca et al., 2021). EECs are not as plastic as once thought and there may exist different sub-types e.g., CaSR-positive L cells or CaSR-negative L cells etc.

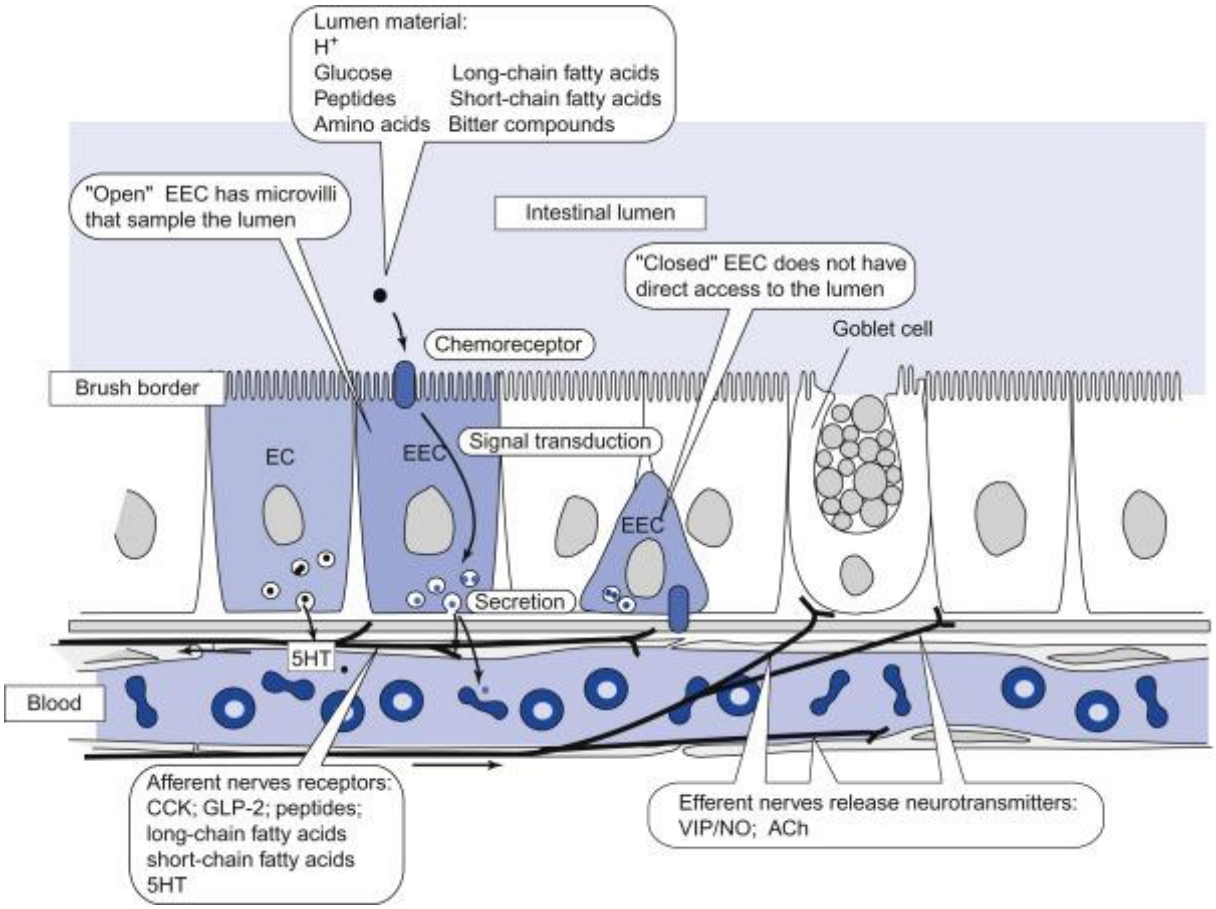


Figure 9: Functions and actions of nutrient-sensing EECs within the colon: sensing luminal nutrients, proteins on nutrient/ chemo-receptors (GPCRs), activation of intracellular pathways and secretion of hormones which can act on afferent nerves or travel through the circulation. Reprinted from (Feher, 2017).

EECs contain secretory vesicles – both large, dense-core vesicles and small clear vesicles. Secretory vesicle content release from EECs may be controlled by neural input; serotonin release from ECs in the rat ileal mucosa was shown to be mediated by adrenergic fibres in the vagal nerve, originating from the sympathetic ganglia (Ahlman and Dahlstrom, 1983). In rats, synapses between afferent neurons and EECs are observed, forming neuroendocrine complexes (Ahlman and Dahlstrom, 1983, Ahlman and Nilsson, 2001). EECs possess vesicle-containing cytoplasmic processes and extensions in CCK, PYY positive cells (though only 30-50%); named “neuropods”, these processes have been shown to make direct connections to nerves and sensory nerve endings (Bohorquez et al., 2015, Pizarroso et al., 2021). These studies offer a potential role of the CNS and central systems in the release of hormones and mediators from the gut. EECs have a role in the gut-brain axis; the vagal afferents that innervate the GI tract are in close proximity to the mucosal epithelium and are able to convey stimuli to the brainstem (Beyak et al., 2006). CCK released from EECs, and leptin, are able to act on receptors on these vagal afferents and activate them to reduce food intake and gastric motility (Peters et al., 2006).

The small clear vesicles within EECs, also called synaptic-like microvesicles (SLMVs), can transport amines across the vesicular membrane, against the concentration gradient, through biogenic amine transporters (Weihe and Eiden, 2000). The vesicular monoamine transporter (VMAT1) fulfils this role for serotonin in serotonin-secreting EECs (Weihe and Eiden, 2000). The contents of SLMVs are exocytosed from the basolateral side in response to cellular plasma membrane depolarisation caused by activation of transient receptor potential cation channel subfamily M member 5 (TRPM5), a cation channel that transduces the signals from sweet, bitter, savoury stimuli (310) on intestinal EECs and causes a rise in intracellular calcium levels (Ashcroft et al., 1994, Franklin and Wollheim, 2004). Hormones are exocytosed across the basal lamina, where they can affect vagal afferents in the lamina propria (Duca and Lam, 2014). This adds to evidence for the role of EECs in functioning as conveyors of electrochemical as well as hormonal signals.

1.5.1 L cells

L cells make up the second largest EEC population (after ECs) in the large intestine and have the highest expression in the colon (Sjolund et al., 1983). L cells are “bottle”/ “flask” triangular shaped cells, with apical processes protruding into the lumen and sometimes a basal process along the basement membrane (Gunawardene et al., 2011). These luminal processes feature microvilli and it is thought that this feature allows L cells to sense the presence of nutrients (Holst, 2007). The basal processes of L cells feature synaptic structures, express pre-synaptic proteins and enzymes for the neurotransmitter dopamine and can directly contact nerves (Latorre et al., 2016, Kaelberer and Bohorquez, 2018). L cells co-secrete the anorectic hormones PYY and GLP-1 (Habib et al., 2013), and indeed these two molecules have been found within the same granules within L cells (Holst, 2007). When isolated colonic cells were examined by flow cytometry, all cells that expressed PYY also co-expressed GLP-1, with the population of cells expressing only one hormone being insignificant (Habib et al., 2013). Fats, carbohydrates, and proteins have been shown to promote the release of PYY and GLP-1 (Duca and Lam, 2014). As previously discussed, MCR4 is expressed in the brainstem, hypothalamus and vagal afferent nerves, and it is also the second most highly expressed GPCR on PYY and GLP-1 containing L cells (Panaro et al., 2014). Mice with a loss of function mutation in the MCR4 are obese and exhibit hyperphagia and hyperinsulinemia (Huszar et al., 1997). The MCR4 agonist α-MSH given intraperitoneally, was shown to increase fasting plasma PYY and GLP-1 levels in mice (Panaro et al., 2014). The endogenous ligand of MCR4 in the colon, however, remains elusive (with γ-MSH, β-endorphin and adrenocorticotropic hormone likely candidates (Tanaka et al., 1982)) but could allow clarification of the MC4R-L cell satiety pathways.

GLP-1 release from L cells is significantly decreased in the morbidly obese (Holst et al., 1983), and this decrease is independent of free fatty acid levels (Toft-Nielsen et al., 2001). In addition, the L cell density of obese subjects is higher in those on a high-fat (>30%) diet, whilst high-fat diet mice had increased numbers of GLP-1 positive cells, but unchanged numbers of PYY positive cells in the colon (Aranias et al., 2015). Obesity and a diet associated with obesity can therefore affect both the expression and activity of L cells. As previously mentioned, GLP-2 is also released from L cells in response to nutrients and neural

signals (Brubaker and Anini, 2003) however, evidence of its role in obesity is lacking, though a study in obese mice showed that a GLP-2 agonist repaired abnormal gut permeability and inflammation (Cani et al., 2009).

L cells are likely to be the most important EEC sub-type involved in satiety and nutrient sensing, through the action of the two powerful anorectic hormones PYY and GLP-1 released from these cells. It is the action of these hormones and the nutrient interactions with L cells that are thought to be the main drivers of increased satiety post-RYGB surgery, as described earlier in the Obesity section.

1.5.2 Enterochromaffin cells

ECs are the most common EEC cell type in the GIT, making up >70% of EECs in the proximal large bowel (Ahlman and Nilsson, 2001) and producing 95% of the serotonin (5-hydroxytryptamine - 5-HT) in the body (Spiller, 2008). ECs are pyramidal in shape, often with apical processes that reach into luminal spaces (Gunawardene et al., 2011). ECs have been shown to be able to act as chemosensors, able to transduce signals from nutrient stimulation to primary afferent nerve fibres, via serotonin (Bellono et al., 2017). EC cells store and release serotonin, a neurotransmitter which responds to tastants (taste-stimulating molecules expressed in food) such as SCFAs, glucose, amino acids (AAs) and lipids (Reigstad et al., 2015, Bertrand and Bertrand, 2010, Martin et al., 2017). ECs express transporters for monosaccharides, salts, and receptors for tastants (T2R) such as glucose, artificial sweeteners and sodium, and secrete serotonin in response (Rasoamanana et al., 2012).

As previously discussed, serotonin is an important hormone for the modulation of satiety and food cravings, and several serotonergic drugs have seen some success in driving weight loss. Studies are unclear as to the exact role of serotonin-positive EC cells: some argue serotonin is released from ECs upon mechanical and chemical stimulation, whilst others argue that serotonin-positive neurons expressed centrally regulate gut motility (Latorre et al., 2016).

ECs and serotonin have been shown to play an important role in nutrient sensing and obesity. EC numbers are elevated in the duodenal mucosa of obese humans compared to

controls (Young et al., 2018), indicating a link between EC expression and nutrient intake (Peiris et al., 2018). The high-affinity, low-capacity glucose transporter GLUT1 is highly expressed in the colon despite limited glucose reaching the colon under normal conditions (Martin et al., 2017). This might indicate a very sensitive response to glucose that does reach the colon, for instance in an obese diet that consists of more glucose-rich foodstuffs.

EC cells and serotonin are likely to play a key role in satiety and obesity. Research has shown that EC expression is changed in obesity and their activity in releasing serotonin is also changed. Under normal conditions serotonin has been shown to act as an appetite suppressor and increases energy expenditure in brown adipose tissue (Morrison et al., 2014). However, in obesity there is generally an increased release of this satiety hormone (Yabut et al., 2019). Its role in regulating mood, appetite control and satiety in general could be altered in the obese state, or it could be that the body becomes resistant to the satiating effects of serotonin.

1.5.3 Tuft cells

Tuft cells are a chemosensory EEC-type with roles in colorectal cancer, metabolic signalling and type 2 immunity (Steele et al., 2016). They are a rare form of EEC (0.4 – 2% of total EECs) (Gerbe et al., 2016)) found in epithelial tissues such as the pancreas, lungs and GIT (mainly stomach). Tuft cells express nutrient receptors (and associated signalling proteins) which can detect sweet, bitter and savoury stimuli (Gerbe and Jay, 2016) as well as glutamate and aliphatic non-essential amino acids (Nelson et al., 2002). Tuft cells express α-gustducin which has been shown to be involved in incretin hormone release (Janssen and Depoortere, 2013), releasing GLP-1 in response to sugars and sweeteners (Jang et al., 2007).

Tuft cells in the stomach can detect bitter tastants as a defence against potentially toxic food (Harmon et al., 2021). This leads to a short-term increase in ghrelin which can temporarily increase appetite, followed by a sharp decrease in food intake which correlates with decreased gastric emptying (Janssen et al., 2011). Bitter agonists can also inhibit stomach contractions in order to prolong the presence of nutrients in the stomach, increasing early satiety and reducing overall food intake (Janssen et al., 2011).

In obese patients the expression levels of tuft cell chemosensory elements in the gastric mucosa are increased (α-gustducin, PLCβ2 and TRPM5) or decreased (TAS1R3), compared to non-obese subjects (Widmayer et al., 2012). Tuft cells release acetylcholine (ACh) when activated by the bitter/umami (savoury) taste transduction pathways (von Moltke et al., 2016). ACh is the primary neurotransmitter of the ENS (Reichardt et al., 2013), with roles in proliferation, mucus secretion, cytokine production and migration of cells within the epithelium (Keely, 2011). Studies have shown that obesity and type 2 diabetes are associated with lower levels of ACh, due to overexpression of ACh-degradation enzymes (Sfera et al., 2017). The expression of ACh in the gastrointestinal tract is mostly attributed to tuft cells (Eberle et al., 2013), therefore tuft cells are likely to be important cells in both nutrient sensing and interacting with the ENS. Studies in DIO obese mice have demonstrated increased concentrations of 5-HT and ACh compared to normal diet mice, and heightened levels of ACh have been shown to increase the responsiveness of myenteric neurons (Reichardt et al., 2013). It is possible that the increased gastric emptying and faster colonic transport observed in DIO mice could therefore be due to the role of ACh and 5-HT (Reichardt et al., 2013).

Tuft cells are probably a key EEC in obesity, with roles in nutrient sensing, inflammatory responses and controlling the composition of the microbiome, however they are not as important cells in regulating satiety, which is the focus of this study.

1.5.4 Nutrient-sensing G-protein coupled receptors

Nutrients in the gut are sensed primarily by GPCRs that specifically bind various nutrients. EECs in the colon express similar chemosensing receptors as those expressed in the mouth and tongue (Rasoamanana et al., 2012, Duca and Lam, 2014) – both CaSR and GPR120 are also expressed here (Keast et al., 2021). GPCRs are expressed in the plasma membrane of taste cells, with extracellular and intracellular domains binding nutrients and initiating intracellular signals, respectively (Kobilka, 2007). GPCRS have a hydrophobic seven-transmembrane domain motif (Conigrave and Brown, 2006) and are divided into classes A, B and C according to their sequence homology (Reimann et al., 2012). The rhodopsin-like family A includes receptors responsive to SCFAs; the secretin-like family B includes receptors

for GLP-1 and gastro-inhibitory peptide; the metabotropic glutamate family (C) includes nutrient/ taste receptors T1R, CaSR and GPRC6a (Reimann et al., 2012). Class C receptors can dimerise and indeed the receptors CaSR and T1R depend on this for their function (Reimann et al., 2012). Different GPCRs sense different stimuli that can be present in dietary products or products of bacterial digestion. GPCRs also bind circulating metabolites derived from the host intermediary metabolism, such as lactate and ketone bodies (Husted et al., 2017). Few medium chain fatty acids (MCFAs) and LCFAs reach the colon after digestion in the small intestine, with MCFAs in particular being rapidly hydrolysed (Greenberger et al., 1966); however, the presence of receptors for these fatty acids suggests that even relatively small amounts may have important roles in regulating satiety, and that therefore large changes in their presence in the colon (for instance due to dietary changes associated with obesity), might have large impact on satiety regulation.

The G-protein coupled T1R family taste umami (T1R1/ T1R3) and sweet (T1R2/ T1R3) (Rasoamanana et al., 2012, Li et al., 2002) stimuli. These taste receptors are expressed in the gastrointestinal tissue of rodents; T1R1, T1R2, T1R3, α-gustducin and PLCβ2 (phospholipase C: a signalling protein that generates diacylglycerol (DAG) and inositol trisphosphate (IP3)) in the intestine and T1R1, T1R3 and PLCβ2 in the stomach (Rasoamanana et al., 2012).

The T1R1/ T1R3 heterodimer is expressed in the gut and detects glutamate (mono-sodium glutamate form), aliphatic non-essential L-amino acids and monosaccharides like glucose, fructose, regulating their absorption (258). T1R1/ T1R3 is associated with CCK release through the gut-brain axis to regulate appetite and satiety (24). There is some evidence that the T1R2/ T1R3 heterodimer can control glucose homeostasis through the release of GLP-1 and activation of glucose transporters (24).

T2R/ TAS2R are a family of receptors encoded by 25 different genes, they detect bitter substances and are therefore believed to play a role in defending against external toxins (Latorre et al., 2016, Chandrashekar et al., 2000). This family is expressed on EECs in the gastrointestinal mucosa and activation of these cells releases ghrelin and activates CCK and Y2 receptors expressed on vagal neurons in response to bitter tastants (Latorre et al., 2016).

In rats, stimulation of intestinal TAS2Rs (hTAS2R5, hTAS2R14) leads to GLP-1 and CCK release and reduces food intake, with PYY secretion only apparent with stimulation of the hTAS2R39 receptor and with no effect on food intake (Grau-Bove et al., 2020). The effects of agonist action at TAS2R are therefore heterogenous, with different agonists promoting the release of different hormones.

The calcium sensing receptor CaSR and GPRC6a are also amino acid GPCRs that share both sequence and structural homology outside their tastants (though both sense calcium) (Rasoamanana et al., 2012). The Na^+–glucose transporter S-GLT1 in EECs is responsible for the absorption of glucose and galactose and the transporters GLUT-2 and -5 for fructose (Rasoamanana et al., 2012). Glucose can then potentiate the release of serotonin, PYY and GLP-1 in response to T1R2/ T1R3 sensing; blockade of the receptor in humans impairs their secretion in response to glucose (Rasoamanana et al., 2012). CaSR has been shown to be activated by kokumi ("rich" taste) compounds such as γ-glutamyl peptides (legumes, cheeses, fermented foods) (Keast et al., 2021) and leads to release of GLP-1 and CCK in a dose-dependent manner (Yang et al., 2019).

Activation of nutrient receptors by binding of ligands to the extracellular component of GPCRs causes a cascade of changes that leads to cell activation (Figure 10). Upon receptor-ligand binding, the membrane-bound, heterotrimeric, guanine nucleotide-binding G-proteins, containing α, β, and γ subunits (of which there are many variants), activate, dissociate and stimulate effector pathways (Reimann et al., 2012). G proteins are broadly characterised by the composition of their α subunits and the downstream signalling pathways involved: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (Figure 10) (Reimann et al., 2012):

- $G\alpha_s$ stimulates adenylate cyclase to increase the concentration of cyclic adenosine monophosphate (cAMP) within a cell whilst $G\alpha_i$ inhibits adenylate cyclase to decrease intracellular cAMP.
- $G\alpha_q$ stimulates PLC, leading to the generation of DAG and IP_3, which respectively activate protein kinase C and trigger Ca^{2+} release from intracellular stores.

- $G\alpha_{12/13}$ couples to the activation of the small G-protein Rho (Pfleger et al., 2019).

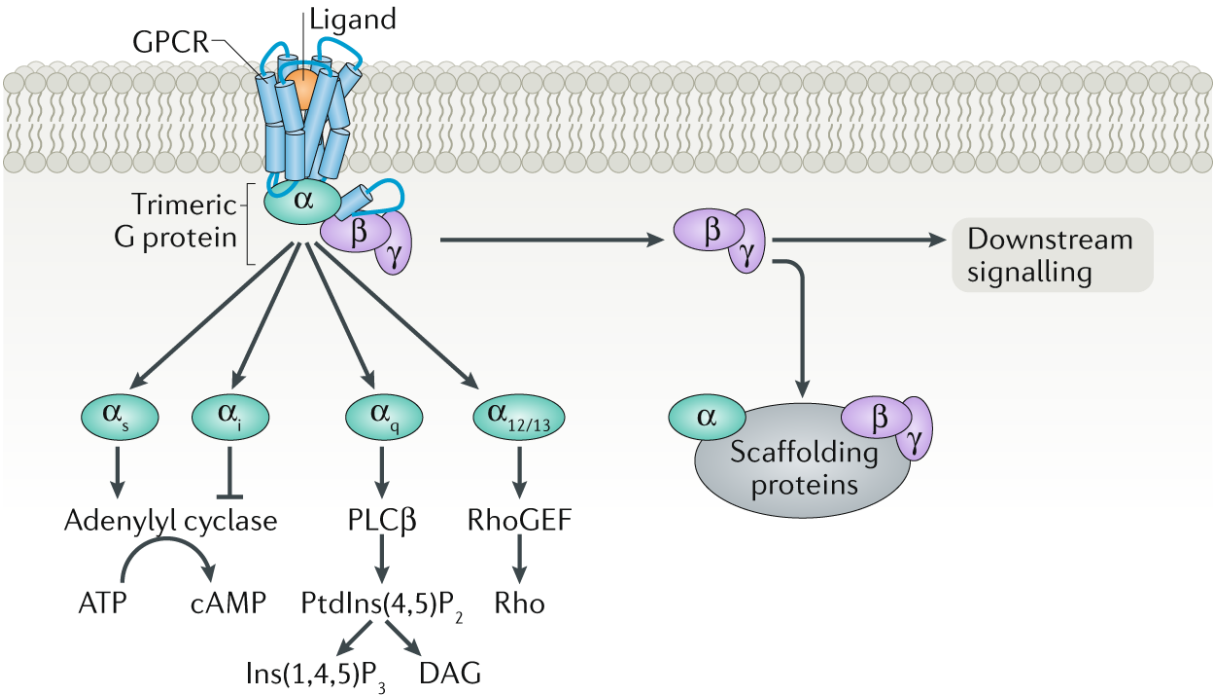


Figure 10: Downstream signalling pathways of GPCRs and families of G proteins. Activation of GPCRs by binding of ligands leads to the activation and dissociation of G proteins which activate and inhibit various internal pathways. Figure reprinted from Fig. 1 (Pfleger et al., 2019).

- **phospholipase Cβ (PLCβ)**
- **inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3; IP3)**
- **phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2).**
- **Rho guanine nucleotide exchange factor (RhoGEF)**

Receptors signal via different types of G-proteins depending on the ligand structure, binding site, membrane potential of the cell and oligomerisation of the receptor (Reimann et al., 2012). Additionally, oligomerisation of the receptor changes the affinity and avidity of ligands and the type of downstream signalling pathway activated (Reimann et al., 2012). Scaffolding proteins such as A-Kinase Anchoring Proteins and WD40 repeat proteins aid compartmentalisation of G proteins and the binding of additional proteins (Pfleger et al., 2019). These nutrient signalling pathways are further complicated by crosstalk between the

different pathways; activation of one receptor can cause the phosphorylation and subsequent desensitisation of another receptor (Reimann et al., 2012).

1.5.4.1 Short chain fatty acids and their receptors

SCFAs are the main by-products of saccharolytic fermentation of indigestible carbohydrates by bacteria of the microbiome (Morrison and Preston, 2016) and act as a local nutrient source as well as cell-signalling triggers (Tolhurst et al., 2012a), affecting blood glucose and lipid levels as well as immune functions (Wong et al., 2006). Cow milk is the main dietary source of SCFAs, but others include starches and fibre (Cook and Sellin, 1998). Their receptors are expressed on EECs (Tazoe et al., 2009), with agonist stimulation shown to cause the release of PYY and GLP-1 (Page et al., 2012).

1.5.4.2 Short chain fatty acids

SCFAs are generated from complex carbohydrates (polysaccharides, oligosaccharides, proteins, peptides, and glycoprotein precursors) by anaerobic microbial breakdown carried out by various microbial species (

Figure 11): humans lack the enzymes to process these compounds (and therefore they are resistant to hydrolysis and digestion in the stomach and small intestine (SI). The breakdown products include formate, acetate, propionate, butyrate and lactate (Macfarlane and Macfarlane, 2003) (typically of 1-6 carbon atoms in length (Schonfeld and Wojtczak, 2016)), gases and heat (Topping and Clifton, 2001).

While acetate is produced by a large number of different bacterial groups, propionate, butyrate and lactate are produced by a small number of organisms using specific substrates (Morrison and Preston, 2016). In the human colon, the *Firmicute* bacterial family is most important in propionate and butyrate production, with *Lachnospiraceae* a source of lactate (Louis and Flint, 2017). Acetate, propionate, and butyrate are present in millimolar

concentrations in the colonic lumen (60, 20 and 20 mM, respectively) (Ang and Ding, 2016b) and present equally across the colon, though this is more likely due to differing absorptive capabilities of different units of the colon (Topping and Clifton, 2001). Bacterial numbers and fermentation are highest in the proximal colon, where the least amount of nutrients have been absorbed (Macfarlane et al., 1992), with the amount of SCFA falling by around half in the distal colon (Topping and Clifton, 2001). The fermentation reactions that occur in the colon, and therefore the by-products created, are highly dependent on the chemical composition of the food source: starch fermentation yields acetate and butyrate whereas pectins and xylans have been shown to yield only acetate (Englyst et al., 1987).

SCFAs are absorbed by enterocytes which use them as a fuel source, and SCFA and MCFAs can diffuse across the colonic epithelium and into the blood stream where they can be metabolised by hepatocytes in the liver (Schonfeld and Wojtczak, 2016) and both can travel across the blood-brain barrier (Spector, 1988). SCFAs are a significant caloric source: ~10% in humans, therefore an increased intake of SCFAs may increase adiposity due to increase calorific intake (Bergman, 1990). In omnivores (including rodents), SCFAs can contribute 20-30% of total calories to a diet (Bergman, 1990), therefore SCFA diet-switching may have a greater effect on body weight in these animals. Changes in the SCFA satiety signalling however are most likely driven by changes in microbial populations. A high fat diet has been shown to promote changes in the microbiome: reducing the numbers of butyrate producing and carbohydrate digesting organisms (O'Keefe et al., 2015) (increased levels of Firmicutes and fewer Bacteroidetes species (Tremaroli and Backhed, 2012)) and this may drive a shift in the role of SCFAs.

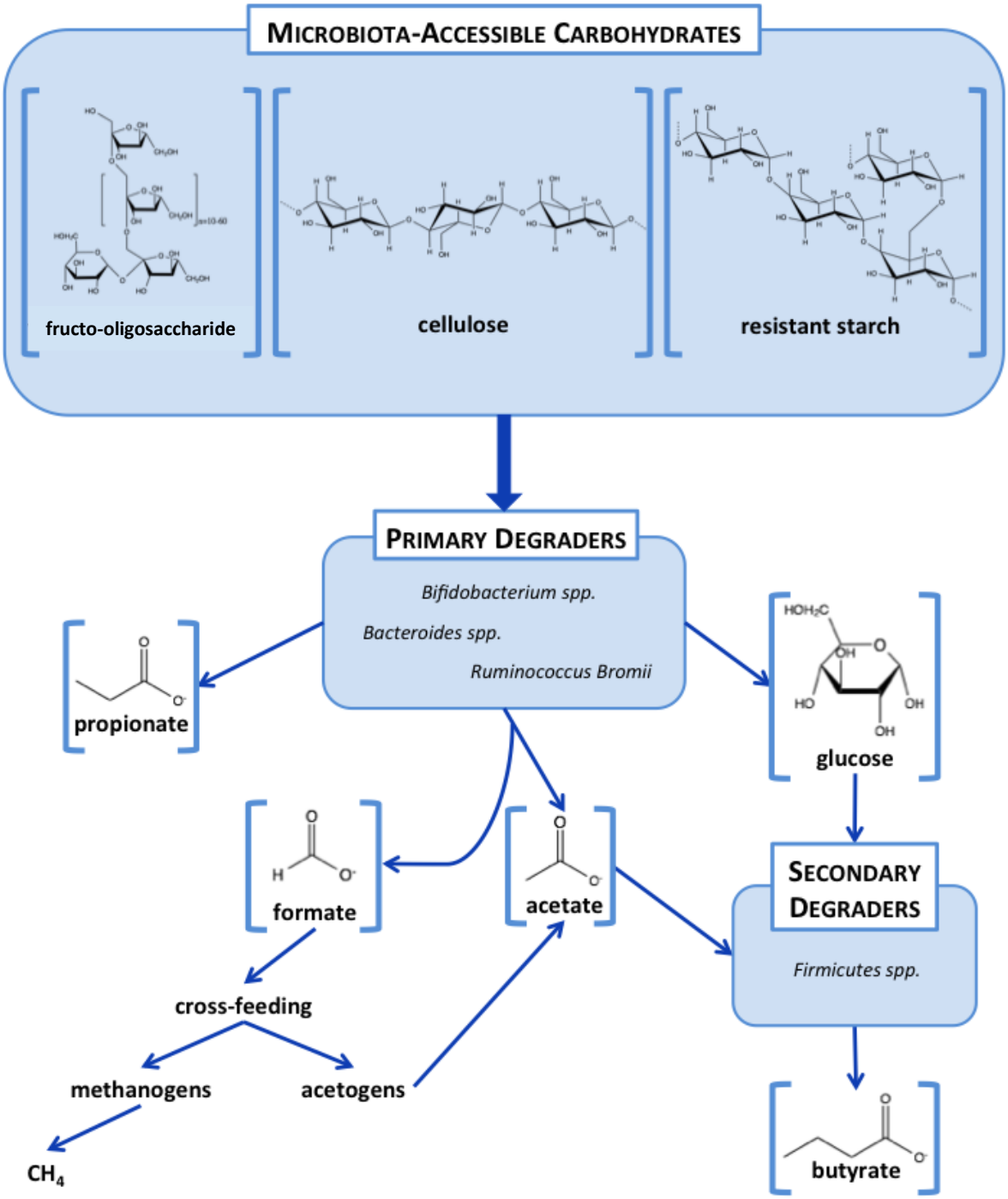


Figure 11: The breakdown of complex carbohydrates by gut microbiota converts these indigestible complexes into digestible nutrients such as the SCFAs propionate, formate, butyrate and acetate, as well as glucose, with carbon dioxide and hydrogen gases released as by-products. Reprinted and modified from (L. Keating).

SCFAs have an important role in satiety as well as obesity. Receptors for SCFAs are expressed on EECs and immune cells (Morrison and Preston, 2016) and SCFAs can act as anorexigenic factors (Larraufie et al., 2018). Acetate has been shown to regulate energy homeostasis; mice lacking the enzyme acetyl-CoA synthetase 2 (which converts acetate into an active form - acetyl-CoA) had 50% less ATP content in skeletal muscle cells, were significantly hypothermic and had reduced exercise capacity and reduced weight gain on a low-carb diet (Sakakibara et al., 2009). Propionate, though less common than acetate in the colon, is better absorbed and activated (Saunders, 1991, Read). Propionate promotes the release of PYY and GLP-1 and significantly reduced food intake in humans given acute dietary supplementation with a propionate ester, as well as increasing plasma PYY and GLP-1 levels (Chambers et al., 2015). The study authors also observed weight loss and decreased intra-abdominal fat and intrahepatocellular lipid content after 24 weeks of dietary supplementation (Chambers et al., 2015). Another study showed that an evening meal of fermentable carbohydrates (brown beans) significantly increased circulating levels of PYY whilst decreasing ghrelin levels at breakfast, compared to a white bread meal (Nilsson et al., 2013). Propionate studies in animals provide some evidence for cholesterol synthesis inhibition (Bush and Milligan, 1971), however, intervention studies in humans failed to provide a consistent result (Wong et al., 2006). Butyrate and the MCFA octanoate, have been shown to inhibit glycolysis and stimulate anabolic glucose formation (Schonfeld et al., 1988), lipogenesis (Takahide et al., 1983) and stimulate oxygen uptake and mitochondrial energization and activity in the rat liver, with octanoate increasing lipogenesis and glycogenesis 2 times more than lactate or pyruvate (Schonfeld et al., 1988). This might explain why SCFA- and MCFA-rich diets increase energy expenditure and decrease adiposity (Baba et al., 1982, St-Onge et al., 2003, St-Onge and Bosarge, 2008). Butyrate is the preferred fuel source of colonic epithelial cells; 70-90% of butyrate is metabolised by these cells (Topping and Clifton, 2001); as a cell energy source it is preferred even over circulating glucose or glutamine (Fleming and Floch, 1986).

There is evidence that certain SCFAs can prevent weight gain and improve energy homeostasis in mice on a high fat diet. Lu *et al.* demonstrated that DIO mice given dietary supplementation of acetate, propionate and butyrate were able to halt diet-induced body-

weight gain, and colonic mRNA expression of GPR43 and GPR41 decreased (Lu et al., 2016). In addition, mRNA expression of PYY, GLP-1 and leptin significantly increased in DIO mice, and dietary supplementation with propionate or with the three SCFAs was able to reduce the changes in the mRNA expression of PYY and GLP-1 and leptin, respectively (Lu et al., 2016).

Different meals and diets have been shown to affect both the microbial populations and their by-products. A diet switch study comparing African Americans against rural Africans showed major changes in the composition of the microbiome, SCFA synthesis and bile acid release (Holmes et al., 2012, O'Keefe et al., 2015). Those on a rural African diet were shown to have higher levels of starch degraders, carbohydrate fermenters and butyrate producers and their metabolites, compared to American diets. Rural Africans consuming their usual diet had significantly higher expression of butyrate synthesizing genes and faecal butyrate concentrations than African Americans; however, this was lost after switching to a high fat, low fibre diet (associated with a 'Western diet'). Americans also had reduced levels of butyrogenesis on a low fibre diet; these levels increased upon diet switch (O'Keefe et al., 2015).

Individuals who are obese or overweight have higher amounts of faecal SCFAs (Schwiertz et al., 2010) compared to lean individuals, which can lead to an overall higher energy intake and fat deposition. Increased levels of *Firmicutes* and fewer *Bacteroidetes* bacterial species have been seen in both obese mice and humans (Tremaroli and Backhed, 2012) which may lead to changes in the SCFA profile, with more butyrate and less formate, acetate and propionate being produced by their respective species. There is evidence that changed SCFA production and microbiome composition contributes to the reduced weight and adiposity seen after murine gastric bypass surgery, as *Bacteroidetes, Verrucomicrobia*, and *Proteobacteria* were enriched and greater propionate and lower acetate production was observed (Liou et al., 2013). SCFAs also have an important role in the cross-talk between the microbiome and the immune system, by conditioning the T-regulatory cell response (Morrison and Preston, 2016), inhibiting macrophage activation (Luhrs et al., 2002) and reducing gut inflammation (Vernia et al., 2000),(Vernia et al., 2003).

1.5.4.3 SCFA receptors

Receptors for short chain fatty acids that I will be examining as part of this study are GPR41, GPR43 and GPR109a. Activation of these receptors has been shown to trigger cell-signalling cascades: GPR41 and GPR43 activation by SCFA metabolites has been shown to release CCK, GLP-1 and PYY and act to increase satiety (Latorre et al., 2016).

GPR41 (encoded by the FFAR3 gene) is expressed on EECs, vagal afferents and mucosal enterocytes (Tazoe et al., 2009). Its main agonists are acetate, propionate and butyrate, and activation promotes the release of GLP-1, CCK and PYY by L cells (Page et al., 2012). GPR41 protein is translated from the bicistronic mRNA that also encodes GPR40, with an internal ribosome entry site switching between encoding of GPR40/ 41 (Halpern et al., 2012). There is evidence that GPR41 has roles in chronic inflammation and host-microbiome interactions (Ang and Ding, 2016b). Mice that are GPR41-/- have lower circulating levels of PYY and reduced calorie harvest efficiency from the diet (Samuel et al., 2008). GPR43 (encoded by the FFAR2 gene) is similar to GPR41 and is expressed on EECs; it acts as a receptor for propionate, pentanoate, butyrate, acetate, formate, and when activated releases PYY and GLP-1 (Page et al., 2012). Acetate, propionate and butyrate are strong agonists for GPR41 and GPR43 (0.5 mM EC50) (Brown et al., 2003), however, this is of much lower potency than for other GPCRs and their agonists – the CCR_2 chemokine receptor has an EC50 of 1 nM for its ligand CCL_2 (Thiele et al., 2011). This may restrict the activation of these receptors to only those locations where SCFA concentrations are at their highest, such as the gut lumen (Ang and Ding, 2016b) or during overfeeding. GPR43 expression has been shown to be modulated by inflammation; treatment of cells with the inflammatory molecules lipo-polysaccharide, tumour-necrosis factor and granulocyte-macrophage colony stimulating factor, raised GPR43 transcript levels (Ang et al., 2015, Senga et al., 2003). This might be of particular significance because, as discussed previously, obesity is associated with increased and chronic inflammation, so this might affect GPR43 expression and therefore PYY and GLP-1 release.

GPR41 and GPR43 share 42% sequence homology (Ang and Ding, 2016b) and have both been shown to be expressed on GLP-1 positive L cells in primary cultures, and mice lacking

these receptors had impaired glucose tolerance and GLP-1 secretion in response to SCFAs (Tolhurst et al., 2012a). The RNA for GPR43 has also been found enriched in ECs (Haber et al., 2017) and Peiris et. al have shown that GPR43 is expressed on human colonic ECs and L cells (Peiris et al., 2021). GPR43 and GPR41 were the only receptors that responded to acetate out of a library of 60 other characterised receptors, though acetate acts more strongly as an agonist (100-fold) on GPR43, with propionate stimulating both receptors equipotently (Le Poul et al., 2003). Acetate and propionate stimulation of these receptors in a cell line led to comparable intracellular calcium release; however, GPR41 did so via the $G_{i/o}$ and GPR43 via the $G_{i/o}$ and G_q G-protein subunits with both increasing phosphorylation of ERK1/2 (Le Poul et al., 2003). Both receptors activated intracellular pathways that led to inositol 1,4,5-trisphosphate generation, intracellular Ca^{2+} release, ERK1/2 activation, and inhibition of cAMP accumulation (Le Poul et al., 2003). When the receptors were stimulated with various SCFAs in a cAMP accumulation assay, using the CHO-K1 cell line, the order of potency of the agonists for GPR43 was propionate > acetate, butyrate, and for GPR41 it was propionate > isobutyrate > butyrate (Le Poul et al., 2003).

GRP43 and GPR41 knock-out mice have impaired L cell activity (Samuel et al., 2008, Tolhurst et al., 2012a) suggesting that these receptors are involved in peripheral mechanisms of satiety. GPR43 has been shown to be protective against DIO in mice in numerous studies, with GPR43 knockout mice having reduced SCFA-triggered GLP-1 secretion and impaired glucose tolerance (Tolhurst et al., 2012a, McNelis et al., 2015), increased lipolytic activity (Ge et al., 2008) and reduced glucose-stimulated insulin secretion (Priyadarshini et al., 2015). GPR41 and -43 have different effects in mouse and human models of human inflammatory diseases, with functional changes in signalling (Brown et al., 2003) – whether this effect extends to SCFA signalling is currently unknown.

GPR109a (encoded by the HCA2 gene) is expressed on EECs, enterocytes and colonocytes (Husted et al., 2017),(Wong et al., 2015) and is expressed at its highest level in the distal colon (Thangaraju et al., 2009). It specifically binds butyrate in the colon (Blad et al., 2012), making it an important mediator of the biological effects of butyrate (Singh et al., 2014). GPR109a is also a receptor for the vitamin niacin, produced by gut microbiota, which has been shown to suppress intestinal inflammation, with niacin being explored as an anti-

tumour, anti-inflammatory drug as well as having anti-lipolytic effects (Thangaraju et al., 2009). Niacin deficiency in humans causes intestinal inflammation, diarrhoea, dermatitis and dementia (Hegyi et al., 2004). GPR109a has also been proposed to increase glucose uptake by enterocytes (Husted et al., 2017).

To conclude, SCFA are the by-products of bacterial digestion, and activation of their related receptors GPR41, GPR43 and GPR109a has been shown to promote postprandial satiety via the activation of PYY and GLP-1.

1.5.4.4 Medium chain fatty acids and their receptors

MCFAs are fatty acids of typically 7-12 carbon atoms in length (Schonfeld and Wojtczak, 2016). These include capric/decanoic, caprylic/octanoic, lauric and myristic acids, and are commonly found in various plant oils (lauric acid makes up 50% of the fat of coconut oil (Xiang et al., 2019)) and some animal fats (Turner et al., 2009) ,(Zacek et al., 2019), with MCFAs making up 6-17% of fatty acids in (bovine) dairy milk (Marten et al., 2006).

1.5.4.5 Medium chain fatty acids (MCFAs)

MCFAs can form medium-chain triglycerides by the combination of MCFAs and glycerol, with octanoic and decanoic acid making up the majority (50-80%, 20-50%, respectively), and caproic and lauric acid being the minority (0.1-2%) (Figure 12) (Ferreira et al., 2014). These are hydrolysed in the small intestine into free fatty acids, and absorbed (Ferreira et al., 2014). MCFAs are a readily available source of mitochondrial energy, requiring neither hydrolysis to be absorbed across the intestinal lumen - as they are able to travel directly to the liver via the portal vein - nor specific transport enzymes to cross the mitochondrial membrane (Foster, 1984). For this reason, they may prove a better target for dietary intervention, by avoiding LCFA-driven lipid deposition in adipocytes. Dietary MCFAs have been shown to enhance thermogenesis and fat oxidation, thus reducing fat deposition in animal and human subjects (Nagao and Yanagita, 2010); they inhibit food intake and weight gain more effectively than LCFAs in rats (Turkenkopf et al., 1982) and mice (Zhou et al., 2017). Increasing chain length of MCFA is associated with improved reductions in food

intake and hunger in human participants given intraduodenal infusions of lauric acid (C12) or decanoic acid (C10) (Feltrin et al., 2004).

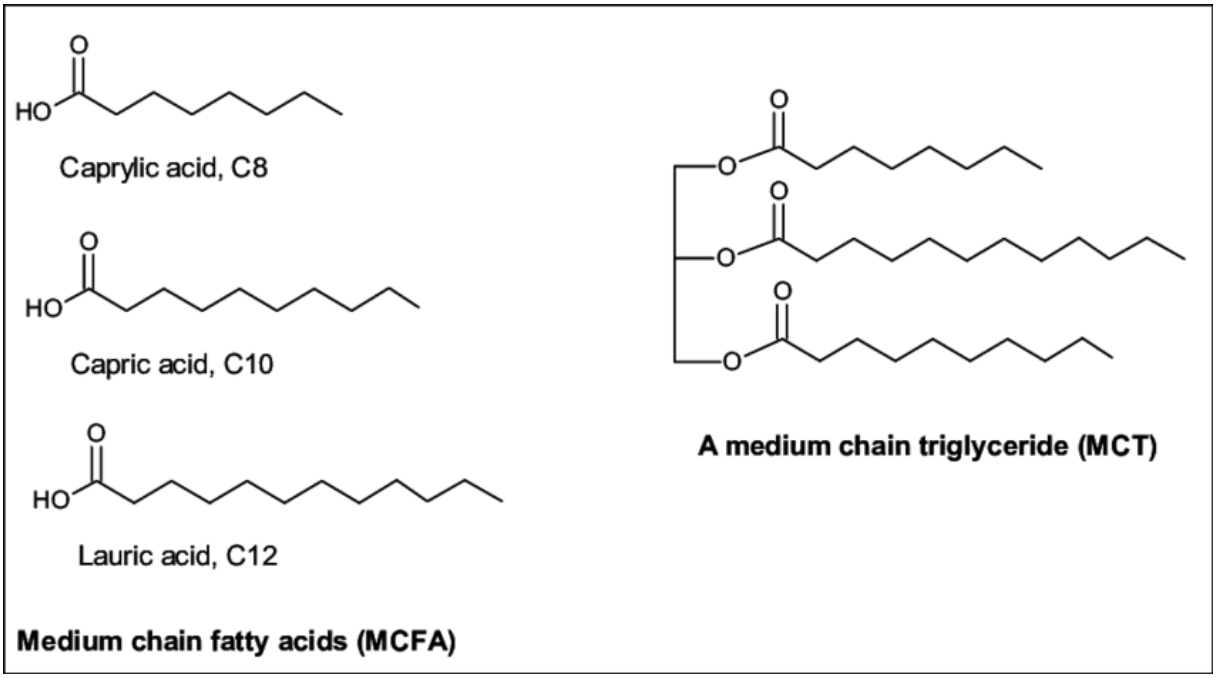


Figure 12: The medium chain fatty acids caprylic, capric and lauric acid can form the medium chain triglyceride structure, by combination with glycerol. C# refers to the carbon length of each fatty acid. Figure reprinted from (Cassiday, 2016).

MCFAs can be converted to ketone bodies in the liver: acetoacetate, 3-hydroxybutyrate and acetone (Morris, 2005), which in turn can be converted to ATP in the mitochondria (Sato et al., 1995) or acetyl-CoA in the brain (supplying 60% of the brain's energy requirements) or acetylcholine precursors in neurons (Morris, 2005). Ketone bodies provide a mechanism for the brain to access the body's fat stores: though fatty acids can cross the blood-brain barrier, the brain cannot oxidise fatty acids for energy (Morris, 2005). Diets high in protein and fat, but low in carbohydrates are often referred to as ketogenic diets due to their ability to produce high levels of ketones (Freeman and Kossoff, 2010). These diets have been shown to have therapeutic potential in epilepsy, as well as other neurological diseases, and their beneficial effects are most likely driven by altered neurotransmission, ketosis and reduced glucose levels (Fernando et al., 2015). Regarding obesity, a meta-analysis review by Bueno *et al.* showed that a ketogenic diet can achieve greater weight loss, improved blood

pressure and cholesterol levels in obese individuals, compared to a low-fat diet (Bueno et al., 2013). The authors postulated that an increased resting energy expenditure and a reduction in insulin levels and improved satiation, drove weight loss (Bueno et al., 2013).

MCFAs are transported via the portal venous system and are rapidly absorbed into the circulation and tissues; they promote satiation and increase energy expenditure, most likely via neuronal mechanisms (Haynes et al., 2020). In the PVN of the hypothalamus, MCFAs enhance α-MSH expression and neuronal activity, indicating activation of the hypothalamic melanocortin system (Haynes et al., 2020). POMC neurons in the hypothalamic ARC have been shown to express the MCFA receptor GRP40, and octanoic acid was shown to regulate the electrical excitability of these neurons, inducing differential membrane activity in different mice (depolarization/ hyperpolarization) (Haynes et al., 2020). The MCFA octanoic acid (or caprylic acid – found in milk (Beare-Rogers et al., 2001)) was shown in mice to rapidly cross from the gut to the hypothalamus where it is oxidised and increases POMC neuron firing rate (Haynes et al., 2020). This is likely to be the driver behind the subsequent (but transient) reduced food intake and increased energy expenditure observed (Haynes et al., 2020). In addition, the MCFA trioctanoic acid was shown in mice to be able to enter the brain and cerebrospinal fluid and rapidly oxidise, reducing food intake and increasing post prandial energy expenditure (Haynes et al., 2020). On the other hand, LCFAs (oleic, palmitic acid) were preferentially stored, rather than oxidised, by hypothalamic neurons, and this led to increased neuronal intracellular triglyceride storage (Haynes et al., 2020). Rats fed a high-fat diet had increased body weight and impaired glucose tolerance levels, but levels of fatty acids (SCFA, MCFA, LCFA) in the blood or spinal fluid were unchanged compared to normal chow fed rats (Haynes et al., 2020). A trial by Peiris *et al.* did not show a significant change in GLP-1 plasma levels in humans given a dietary MCFA nutrient treatment, however they did show a lowered calorific intake (~12%), and the authors postulated changes in circulating PYY levels and paracrine function of GLP-1, to be drivers of this change (Peiris et al., 2021). Another study gave 10 overweight human subjects a formula drink containing 72% MCFA and 22% n-3 long-chain polyunsaturated fatty acids (no other LCFAs). They demonstrated increased fatty acid oxidation and release from adipocytes, leading to a significantly reduced body weight and fat mass, compared to a LCFA-heavy formula (Beermann et al., 2003). The

main fatty acids of this formula drink were: caprylic acid (44.8%); capric acid (27.3%), and the n-3 fatty acids eicosapentaenoic acid (14.4%), and docosahexaenoic acid (5.6%) (Beermann et al., 2003). Obese individuals have low fat oxidation, and this can drive obesity (Larson et al., 1995), therefore being able to “rescue” fat oxidation by a dietary shift away from dietary LCFAs may prove an important clinical tool.

1.5.4.6 MCFA receptors

GPR40 (or FFAR1/ FFA1) and GPR120 are two receptors for MCFAs (Page et al., 2012, Peiris et al., 2018) that also show some affinity for LCFAs (Hirasawa et al., 2005), (Itoh et al., 2003b).

GPR40 (FFAR1 gene) belongs to the same family as GPR41, GPR43 and is expressed at high levels within the pancreas and brain (e.g. hypothalamus and amygdala) (Haynes et al., 2020, Freitas and Campos, 2021) and to a lesser extent within the intestine, on colonic EECs (Edfalk et al., 2008) (Briscoe et al., 2003). GPR40 is most strongly activated in the HEK293 cell line by the LCFAs docosahexaenoic acid, eicosapentaenoic acid and α-linolenic acid, and the MCFAs octanoic acid, decanoic acid and lauric acid, while SCFAs (<C6) failed to evoke a response (Briscoe et al., 2003). GPR40 activation triggers a signalling cascade via the $G\alpha_q$ subunit, activating PLCβ and the generation of IP3 and DAG and increasing intracellular calcium levels (Pujol et al., 2018a). MCFAs have a moderate affinity for the β-cell-expressed GPR40, with decanoic acid inducing glucose stimulated insulin secretion and indirectly increasing GLP-1 and GIP1 secretion in the gut (Briscoe et al., 2003).

Colonic EECs that express GPR40 have also been shown to express ghrelin, GIP1, GLP-1, CCK, PYY, substance P, serotonin, and secretin (Edfalk et al., 2008). In the colon, the EEC subtype I cells have been shown to express GPR40 and release CCK in response to dietary fat (Liou et al., 2011b). Activation of GPR40 has been shown to have beneficial effects on glucose homeostasis, by potentiating glucose-stimulated insulin secretion in pancreatic β-cells, and through increased GLP-1 and GIP1 secretion in the gut (Pujol et al., 2018b). Research observations are somewhat contradictory as to the role of GPR40 in energy regulation: some studies showed that mice without GPR40 were resistant to metabolic

diseases caused by a high fat diet (e.g. hyperinsulinemia, hyperglycaemia) (Steneberg et al., 2005) and showed reduced GIP and GLP-1 secretion and concomitant insulin secretion and glucose secretion (Edfalk et al., 2008); other studies did not support this (Lan et al., 2008); whilst others showed that mice that overexpressed GPR40 had improved glucose tolerance and were resistant to diabetes (Nagasumi et al., 2009).

GPR40 is highly expressed in the islets of the pancreas (2-100 times more than in the pancreas overall) in islet β-cells (Briscoe et al., 2003). The receptor is therefore likely to play a role in insulin production; genetically obese *ob/ob* mice have increased pancreatic GPR40 expression and display β-cell hyperplasia (Briscoe et al., 2003). Prolonged exposure of β-cells to high levels of fatty acids (due to an obese diet or insulin resistance) can reduce their ability to secrete insulin in response to glucose (Carpentier et al., 2000), and this may be driven by binding of these fatty acids to GPR40.

GPR40 is highly expressed throughout much of the brain, particularly in the substantia nigra and medulla oblongata (Briscoe et al., 2003), in areas associated with dopamine pathways (Zhang et al., 2017) and controlling autonomic activities, including digestion (2020). GPR40 could have a role in mediating satiation through mechanical digestive functions and control of food intake impulsivity. Octanoic acid activates POMC neurons in the arcuate nucleus via GPR40, as previously discussed (Haynes et al., 2020). GPR40 is co-localised with NPY and POMC-expressing neurons (Freitas and Campos, 2021); omega-3 fatty acids have been shown to trigger GPR40-dependent POMC-neurogenesis in obese mice to levels comparable to non-obese mice (Nascimento et al., 2016). GPR40 therefore has roles in regulating energy homeostasis in the periphery as well as centrally, in the CNS.

GPR120 is expressed on colonic epithelial cells (including ECs (Haber et al., 2017)), has MCFAs and LCFAs (including polyunsaturated omega-3 fatty acids - PUFAs) as endogenous agonists and when activated has been shown to cause the release of GIP, CCK and GLP-1 (Page et al., 2012). GPR120 activation also has other roles: lowering glucose levels in a GLP-1 dependent manner in mice (Sundstrom et al., 2017), adipogenesis (Gotoh et al., 2007), thermogenesis (Quesada-López et al., 2016) and anti-inflammatory effects in macrophages,

adipocytes, hypothalamic neurons (Ulven and Christiansen, 2015). The GPR120 agonist TUG-891 has been shown to be potent and selective (Hudson et al., 2013) and, along with lauric acid, is able to induce the Calmodulin-dependent protein kinase II (CaMKII) - but not pERK - pathway in L and EC cells, leading to PYY and GLP-1 release (Peiris et al., 2021). Abolishing GPR120 expression in the human STC-1 cell line impaired secretion of CCK and membrane depolarisation in response to fatty acids; CCK (Rasoamanana et al., 2012) and GLP-1 (Hirasawa et al., 2005) secretion is therefore dependent on GPR120 activation.

GPR120 expression in adipose tissue has been shown to be significantly higher in obese human participants compared to lean participants (Ichimura et al., 2012). Furthermore, a deleterious, inhibitory mutation in the GPR120 gene predisposes to obesity in European populations, most likely due to reduced intracellular calcium levels and GLP-1 secretion in response to α-linolenic acid (Ichimura et al., 2012). PUFA-enriched diets have been shown to lower ghrelin levels, increase CCK levels and lower hunger ratings (Polley et al., 2019) and flaxseed oil-enriched diets decrease body weight gain and food intake in obese mice and humans (Oliveira et al., 2015, Zhao et al., 2016). Obese mice fed a high fat diet with/ without PUFAs demonstrated reduced inflammation and enhanced insulin sensitivity after enrichment with PUFAs in the control obese group, but not the GPR120 knockout group (Oh et al., 2010). On a normal fat diet, GPR120 knockout mice had moderate insulin resistance, glucose intolerance and hyperinsulinemia, but no changes to food intake or bodyweight, whereas on a high fat diet, mice gained weight (Oh and Olefsky, 2012). This is likely due to hypothalamic GPR120 signalling and its anti-obesogenic effects: centrally, GPR120 activation reduced food intake and anxiety in mice in one study (Auguste et al., 2016); in another study in DIO mice, diet substitution with PUFAs (flax and olive oil – oleic and octadecatrienoic acid respectively) reduced hypothalamic inflammation and systemic insulin resistance and adiposity (Cintra et al., 2012). The latter study further demonstrated reduced food intake and body mass gain, reduced hypothalamic leptin and insulin resistance and increased expression of anorexigenic, thermogenic POMC and CART in obese rats following intracerebroventricular injection with LCFAs (linolenic acid, oleic acid) (Cintra et al., 2012). These effects were shown as GPR120-dependent in these studies, however, other studies showed no role for the receptor in appetite control (Dragano et al., 2017, Cintra et al.,

2012). GPR120 is expressed in NPY-positive cells in the ARC in rats, and upon PUFA stimulation, rapidly initiates intracellular signal transduction via scaffolding and binding proteins (Figure 10): this may act to counter leptin resistance characteristic of DIO (Cintra et al., 2012). In a recent study by the group, when obese humans were given GPR84 and GPR120 agonists (MCFAs: diindolylmethane, alpha linolenic and lauric acid) pre-prandially in capsules, they reduced calorie intake; the effect was most likely due to the observed increased levels of postprandial PYY (Peiris et al., 2021). Peiris *et al.* also observed no significant change in GLP-1, serotonin or ghrelin levels compared to the placebo group (Peiris et al., 2021), so it is likely that GPR120 activation does not change GLP-1 and serotonin activity.

Our group has also previously shown that the mRNA expression of GPR40 and GPR120 is up-regulated in colons of obese compared to lean mice, with GPR40 expression significantly increasing in in mice who had undergone gastric bypass surgery, whereas GPR120 expression did not significantly change (Peiris et al., 2018). A study in humans has also demonstrated increased expression of GPR40 and GPR120 in obese and overweight individuals, in the duodenum (Little et al., 2014).

1.5.4.7 Long chain fatty acids and their receptors

LCFAs include eicosatrienoic, eicosapentaenoic, oleic, linoleic, linolenic acid (Page et al., 2012) and docosahexaenoic acid (Nakamoto et al., 2013). These products are typically >16 carbon atoms in length (Beermann et al., 2003) and are expressed in sunflower oil (oleic, linoleic acids) (Cleland et al., 1996), walnuts and peanuts (linolenic, eicosatrienoic acids) (King et al., 2008) and fish oil (eicosapentaenoic, docosahexaenoic acids) (Turpeinen and Merimaa, 2011). The LCFA receptors I will be investigating in our studies are GPR40 and GPR120, as discussed above.

1.5.4.8 Long chain fatty acids (LCFAs)

LCFAs can be divided into saturated (SFA) and unsaturated fatty acids. The latter can be monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids. Dietary saturated fats occur in all fat-containing foods and typically have 12-18 carbon atoms (Nettleton et al., 2017); unsaturated fatty acids are typically found in plants, oils, nuts and seeds and are >18 carbon atoms in length (Chan, 2014) (Figure 13).

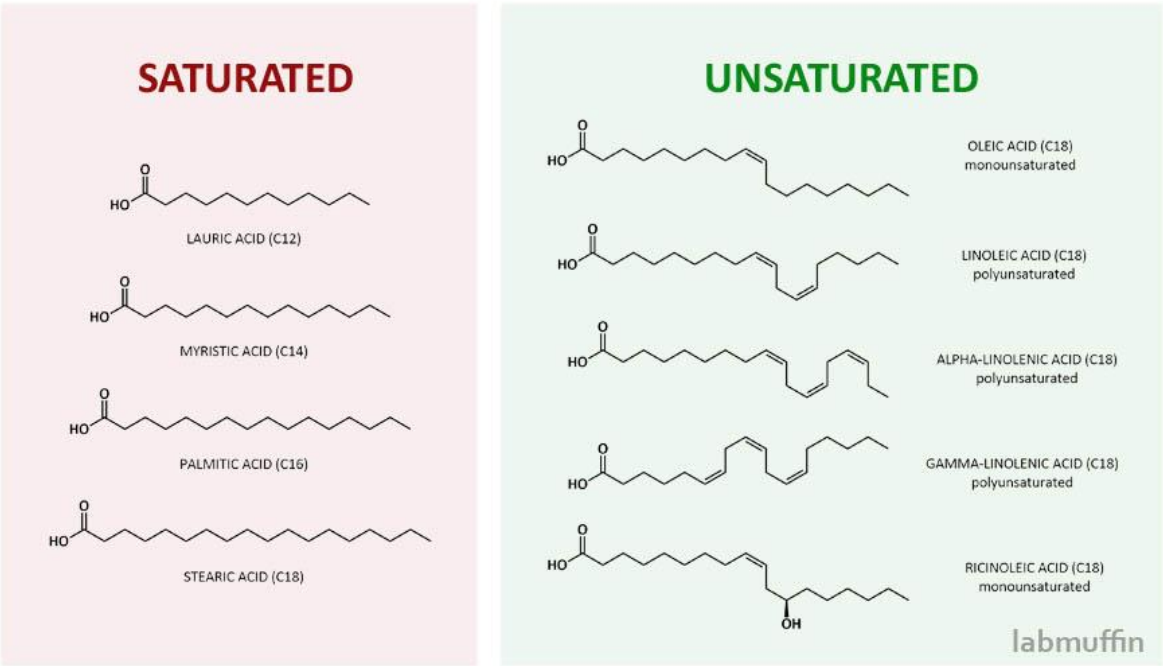


Figure 13: The structures of saturated and unsaturated LCFAs. C# refers to the carbon length of fatty acids. Of note are the double bonds in the chains of unsaturated fatty acids (=). Reprinted from (Wong).

LCFAs induce CCK release, with only fatty acids of chain lengths greater than C10 (10 carbon atoms) able to do so effectively (Matzinger et al., 2000). Stearic, oleic and linoleic intestinal infusions all increased plasma CCK and reduced food intake in human participants, with linoleic acid able to do so to the greatest extent (French et al., 2000). LCFAs also induce the release of PYY in rats (intrajejunal infusions) (Dailey et al., 2010) and humans (naso-ileal catheter) (Maljaars et al., 2009). The role of LCFAs in satiety and food preference in the human colon is, however, poorly understood. Despite the fact that PUFA-enriched diets are associated with reduced ghrelin and hunger levels and increased CCK plasma expression

(Polley et al., 2019) the expression of PYY, GLP-1 and CCK (Edfalk et al., 2008) or CCK and GLP-1 (Page et al., 2012) in GPR40, GPR120 positive EECs, respectively, is not increased.

Unsaturated fatty acids (e.g. linoleic acid, oleic, arachidonic and eicosapentaenoic acid) are more potent than saturated fatty acids (lauric acid) in stimulating the secretion of GI peptides, with saturation being more important than chain length (Rasoamanana et al., 2012). In rats, the presence of linoleic and oleic acids in sweet, salt, sour and bitter solutions can be detected and enhances these solutions' palatability compared to these same solutions without these free fatty acids (FFAs) (Pittman et al., 2006). This in turn may drive increased consumption of foods high in these FFAs and may drive food preference in humans; however, although healthy, fit humans have been shown to be able to detect fatty acids, they have so far not been shown to be attracted to their taste (Chale-Rush et al., 2007).

1.5.4.9 LCFA receptors

GPR120 and GPR40 are receptors for LCFAs (Nakamoto et al., 2013, Oh et al., 2010) and are expressed (GPR120 highly so) within the colon (Symonds et al., 2015).

GPR40 potentially has a role in GLP-1 and GIP release; mice who are GPR40$^{-/-}$ show a reduced release response of these hormones to dietary fat (Reimann et al., 2012). GPR120 is expressed on both L cells and EC cells (5 times higher) within the human colon (Peiris et al., 2021). In the STC-1 cell line, silencing RNA targeting GPR120 led to impaired α-linolenic acid-induced GLP-1 and CCK release; an effect which was not seen with siRNA against GPR40 (Reimann et al., 2012).

In mice, GPR40, GPR120 and GPR119 are highly expressed on GLP-1 positive cells of the small intestine, and co-stimulation of GPR40 and GPR119 - but not GPR120 - with triglycerides leads to increased GLP-1 release (Ekberg et al., 2016). Triacyl glycerols (TAGs) showed a mild reduction in the incretin response in GPR120 knockout animals in this study (Ekberg et al., 2016), whilst other studies in mice have shown that GPR120 does not affect GLP-1 release (Paulsen et al., 2014, Xiong et al., 2013).

The function of GPR120 and GFPR40 as LCFA receptors is generally not as well studied in humans or in the colonic setting (with much research focusing on the liver and adipocytes) - compared to their function as MCFA receptors, and there is a paucity of data compared to the other receptor agonists I will be examining.

1.5.4.10 Diet derived amino acids and their receptors

AAs are defined as organic substances with amino and carboxyl groups. AAs share a basic structure (Figure 14), differing only in the side chains (Figure 15). AAs (except glycine) can have L- and D-isoforms and most D-AAs can be converted into L-AAs by oxidases and transaminases (Baker, 2009). A lot of the research on the role of AAs in nutrition and diet (e.g., in growth and muscle/ fat synthesis) originates from studies in pigs or cows, due to the interests of the meat industry.

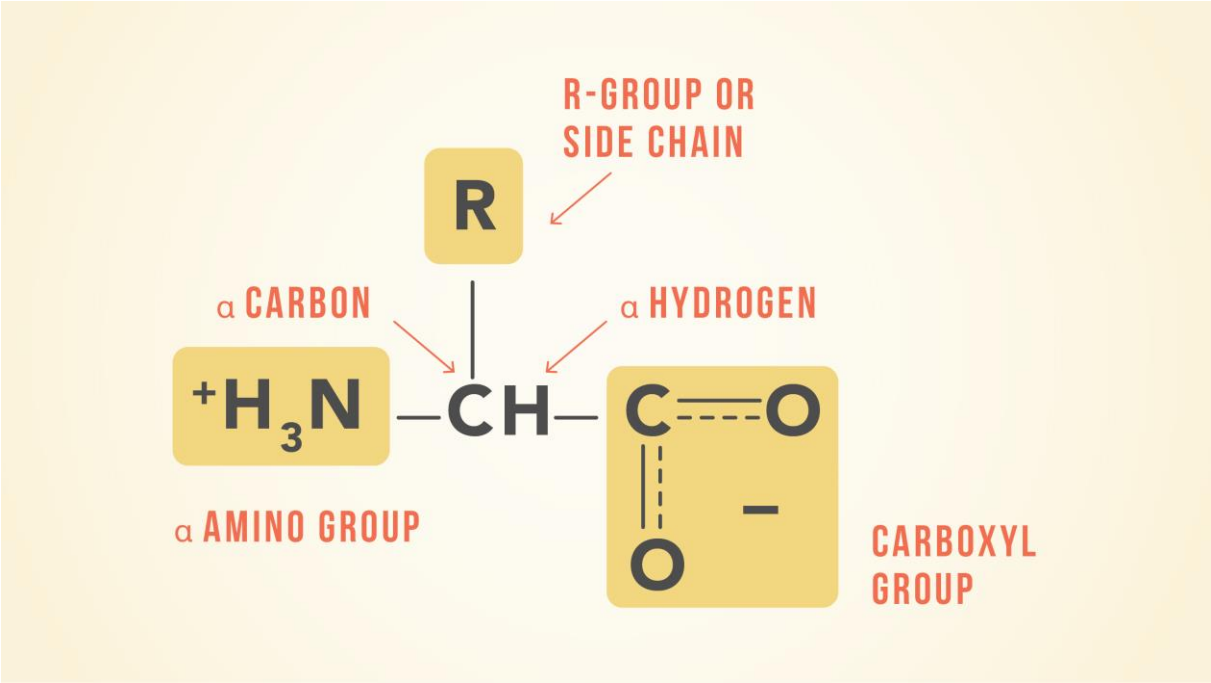


Figure 14: Amino acids share a common central structure of an amino and carboxyl group bound to a carbon and hydrogen centre, with a side chain providing variation. Reprinted from https://www.technologynetworks.com/applied-sciences/articles/essential-amino-acids-chart-abbreviations-and-structure-324357.

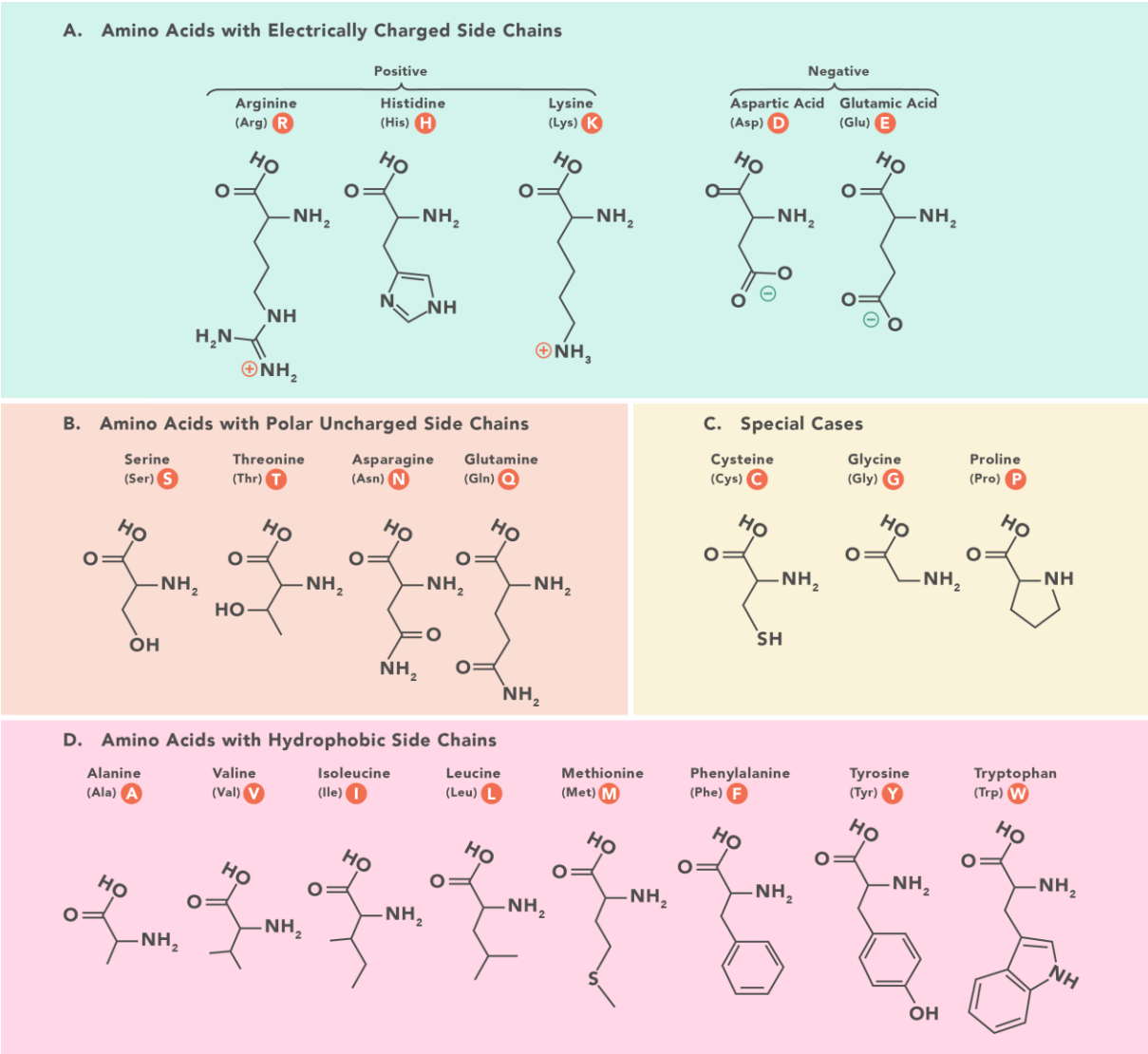


Figure 15: The structures and classification of amino acids. Amino acids can be subdivided by the properties of charge, hydrophobicity and polarity. Letters in red circle refer to the common acronym for each amino acid. Reprinted from https://www.technologynetworks.com/applied-sciences/articles/essential-amino-acids-chart-abbreviations-and-structure-324357.

1.5.4.11 Amino acids

AAs are required for the synthesis of proteins in the body, including hormones and neurotransmitters (Allowances, 1989). Animal (and plant) proteins are made up from around 20 common AAs (Allowances, 1989). AAs can act as cell signalling molecules, gene expression regulators, metabolic regulators and are involved in protein phosphorylation

cascades (e.g. the cellular processes regulating, the phosphorylation of the kinase mTOR1 by glutamine, arginine, leucine) (Wu, 2009). There are nine AAs that humans cannot synthesise naturally and so must acquire from the environment. These are the 'essential' L-amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Allowances, 1989). Some important AAs relevant to this current study, their functions and dietary sources are listed in Table 3.

Plasma levels of AAs change in obesity - levels of tyrosine, phenylalanine, alanine, glutamate and glutamine increase and glycine levels decrease in obese (insulin resistant) individuals (Newgard et al., 2009): these changes are associated with glycemia and insulin sensitivity (Wurtz et al., 2012). Higher plasma levels of phenylalanine, isoleucine, tyrosine and valine (Floegel et al., 2013) and decreased levels of glycine (Palmer et al., 2015) are associated with increased risk of type 2 diabetes. Plasma concentrations of glutamate, serine, glycine, correlated with hepatic insulin resistance and levels were higher in obese subjects; the authors postulated that this was driven by an altered activity of glutamate dehydrogenase (which converts leucine and glycine into glutamate) (Gaggini et al., 2018).

Table 3: Key amino acids, their function in relation to satiety, nutrition and homeostasis and their diet source and associated receptor(s)

Amino acid	Function/ effects	Dietal source	Receptors/Other targets
Phenylalanine	• Metabolised into tyrosine: precursor for dopamine, thyroid hormones (Kim et al., 2007) • CaSR-dependent increased GLP-1 release in murine STC-1 cells(Wang et al., 2019a)	meat, grains, dairy products and fish (Conigrave et al., 2000b, Górska-Warsewicz et al., 2018)	CaSR (Conigrave et al., 2000b)
Tryptophan	• Biosynthetic precursor of serotonin, melatonin (Kim et al., 2007)	meat, grains, dairy products and fish	CaSR (Conigrave et al., 2000b)

	• Biosynthesis of serotonin is catalysed by tryptophan hydroxylase: L-tryptophan > 5-hydroxytryptophan (Birdsall, 1998)	(Conigrave et al., 2000b, Górska-Warsewicz et al., 2018)	
Histidine	• Cell adhesion and migration • Complement activation, immune complex clearance, phagocytosis of apoptotic cells (Jones et al., 2005)	meat, grains, dairy products and fish (Conigrave et al., 2000b, Górska-Warsewicz et al., 2018)	CaSR (Conigrave et al., 2000b), Cationic amino acid transporter Slc7a (Hou et al., 2020)
Cysteine	• Glutathione, homocysteine and taurine production (Kim et al., 2007) • Stimulation of glucose, fatty acid oxidation (Li et al., 2009)	meat, grains, dairy products and fish (Conigrave et al., 2000b, Górska-Warsewicz et al., 2018)	CaSR (Conigrave et al., 2000b)
Glutamine	• Dietary supplementation increased expression of genes related to intestinal oxidative defence • Body weight gain (Wang et al., 2008) • Glutamate production • Epithelial cell fuel source (Wang et al., 2009) • Gluconeogenesis (Wu et al., 2007) • Insulin release from pancreatic β-cells (Newsholme et al., 2003) • Increases cAMP levels in a glucagon expressing cell line (GLUtag), induces L cell GLP-1 release (Nakamura et al., 2020)	animal protein (11%-22% of protein by weight) plant protein (40%) e.g. tomatoes, beets, strawberries, high gluten foods (Giacometti et al., 1979) MSG (additive) (Giacometti et al., 1979)	TAS1R3 (Nakamura et al., 2020)

Glutamate	• Precursor for intestinal synthesis of glutathione, arginine and proline (Reeds et al., 1997) • Alternate enterocyte fuel source (low glutamine) (Hasebe et al., 1999) • Dietary supplementation increases muscle protein synthesis and body-weight gain in pigs (Wu et al., 2007) • Central and peripheral neurotransmitter (Newsholme et al., 2003) • Immune regulation (Li et al., 2007) • Decreases cAMP levels in (GLUtag) cells (Nakamura et al., 2020)	Deamidation of glutamine by deaminase in the kidney and gut (Tapiero et al., 2002)	ionotropic glutamate receptors, e.g. N-methyl-D-aspartate receptor (beta cells) (Maechler, 2017) (STC-1 EECs) (Fukunaga et al., 2010)
Arginine	• Stimulates GLP-1 secretion (rat intestine) • Intragastric administration in DIO mice: increased insulin and GLP1-1 levels (Ding et al., 2018) • Increased nitric oxide oxidation of energy stores; reduced fat mass in obese, diabetic rats (Fu et al., 2005) • Decreased fat mass: long-term oral administration in adult obese humans, type II diabetes (Lucotti et al., 2006) • Improved systemic insulin sensitivity • Lymphocyte, T cell development (Newsholme et al., 2005) • High dietary levels can cause gut dysfunction (diarrhoea, villus atrophy) (Zhan et al., 2008)	Meat, bread, milk/ milk products (Oomen et al., 2000)	GPRC6a (Górska-Warsewicz et al., 2018, PubChem, 2019b)
Glycine	• Purine nucleotides, glutathione and haem synthesis (Li et al., 2007)	Meat (Wu, 2020), soy, pea protein (Liu et al., 2019), vegetables,	N-methyl-d-aspartate receptor (beta cells) (Maechler, 2017) (STC-

	• Anti-inflammatory in response to pathogen infection/ colitis (Li et al., 2007)	carbohydrates (Trefflich et al., 2020)	1 EECs) (Fukunaga et al., 2010)
Lysine	• Immunoprotective: cytokine production, lymphocyte proliferation (Li et al., 2007) • Competes with arginine for cellular uptake, can inhibit cellular activity of arginine, including nitric oxide synthesis indirectly (Wu and Meininger, 2002) • 30% of lysine oxidation occurs in the intestine (van Goudoever et al., 2000) • Insulin release from pancreatic β-cells (Newsholme et al., 2003) • Inhibits serotonin binding to 5-HT4 receptor: reduced anxiety, diarrhoea, stress in rats	Pea, whey casein proteins (Liu et al., 2019), wheat, corn, or rice, peanuts, beans (Matthews, 2020), meat/ meat products (Gorska-Warsewicz et al., 2018)	5-HT_4 receptor(Smriga and Torii, 2003)
Threonine	• Mucin synthesis and maintenance of gut barrier integrity (Bertolo et al., 1998) • Activates *Drosophila* EECs (Park et al., 2016)	Whey, casein proteins (Liu et al., 2019), meat/ meat products, grains, dairy (Gorska-Warsewicz et al., 2018)	Receptor not yet identified
Alanine	• inhibits pyruvate kinase (hepatic) (Meijer, 2003): regulates gluconeogenesis and glycolysis	Whey, pea protein (Liu et al., 2019), meat/ meat products, grains, dairy (Gorska-Warsewicz et al., 2018)	Receptor not yet identified

1.5.4.12 Amino acid sensing receptors

1.5.4.12.1 Calcium sensing receptor

CaSR binds calcium and the AAs phenylalanine, tryptophan, histidine and cysteine (Conigrave et al., 2000b) which are present in meat, grains, dairy products and fish (Conigrave et al., 2000b, Górska-Warsewicz et al., 2018), as well as other synthetic agonists (Villarroel et al., 2014). CaSR is expressed on L cells of the distal small intestine (Tang et al., 2016) as well as being widely expressed both on epithelial cells and neurons of the stomach and small and large intestine (Conigrave and Brown, 2006). In the stomach, CaSR has a role in controlling gastric acid, pepsinogen and mucus secretion release (Conigrave and Brown, 2006). In the colon, it is expressed on myenteric neurones and in the apical and basal aspects of epithelial cells of crypts (Chattopadhyay et al., 1998). CaSR activation alters fluid absorption and secretion via cAMP-controlled pathways (Chattopadhyay et al., 1998). Extracellular calcium has also been shown to inhibit colonic epithelial cell proliferation, likely through a CaSR-mediated mechanism (Kállay et al., 1997).

CaSR is a particularly large GPCR and has binding sites for calcium distinct from the binding sites for amino acids (Conigrave and Brown, 2006). Under a stable concentration of Ca^{2+} at 1 mM, the receptor acts as an aromatic L-amino acid receptor; below 1 mM, CaSR acts as a Ca^{2+} receptor (Conigrave and Brown, 2006). Therefore, CaSR is able to switch 'modes' to respond to AAs (allosteric activators) or elevated calcium levels and therefore alter the biological response (Conigrave and Brown, 2006). There is also some evidence that both calcium and AAs may act as co-agonists of CaSR (Conigrave et al., 2000a, Young and Rozengurt, 2002), with extracellular calcium shown to enhance L-tryptophan binding (EC50 0.5mM to 0.12mM). In response to high levels of plasma calcium, CaSR regulates circulating calcium by inhibiting the secretion of the parathyroid hormone (Villarroel et al., 2014) which regulates calcium homeostasis, bone regulation (Garrett and Emerson, 2008). Obese subjects have been shown to have a high sensitivity to calcium, with greater activity of the receptor and increased serum levels of parathyroid hormone (Hultin et al., 2010).

Activation of CaSR expressed on EEC cells has been shown to release gastrin, CCK and GLP-1 (Tang et al., 2016, Reimann et al., 2012). Additionally, L cells from mice deficient in

CaSR have impaired release of CCK and Ca^{2+} in response to L-phenylalanine (Reimann et al., 2012). CaSR expression is found in murine STC-1 cells and stimulation with L-phenylalanine increases CCK release, whilst a CaSR antagonist prevents CCK release (Hira et al., 2008). CaSR has a variety of roles in the colon; regulating secretion, absorption, motility, epithelial integrity, and the immune system (Tang et al., 2016) as well as maintaining calcium homeostasis in cells (Conigrave and Brown, 2006). Activation of colonic CaSR leads to a rapid increase in intracellular Ca^{2+} levels in surface and crypt cells, in a manner consistent with activation of GPCR pathways (Cheng et al., 2002). This increase can be blocked by inhibiting phosphatidylinositol-phospholipase C-inositol 1,4,5-trisphosphate (PI-PLC-IP3): this increase in intracellular Ca^{2+} is not caused by entry of calcium but by receptor mediated release from cell stores (Hebert et al., 2004). Crypt fluid secretion that occurs under normal conditions (Singh et al., 1995) can be reversed (crypt fluid secretion absorption) with activation of CaSR by forskolin (Cheng et al., 2004) and subsequent activation of calmodulin-sensitive phosphodiesterase activity, which metabolises intracellular cAMP (Hebert et al., 2004).

CaSR has a role in regulating satiety through postprandial secretion of GLP-1, GIP, and insulin, with gastric ghrelin-secreting cells also expressing CaSR (Tang et al., 2016). CaSR regulates the movement of fluid in the colon that aids digestion by regulating the absorption of NaCl and SCFAs (Tang et al., 2016). These secretions are mediated by the ENS, and CaSR agonists have been shown to function as inhibitors of ENS activity (Tang et al., 2016). Both oral and duodenal CaSR agonist (γGlu-Cys, protamine, and poly-d-lysine hydrobromide) administration to rats have been shown to attenuate glycaemic responses to an oral glucose tolerance test, with duodenal administration decreasing gastric emptying, most likely through the actions of CCK, GLP-1, and serotonin (Muramatsu et al., 2014). In mice, CaSR mRNA expression in the antrum of the stomach increased in high fat DIO mice compared to standard diet mice (Nunez-Salces et al., 2020); however, the role of CaSR in obesity in the human colon is less clear.

1.5.4.12.2 GPRC6a

GPRC6a is expressed on L cells (Oya et al., 2013) and binds the amino acids L-arginine and L-lysine, which are found in plant and animal proteins and dairy products (Górska-Warsewicz et al., 2018, PubChem, 2019b) and L-ornithine, found in wild rice and grapes (PubChem, 2019a). GPRC6a has a similar structure to CaSR and is also able to induce gastric acid and pepsinogen secretion in the stomach mucosa and pancreas (Rasoamanana et al., 2012). In addition, GPRC6a also shares a calcium binding site with CaSR; the two receptors differ mostly due to their AA agonists (Rasoamanana et al., 2012).

GPRC6a has been suggested to have roles in mediating extracellular calcium-sensing responses in tissues, and nutrient-sensing (Wellendorph et al., 2009). GPRC6a causes the release of GLP-1 and adiponectin, affecting insulin release, slowing gastric emptying and inhibiting glucagon release (Pi et al., 2017). GPRC6A null mice have organ-specific defects in glucose and fat metabolism and are more susceptible to high fat DIO (Pi et al., 2017), however there is no impact on body-weight on a chow diet, though adiposity is increased (Pi et al., 2008). It is likely that the receptor plays a role in energy metabolism: mice who are receptor-deficient demonstrated a 50% increase in voluntary wheel running when compared to wild-type littermate mice (Pi et al., 2008).

The exact roles of GPRC6a are unclear in humans, with single nucleotide polymorphisms in the gene for the receptor being associated with insulin resistance, cardiovascular disease and dementia (Pi et al., 2017).

1.5.4.13 GPR119

GPR119 is a GPCR for glycerols, it is expressed on L cells (Tough et al., 2018b), and activation has been shown to induce the release of GLP-1, PYY and GIP1 (Nunez et al., 2014, Tough et al., 2018b). GPR119 also senses lysophosphatidylcholine and fructose (Hansen et al., 2012, Gonzalez-Granda et al., 2018). GPR119 agonists have shown some value therapeutically in the treatment of T2D, lowering HbA1c levels, fasting and postprandial

glucose, and significantly increasing PYY plasma levels (with no change in GLP-1 levels) (Yamada et al., 2018).

2-monoacylglycerol is an agonist for GPR119, increasing cAMP levels of cells and increasing plasma levels of GLP-1 (but not PYY or CCK) in non-obese individuals (Hansen et al., 2011). Indeed, GPR119 has been shown to be just as important in sensing TAGs as GPR40 (with GPR120 playing a more minor role) as well as stimulating hormone release (Ekberg et al., 2016). TAG is formed from the esterification of a glycerol backbone with 3 fatty acids and their breakdown products stimulate GPCRs on EECs, with TAGs being hydrolysed in the gut into fatty acids and monoglycerides (Bayly, 2014). These fatty acids include LCFAs which are released by the digestion of TAGs by pancreatic lipase (Janssen and Depoortere, 2013), and as previously discussed these induce the release of hormones by stimulating GPR40 and GPR120 on EECs.

In a study by Tough *et al.*GPR119 activity in the human and murine colonic tissue has been shown to be both PYY- and glucose-dependent: a synthetic GPR119 agonist, 2-monoacylglycerol and oleoylethanolamide were able to activate (depolarise) L cells and slow colonic transit and this could be abolished by treating tissue with a Y_1 or Y_2 receptor antagonist, respectively (Tough et al., 2018b). Though Tough *et al.* did not directly measure PYY release (instead relying on PYY knockout mice tissue and Y_1, Y_2 receptor modulation experiments), they saw maximal response to GPR119 and MCR4 agonists (though reduced) in the distal and ascending colon regions (Tough et al., 2018b). These results contrast with those of Hansen *et al.* who saw no change in plasma levels of PYY (Hansen et al., 2011). Hansen *et al.* delivered oleic acid and 2- monoacylglycerol directly to the proximal jejunum, whereas Tough *et al.* bathed colonic biopsy tissue with solutions of the agonists. The absence of PYY plasma release observed by Hansen *et al.* could therefore be due to the observation by Tough *et al.* of a gradient of GPR119 activation from the jejunum (minimal) to the distal colon (highest), where GLP-1 responses are minimal in the mouse (Tough et al., 2018a) and in human tissue GPR119-induced GLP-1 responses were only present after blocking Y_1, Y_2 receptors (Cox et al., 2010).

1.5.4.14 Prostaglandin E receptor 4

Prostaglandin E_2 receptor 4 (PTGER4 or EP4) is a GPCR that senses PGE_2, which is a derivative of arachidonic acid (Yasui et al., 2015), a LCFA found predominantly in meat, especially chicken and beef, and also in eggs (Keim and Branum, 2015).

Arachidonic acid can be synthesized from linoleic acid and can also be derived from phospholipids (by the action of the enzyme phospholipase A2). It is metabolised by cyclooxygenases (COX) and PGE synthases into prostanoids, including PGE_2 (Yasui et al., 2015). Overexpression of COX-2 is associated with numerous malignancies; it is expressed 80-90% more in colonic cancer tissue compared to normal tissue (Voutsadakis, 2007). Prostaglandins consist of 20 carbon atoms and a 5-carbon ring and are expressed in almost all cells and tissues (Karpisheh et al., 2019) and can act in both a paracrine and autocrine manner (Karpisheh et al., 2019).

PGE_2 has four GPCRs: EP1, 2, 3 and 4 (Yasui et al., 2015), expressed on the surface of colonic epithelial cells as well as in intestinal macrophages and the lamina propria (Olsen Hult et al., 2011). Each EP receptor signals via distinct pathways (Fulton et al., 2006). EP4 stimulation induces the formation of $G_{\alpha s}$-$G_{\beta\gamma}$ complexes, the activation of adenyl cyclase and upregulation of cAMP, and PKA activation (Fulton et al., 2006). PKA can activate the cAMP response element binding protein, which leads to increases in the expression of c-fos, somatostatin and corticotropin-releasing hormone in HEK293 cells (Fulton et al., 2006). EP4 also stimulates the ERK pathway and inhibits NF-κB induction (Fulton et al., 2006). PGE_2 has been shown in mice to promote an anti-inflammatory macrophage phenotype, reducing their accumulation in adipose tissue and the secretion of the pro-inflammatory monocyte chemoattractant protein 1 (Yasui et al., 2015).

Obesity has been shown to promote the shift in macrophages from the anti-inflammatory M2 to the pro-inflammatory M1 state, which facilitates insulin resistance in adipose tissue (Yasui et al., 2015). Activating EP4 signalling increased the number of M2 macrophages and promoted a switch away from the M1 phenotype (Yasui et al., 2015). EP4 signalling therefore has an important role in mediating chronic inflammation - a common state in obesity and one that precedes the development of diabetes (Yasui et al., 2015). EP4

agonists have been shown to improve glucose tolerance and increase insulin sensitivity. This can lead to an amelioration of obesity-induced abnormal glucose tolerance and insulin resistance; however, there was no effect on body weight or food intake (Yasui et al., 2015).

1.5.4.15 G-protein-coupled bile acid receptor

The G-protein-coupled bile acid receptor (GPBAR1 - or TGR5) is a GPCR that detects bile acids. The EC50 potency rank is: taurolithocholic acid > lithocholic acid > decoxycholic acid > chenodeoxycholic acid > cholic acid (Guo et al., 2016b, Guo et al., 2016a). Linolenic (Katsuma et al., 2005b) and oleanolic (Sato et al., 2007) acid have also been shown to be weak TGR5 ligands. TGR5 is expressed throughout the GIT (Xie et al., 2021), stomach, liver and lungs (Guo et al., 2016a). TGR5 activation enhances serine/ threonine kinase phosphorylation, increases NO production (Kida et al., 2013), activates the mTOR pathway (Perino et al., 2014) and antagonises the NF-κB family pathways (Guo et al., 2016a).

Bile acids are acidic sterols synthesised from cholesterol (and conjugated with glycine/ taurine) in the liver and stored in the gall bladder (Russell and Setchell, 1992). Bile acids are released into the gut lumen by the gall bladder in order to emulsify dietary lipids (Reimann et al., 2012), allowing for their transport and digestion (Xie et al., 2021), with around 95% being reabsorbed back from the intestine (Trefflich et al., 2020) and transported back to the liver (enterohepatic circulation) (Russell and Setchell, 1992). Bile acids have a role in signalling via GPBAR1 expressed on colonic EECs (especially L cells (Reimann et al., 2012)), as well as cells in the small intestine, liver, lung and stomach (Guo et al., 2016b). Bile acid activation of TGR5 rapidly increases cAMP production (Maruyama et al., 2002).

Bile acids have also been found in the CNS and PNS; in the PNS they have been shown to regulate energy intake (Xie et al., 2021). GPBAR1 activation induces cAMP production which increases the responsiveness of L cells to glucose and increases GLP-1 release in the human colon (Reimann et al., 2012, Adrian et al., 1993) and the murine EEC line STC-1 (Katsuma et al., 2005b). Postprandial bile acid levels have been correlated negatively to ghrelin and positively to PYY and GLP-1 levels (Xie et al., 2021). Poole *et al.* proposed that TGR5 is important in mediating the ileal brake by delaying gastric emptying, releasing GLP-1 and activating inhibitory neurons (Poole et al., 2010). Obese individuals have higher levels of

plasma bile acids (Xie et al., 2021) and oral administration of bile acids increases PYY release and decreases appetite in obese humans (Roberts et al., 2011), substantiating the hypothesis that the obese body is deficient in bile acids – not resistant. Patients who have undergone gastric bypass surgery have higher levels of serum bile acids than pre-surgery obese and non-obese individuals, and this is evident 2-4 years after surgery (Patti et al., 2009). Serum levels of bile acids are inversely related to postprandial levels of triglycerides and glucose and levels of GLP-1 and insulin secretion are also increased in bypass patients (Patti et al., 2009). Mice given a high fat diet with cholic acid had increased energy expenditure in brown adipose tissue and were resistant to weight gain and protected from insulin resistance (improved glucose tolerance), compared to mice on a high fat diet without cholic acid (Patti et al., 2009). These effects are driven by increased cAMP production from the binding of bile acids to TGR5 (Patti et al., 2009).

The cell line STC-1 was shown to strongly release GLP-1 in response to TGR5 stimulation, in a dose-dependent manner (Katsuma et al., 2005b). In the human colon, TGR5 stimulation has been shown to increase levels of plasma PYY (Adrian et al., 1993). GPBAR1 has also been shown to have an anti-inflammatory role during LPS induction and cellular injury, by supressing Toll-like receptor 4 activation in mice, including gastric inflammation. In human monocytes, however, GPBAR1 activation enhanced the inflammatory profile of these cells (Guo et al., 2016b).

Farnesoid X receptor (FXR) is another bile acid receptor expressed in the ileum of the SI and regulates bile acid synthesis and transport (Jiang et al., 2021), and treatment of obese mice with a FXR antagonist reverses high-fat diet-induced and genetic obesity, insulin resistance and hepatic steatosis (Jiang et al., 2015). For this review, we will be focusing on GBAR1.

1.5.5 Intracellular pathways activated by nutrient sensing GPCRs

pERK and CaMKII are cell activation markers of EECs, as demonstrated by Symonds *et al*. (Symonds et al., 2015). They become phosphorylated after GPCR activation, causing changes in secretion, gene expression and cell metabolism (Belcheva and Coscia, 2002).

GPCRs are phosphorylated by serine/threonine kinases such as protein kinase A and C, as well as by GPCR kinases (Lefkowitz, 1998). There is evidence that different ligands induce differential phosphorylation; different phosphorylation patterns of the β2-adrenergic receptor by different GPCR kinases induce different cellular functions (Nobles et al., 2011). This is termed the "barcode" hypothesis and may allow the same nutrient receptor to respond in drastically different ways to different ligands by phosphorylating at different cellular locations and timepoints.

CaMKII is activated by increases in intracellular calcium caused by its release from intracellular stores upon activation of phospholipase C, an enzyme activated by GPCR activation (Figure 10)(Pfleger et al., 2019). Increased numbers of CaMKII and pERK immunoreactive cells (markers of cell activation) were observed in the mouse colonic mucosa after stimulation with the CaSR agonists phenylalanine and tryptophan (Symonds et al., 2015), as well as GPR40, TGR5 agonists (Peiris et al., 2018). This was also observed in human colonic tissue exposed to lauric acid (a nutrient demonstrated to stimulate hormone release), with increased numbers of immunoreactive pERK cells (Symonds et al., 2015). pERK and CaMKII immunoreactive cells were further observed uncommonly within crypts and more commonly towards the apical surface of mucosal villi, where mature cells are more likely to be exposed to nutrients (Symonds et al., 2015, Peiris et al., 2018). pERK and CaMKII activity were also shown to be cell-specific, with serotonin-containing cells (ECs) activating the CaMKII pathway, and PYY-/ GLP-1-containing cells (L cells) activating the pERK pathway (Peiris et al., 2021). Peiris *et al.* demonstrated that human EECs stimulated with the GPR120 agonist TUG891 did not change levels of pERK, but did increase CaMKII – this same effect could not be replicated with lauric acid (GPR84 agonist) (Peiris et al., 2021). This further indicates the existence of specific phosphorylation pathways for different GPCRs, that might lead to different downstream effects. Peiris *et al.* have shown that different nutrient stimuli can cause the release of different mediators from EECs in colonic biopsies; lauric acid activated PYY, GLP-1 and serotonin release, whereas TUG891 activated PYY and GLP-1 release, with a combination of lauric acid and TUG891 increasing PYY and GLP-1 release 2-4 times more than either nutrient alone, without an increase in serotonin release (Peiris et al., 2021). GPCRs are therefore able to activate distinct and parallel intracellular signalling

cascades in response to multiple stimuli and this may aid in the translation of the composition of a nutrient load in the colon into a GLP-1, PYY or serotonin response.

To conclude, nutrients bind selectively to GPCRs, causing cell activation and the release of mediators, hormones or peptides. Nutrients can be categorised based on their chemical components and the nutrient receptors that they preferentially bind to, and act as agonists upon.

1.6 Study aims and hypothesis

1.6.1 Hypothesis

Obesity is often caused by an increase in food intake as well as a change in diet. In the GIT this leads to changes in the activation of nutrient receptors as well as mechanosensory systems. As previously discussed, the composition of the diet is able to alter satiety: for example, in obese humans, increasing the MCFA/ PUFA (GPR40/ GPR120 agonists) intake decreased calorific and increased postprandial levels of PYY (Peiris et al., 2021). Meanwhile GPR43 and GPR41 knockout mice have impaired glucose tolerance and GLP-1 secretion in response to SCFAs (Tolhurst et al., 2012a).

Looking at the evidence in the literature, we therefore hypothesis that overstimulation of nutrient receptors negatively affects nutrient sensing in the colon, driving reduced satiety and increased food intake. We also expect that altered expression of nutrient receptors and subsequent changes to cell activation and release of hormones and peptides, or a combination of these factors, act to reduce satiety and are a key pathophysiological feature in the obese state.

1.6.2 Aims

The overall aim of this study was to elucidate the effects that overstimulation of nutrient receptors, a feature of obesity, have on nutrient sensing in the colon: on EEC phenotype expression, expression of nutrient receptors and the ability of cells to be activated by nutrients.

Specific aims:

1. Examine nutrient receptor expression in a cohort of human subjects with varying BMIs
2. Investigate how enteroendocrine cell type expression/ phenotype changes with BMI
3. Examine how activation of enteroendocrine cells is changed with nutrient receptor stimulation
4. Examine the ability of EECs to release mediators in response to cellular activation

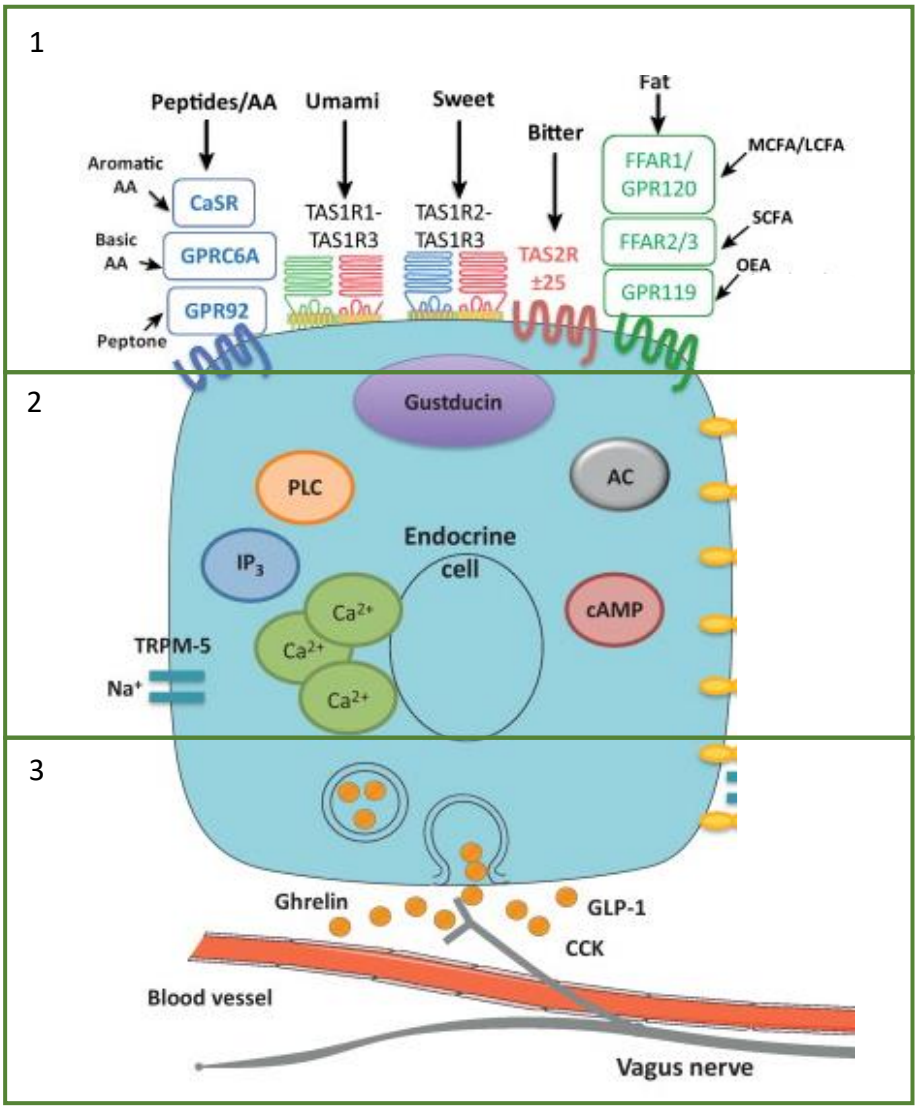


Figure 16: Aims visualised within an EEC. Adapted from (Janssen and Depoortere, 2013).

Aim 1: Examine nutrient receptor expression in a cohort of human patients with varying BMIs.

Aim 2: Examine how EEC expression changes with BMI

Aim 3: Examine how activation of EECs is changed with BMI

Aim 4: Examine release of mediators (hormones, neuropeptides) by cells in the colon in response to varying BMI.

2 Chapter 2: Characterisation of the mRNA and cellular expression of nutrient-sensing receptors and appetite-regulating hormones, peptides in the colon of healthy vs. overweight/ obese individuals

2.1 Introduction

Obesity is associated with increased calorie intake and blunted post-prandial release of satiety hormones: circulating PYY (le Roux et al., 2006b, Alvarez Bartolome et al., 2002) and GLP-1 (Muscelli et al., 2008, Ranganath et al., 1996) are reduced post-prandially - though evidence for GLP-1 levels is less clear, with some studies showing no change (Knop et al., 2012, Vilsboll et al., 2003), and changes in other hormones like CCK remaining uncertain. Obesity is associated with a change in the composition of ingested foodstuffs and nutrients, usually being biased towards junk/fast foods - low in fibre but high in carbohydrates (Leech et al., 2015) – and an increased intake of SCFAs (Schwiertz et al., 2010).

Most studies looking at nutrient sensing in the GIT either do not focus on the colon or use animal, not human, tissue. The GPCRs GPR40 and GPR120 have been shown to be more highly expressed in the pancreas (Briscoe et al., 2003) and colon (Peiris et al., 2018) of obese mice and the adipose tissue of obese humans (Ichimura et al., 2012). GPR41, GPR43, GPR40, GPR120, GPR119 have been shown to be more highly expressed in the colons of DIO mice (Peiris et al., 2018, Lu et al., 2016). Data on expression of these GPCRs in the human colon is however, lacking. Previous work by the group has shown that humans express, at the mRNA level, GPR120, GPRC6a, GPR40, GPR41, GPR43, TGR5 and CaSR in the colon and that PYY and GLP-1 co-express with GPR41 in mice and CaSR in humans (protein level analysis) (Symonds et al., 2015). This previous study also demonstrated that murine colonic mucosal cells have increased expression of pERK and pCaMKII upon stimulation with phenylalanine and tryptophan (Symonds et al., 2015). However, how expression of these GPCRs, hormones, activation markers might change with obesity was not studied. Understanding how the expression of nutrient receptors might change with changing BMI is critical for the development of new treatments for obesity: if a GPCR is downregulated, the body might be resistant to treatment with its ligand.

Though plasma levels of satiety hormones have been shown to be altered in obesity, changes at the cellular or mRNA level are not well studied in the human colon. Part of the difficulty in studying hormone expression is their bioavailability. GLP-1 and serotonin are produced from the translation and post-translational modifications of the glucagon/ pro-glucagon gene (into GLP-1, GLP-2 in the intestine) (Holst, 2007, De Silva and Bloom, 2012) and the activity of the TPH1 enzyme, respectively (France et al., 2016, Watanabe et al., 2010), therefore there is no direct way of measuring their mRNA expression. In addition, mRNA expression and hormone release are often not correlated: there is evidence that serotonin plasma levels are increased in obese individuals without a resulting change in colonic TPH1 mRNA expression (Young et al., 2018). In the case of leptin, it is mostly produced in the adipose and stomach tissue (Zhang et al., 1994) rather than the colon. For this reason, I measured the expression of its receptor, which is expressed in colonic tissue (Hardwick et al., 2001) and may drive leptin resistance in the obese state (Fogteloo et al., 2003, Heymsfield et al., 1999). Though plasma levels of PYY and GLP-1 have been shown to be reduced in obesity (le Roux et al., 2006b, Alvarez Bartolome et al., 2002, Muscelli et al., 2008, Ranganath et al., 1996), even 12 months after weight loss (Sumithran et al., 2011), information on their expression at the genetic level is lacking.

Understanding any changes in the expression phenotype of hormone/ peptide expressing cells (e.g., PYY-expressing L cells, serotonin-expressing EC cells) is important in understanding why plasma levels of a hormone might change, or in the case of leptin, why a higher plasma post-prandial release of a hormone does not lead to increased satiety. Decreased cellular mRNA and protein expression of both hormones and nutrient receptors could drive reduced ligand-receptor signalling at nutrient receptors, reduced cellular activity and a corresponding reduction in hormone release and plasma levels. It is therefore important to assess mRNA and protein expression simultaneously to elucidate the signalling pathways from GPCR to release of mediator.

To address these knowledge gaps, I examined changes in the expression of GPCRs, hormones and peptides at the mRNA and protein level between overweight/ obese and non-obese individuals. Our aim was to characterise any changes to the molecular mechanisms that regulate appetite via nutrient-sensing GPCRs expressed on hormone-

expressing EECs. Based on previous studies, I hypothesised that the mRNA and cellular expression of satiety hormones (PYY, GLP-1, serotonin) will be lowered in obesity, and will be associated with altered expression of nutrient-sensing receptors.

A paper entitled “Effect of Obesity on the Expression of Nutrient Receptors and Satiety Hormones in the Human Colon” was published in Nutrients, 2021, based on the findings from this chapter.

2.2 Methods

2.2.1 Human Tissue Collection

Tissue specimens were collected from two centres: The Royal London Hospital, in London, the UK, and the Maastricht University Medical Center (MUMC), in Maastricht, the Netherlands.

In London, non-inflamed, non-cancerous (morphologically normal), full thickness and mucosal samples were taken from the ascending, transverse and descending colon of patients (N=23) undergoing gastrointestinal cancer surgery(NREC 09/H0704/2). Full thickness samples were immediately fixed upon collection in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.45) at 4°C overnight.

In Maastricht, biopsies were taken from the right sided proximal and sigmoid colon of patients with irritable bowel syndrome (IBS) (N=30) participating in the Maastricht IBS cohort study and undergoing routine colonoscopy (NL24160.068.08 / METC 08-2-066). In addition, biopsies were taken from the sigmoid colon of healthy controls (N=7) participating in an interventional study (placebo group). Inclusion and exclusion criteria of the Maastricht IBS cohort and the healthy control group are described elsewhere (Tigchelaar et al., 2017a). Biopsies were placed in Eppendorf tubes and immediately flash frozen in liquid nitrogen to be stored at -80°C until RNA isolation. From these patients I obtained cDNA from sigmoid and proximal colon samples (sigmoid colon N=43, proximal colon N=24), except for healthy controls for whom only sigmoid samples were obtained.

All participants gave written informed consent prior to inclusion. The studies were approved by the East London and The City HA Local Research Ethics Committee [NREC 09/H0704/2] and the University of Maastricht Medical Ethics Committee, respectively, and were performed in compliance with the revised Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, 2013). The Maastricht study is registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT00775060). Patient and healthy control characteristics are presented in Figure 17 and Figure 18.

A

	Female		Male	
N: (%)	10	(33%)	20	(67%)
Age: mean, +/- SD (years)	62.4	±19.2	61.5	±18.1
BMI: mean, +/- SD	26.2	±4.1	25.7	±5.6

B

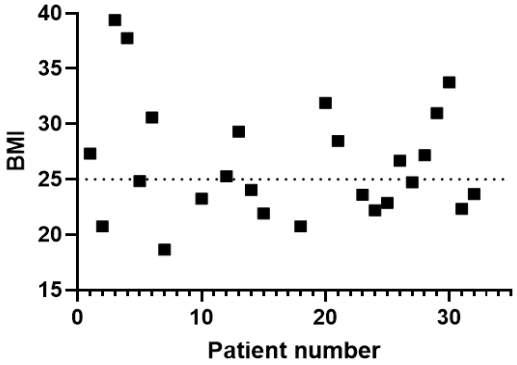


Figure 17: Patient demographics of patients from the Royal London Hospital. A: Patient demographic information (N=32). B: BMI (body-mass index) data from individuals from whom colonic tissue was collected is plotted alongside their assigned patient number. Dotted line signifies the clinical cut-off for obesity.

A

	Female		Male	
N: (%)	47	69.1%	21	30.9%
Age: mean, +/- SD (years)	44.8	±16.2	34.7	±8.9
BMI: mean, +/- SD	25.8	±5.2	24.3	±5.2
IBS subtype: N, (%)				
Constipation	18	38.3%	0	0%
Diarrhoea	11	23.4%	2	9.5%
Mixed	15	31.9%	9	42.9%
None (healthy control)	3	6.4%	10	47.6%

B

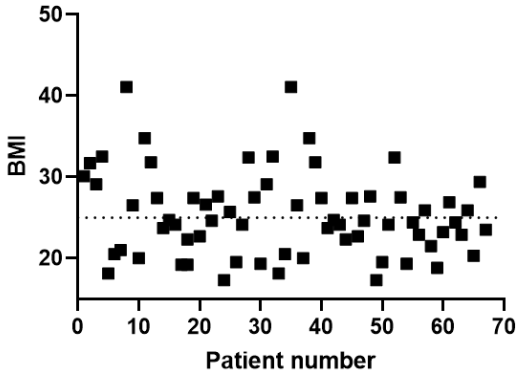


Figure 18: Patient demographics of patients recruited at the University of Maastricht, NL. A: patient demographic information (N=67). B: BMI data from individuals from whom colonic tissue was collected is plotted alongside their assigned patient number. Dotted line signifies the clinical cut-off for obesity.

2.2.2 Gene expression studies

Quantitative real-time reverse transcriptase PCR (RT-PCR) was used to assess the relative expression of nutrient GPCRs and hormones/peptides in human colonic tissue.

2.2.2.1 RNA extraction

In London, RNA was extracted using a RNeasy Mini kit (74106 Qiagen). Whole tissue was obtained as previously described and blended with a TissueRuptor (Qiagen) in buffer RLT solution (provided with kit, lysis buffer). The resulting tissue homogenate was spun for 3 minutes at 1300 rpm to remove debris. The solution was then spun through provided spin columns (15s, 1300 rpm) and DNAse 1 enzyme added (1/8 ratio with buffer RDD – aids column DNA digestion), to remove genomic DNA contaminants, for 15 minutes at room temperature. The solution was washed with buffer solutions by spinning (30 s, 13000 rpm) the column until finally RNA solution was spun out of the column with 15 µl RNAse free water. RNA quantity and quality were assessed using NanoDrop (DS-11, DeNovix). 1 µl of RNA solution was pipetted onto a Nanodrop machine which calculated the ng/µl of RNA in the solution as well as the ratio of sample absorbance at 260 and 280 nm. A concentration of 800 – 1000 ng/µl and 260:280 ratio of 1.8-2.2 was deemed optimal.

cDNA from Maastricht colonic biopsies were sent to London for qPCR analysis. Their protocol, in brief, was to isolate total RNA from frozen biopsies using TRIzol reagent (Invitrogen, Carlsbad, USA) and then purify with the RNeasy Plus Mini Kit (see above for details). The quantity and purity of the RNA samples was determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). A concentration greater than 5 µg/µl total RNA was used for subsequent PCR experiments.

2.2.2.2 cDNA conversion

In London, RNA samples were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Thermo Fisher Scientific). Briefly, a master mix of RNA, genomic DNA wipe-out solution and water were incubated for 2 minutes at 42°C. To this master mix was added reverse transcriptase enzyme, buffer and

primer mix and this was incubated for 15 minutes at 42°C, then 95°C for 3 minutes. Converted cDNA was then immediately used in subsequent experiments.

RNA samples from Maastricht were reverse transcribed using MultiScribe Reverse Transcriptase (Thermo Fischer Scientific). RNA was diluted x100 with RNAse free water and amplified: each reaction contained 12.5 µl SYBR Green Supermix, 1 µl of 10 µM gene-specific forward and reverse primers, 4 µl diluted cDNA template and 5.5 µl sterile water. cDNA was shipped at -20°C and stored at -80°C in London.

2.2.2.3 High-throughput qPCR array cards

cDNA from biopsies obtained in Maastricht were analysed using TaqMan PCR (Thermo fisher TaqMan Array Micro Fluidic card, Cat# 4342249). For each patient, the relative gene expression of genes of interest and 2 positive controls (18s ribosomal RNA (18s) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated for each plate. These positive control genes are constitutively expressed in all cells: 18s is a component of ribosomes that are common to all eukaryotic cells; GAPDH is an enzyme involved in glycolysis (Barber et al., 2005).

50 µl of Taqman PCR universal master mix II (4440043, containing: AmpliTaq Gold DNA Polymerase, dNTPs (with dUTP), ROX Passive Reference, optimized buffer components), primers and 50 µl of cDNA (30-100 ng/ µl) were mixed in a tube, with each gene primer prepared in a separate Eppendorf. Reservoirs on the plate were filled with 100 µl of this mix (Figure 19).

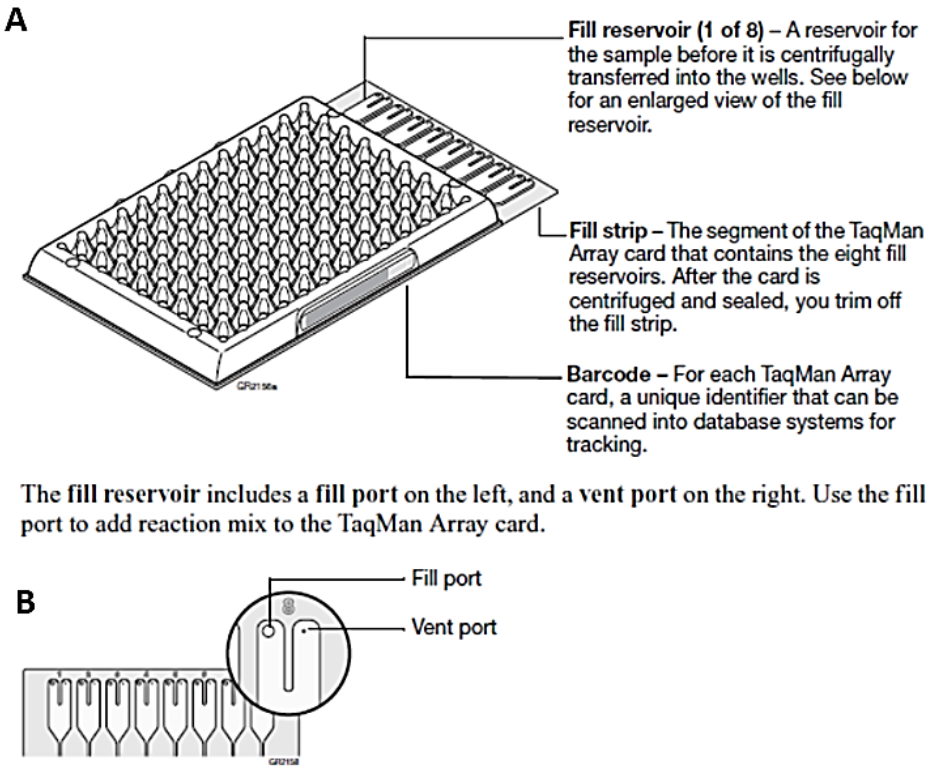


Figure 19: Diagram of Taqman array plate (A) and mechanism for filling wells via fill ports of reservoirs (B). Figure reprinted from TaqMan® Array Micro Fluidic Cards user guide, Applied Biosystems (Part Number 4400263 Rev. C 07/2010).

The filled card was then centrifuged (1200 rpm, 1 minute) and sealed. Plates were run on a ViiA7 machine using the following cycling parameters: 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of 15 seconds of 95°C, then 1 minute of 60°C.

2.2.2.4 Single reaction qPCR

For genes with limited amplification using ViiaA7 qPCR, individual PCR expression studies were performed using Quantitect primers (Qiagen) with SYBR green RT-PCR. To limit failure of detection due to relatively low abundance in tissue, a higher concentration of cDNA was used. Primers were optimised by running plates with three cDNA concentrations (25 ng, 50 ng and 75 ng) from biopsies obtained from the Royal London Hospital. A concentration of 75 ng of cDNA was selected for all studies as amplification was detectable

at this concentration compared to the lower concentrations. SYBR green kits were purchased from Qiagen (Table 4) and target gene expression was determined relative to the endogenous control, 18s ribosomal RNA, using the comparative cycle threshold method, on an Applied Biosystems StepOnePLus real-time PCR system thermal cycling block. In addition to a housekeeping gene control, negative control wells which contained primer and SYBR green master mix, but no template cDNA, were used for each PCR run.

Briefly, 25 µl/ well of a reaction mix was prepared: cDNA (75 ng/ well); 12.5 µl Quantifast SYBR green master mix; Quantitect primer assay 2.5 µl (reconstituted in 10 mM Tris.Cl, 1mM EDTA); RNase-free water. 25 µl of this reaction mix was added to each well of a 96 well PCR plate (4346907, Thermo Fisher). The plate was spun down and run on the cycler: 5 minutes initial activation step at 95°C, then 40 cycle repeats of 10 s at 95°C (denaturation) followed by 30 s at 60°C (annealing and extension of primers, cDNA).

Table 4: Primers for gene expression studies

Gene/ target	Manufacturer	Product code
Cholecystokinin	Qiagen	QT00073871
Cholecystokinin vb (CCK B)	Qiagen	QT02405228
Cholecystokinin va (CCK A)	Qiagen	QT02405221
Calcium-sensing receptor (CaSR)	Qiagen	QT00055944
GPRC6a	Qiagen	QT01033102
IFN-y	Qiagen	QT00000525
18s ribosomal RNA	Qiagen	QT00199367

2.2.2.5 mRNA expression data analysis

The relative expression of mRNA from the sigmoid and proximal colon was plotted separately against BMI. BMI values were used to divide patients into normal weight patients (BMI<25) and obese/ overweight patients (BMI≥ 25). The patient group of BMI <25 are all those patients below or equal to 24.9 BMI. Sigmoidal and proximal colon samples were analysed separately. Table 5 shows the number of samples and the colonic location for each gene.

Table 5: Distribution of patient samples by gene examined by Taqman qPCR

Proximal		Sigmoid
A (n=6, n=6)	GPR43	F (n=9, n=11)
B (n=6, n=6)	GPR41	G (n-9, n=8)
C (n=7, n=6)	GPR109a	H (n-9, n=10)
D (n=6, n=6)	GPR40	I (n-9, n=8)
E (n=7, n=7	GPR120	J (n=12, n=13)
	Calcium sensing receptor	K (n=6, n=7)

The Maastricht and Royal London PCR data were both processed and analysed identically. After data collection, the average Ct value of each well was taken (each sample was run in duplicate) for each gene target and patient. The target genes (Table 4) had their Ct values calculated against the average Ct of 18s ribosomal RNA for each patient (average Ct gene ÷ average Ct 18s) to obtain the ΔCt value. This value was then converted into $2^{-\Delta CT}$ to obtain the relative gene expression for each patient sample. With the 2^{-dCT} values, there was often a very large range of data points. Most commonly this was because of a very high original Ct value.

To quantitatively remove any possible outliers in the data, the interquartile range was calculated in Excel, with any data that fell outside of the lower or upper bounds removed. The first and third quartiles were calculated from the data, and this was used to generate an inter-quartile range (IQR). The lower bound was calculated from the first quartile and IQR, and the upper bound from the third quartile and IQR.

Samples from the Maastricht were from IBS-diagnosed patients and healthy controls; I aimed to determine if the IBS state could affect mRNA expression and be a possible confounding factor, when compared to samples from the RLH. However, due to the low numbers of available healthy control samples (1-2), I was unable to statistically conclude whether this was the case.

2.2.3 Immunohistochemistry

2.2.3.1 Cryopreservation

Full thickness tissue was collected in 4% paraformaldehyde (PFA) solution and fixed at 4°C overnight, then washed in PBS 3x, 5 minutes on a shaker. Tissue was submerged in 30% sucrose, PBS solution at 4°C overnight and stored in 15% sucrose solution (in PBS, Sigma) until tissue had sunk to the bottom of the container. Tissue was then submerged in a solution of 50% OCT (VWR, Cat# 361603E), 50% PBS and kept in the fridge overnight. Tissue was submerged in a mould of OCT embedding compound and flash frozen in liquid nitrogen. Frozen tissue blocks were stored at -80°C.

2.2.3.2 Preparing tissue blocks for cryotome cutting

Frozen blocks of tissue were mounted onto the cryostat block holder which was mounted onto the cutting block of the cryotome (Leica, Ag Protect CM950). Sections of 10 µm were cut and collected on Superfrost Plus microscope slides (VWR, Cat# 631-0108) and frozen at -20°C for a minimum of 24 hours.

2.2.3.3 Immunohistochemistry methodology

Frozen 10 µm sections were re-hydrated with PBS for 5 minutes (Sigma-Aldrich), blocked for 1 hour at room temperature with Trident Universal Protein Blocking Reagent (animal serum free) (Insight Biotechnology, GTX 30963) and primary antibody applied (Table 6, Table 7) for 18 hour at 4 °C. Tissue was then washed 3 x 5 minutes in PBS and incubated for 1 hour with species-specific Alexa Fluor conjugated secondary antibodies (Table 8 (1:200, 1:400)). Slides were cover-slipped with a mounting media containing DAPI; a cell nucleus marker (VECTASHIELD – Vector laboratories H-1500). Negative controls were obtained by omitting the primary antibody. Table 6 lists antibodies that were assayed, often for the first time in human frozen samples, and which failed to provoke any positive signal or were later discontinued by the manufacturer (or both).

Table 6: Primary antibodies used for immunohistochemistry that were unsuccessful in detecting specific binding.

Protein	Species	Clonality	Company	Product ID	Dilution(s)	Discontinued
GPR120	Rabbit	poly	Sigma	SAB4501490	1:200	No
GPR120	Rabbit	poly	Abcam	AB97272	1:200	Yes
GPR120	Rabbit	poly	Abcam	AB188954	1:200, 1:400	Yes
GPR119	Rabbit	poly	Abcam	AB75312	1:100, 1:200, 1:500	No
GPR43	Rabbit	poly	LifeSpan Biosciences	LS-A6598-LSP	1:100, 1:200, 1:500	No
GPR43	Rabbit	poly	Sigma	SAB4501283	1:100, 1:200	No
GPR43	Rabbit	poly	LifeSpan Biosciences	LS-A6599	1:100, 1:200	No
GPR40	Rabbit	poly	LifeSpan Biosciences	LS-B4078	1:100, 1:200	No
GPRC6a	Rabbit	poly	LifeSpan Biosciences	LS-B9471	1:100, 1:200	No

Table 7: Primary antibodies used for immunohistochemistry.

Protein	Species	Clonality	Company	Product ID	Dilution
CaSR	Mouse	mono	Abcam	AB-19347	1:200
PYY	Rabbit	poly	US Biological	P3285-10	1:200
5-HT	Goat	poly	Immunostar	20079	1:200

Table 8: Secondary antibodies used for immunohistochemistry.

Species	Species reactivity	Wavelength	Company	Product ID	Dilution
Chicken	Mouse	488	Invitrogen	A21200	1:400
Donkey	Rabbit	568	Invitrogen	A10042	1:400
Donkey	Rabbit	488	Invitrogen	A21206	1:400
Donkey	Goat	568	Invitrogen	A11057	1:400
Donkey	Goat	488	Invitrogen	A11055	1:400

2.2.3.4 Microscopy

Sections were visualised and imaged on an epifluorescence microscope (Leica DM4000 Epi-Fluorescence Microscope) and images acquired on a monochrome CCD digital camera system (Leica DFC365) using the Metamorph imaging System software. Images were

analysed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018).

Fields of view (FOV) were taken to keep the myenteric plexus and submucosa region consistently in view, as shown in Figure 20. This is where I saw the highest number of cells – most likely because hormone secretion occurs on the basolateral sides of EECs, facing into the lamina propria (Kaelberer and Bohorquez, 2018). In addition, it is also the area of the tissue least likely to be damaged, being in the centre of each section.

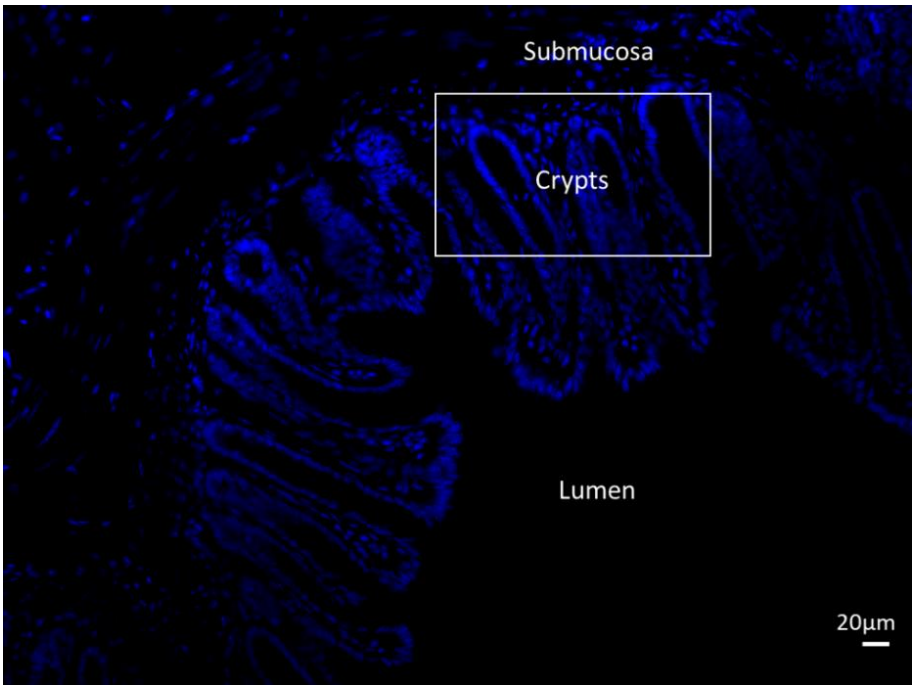


Figure 20: Diagram showing how fields of view were taken. Tissue is human colonic tissue taken at a 20x objective lens, stained for cell nuclei with DAPI (405 wavelength, blue). White box denotes the region where a FOV would be taken (with a 40x objective lens), orientated to keep the submucosa, muscularis externa within field, rather than the lumen.

A total of 15-20 FOVs were taken for each condition, with around ~4500 images collected. Cell counts were performed on stained sections and positively stained cells were counted within crypts. For each FOV, the number of cells and crypts were counted. The number of cells per crypt was then averaged for each condition and patient sample. Cells were counted based on the inclusion and exclusion criteria listed in Table 9.

Table 9: Inclusion and exclusion criteria for immunohistochemistry cell counting.

Inclusion criteria	Exclusion criteria
Whole cells	Parts of cells visible within the FOV
Cells clearly distinguishable with nucleus (shape, size)	Ambiguity in defining as a cell
Cells located within crypts	Cells located outside of crypts
Levels of background low	High background across the FOV
High signal: noise ratio	Low signal: noise ratio
	Structures and cells within FOV are clearly defined

Patients (N=23) were divided into BMI groups of normal weight patients (BMI<25) N=12)) and obese/ overweight patients (BMI≥ 25) N=11)) and the average number of cells/ crypts for: CaSR; serotonin; PYY; CaSR + serotonin; CaSR + PYY co-stained cells, was plotted to determine if increasing BMI affected expression. The proportion of CaSR positive cells that co-localised with PYY or serotonin and whether this proportion altered with changing BMI was also determined.

2.2.3.5 Statistical analysis

Statistical analysis for mRNA expression was performed using unpaired t tests (Mann-Whitney test) , Figure 25, Figure 27, Figure 28). as well as for cell counting (IHC image analysis) (Figure 26, Figure 29, Figure 30)(GraphPad Prism, V9, GraphPad Software, Inc), with $p<0.05$ considered statistically significant. Data is expressed as mean ± SEM in bar charts. Unpaired *t* tests are used to compare the means of our two different (independent) populations (BMI <25 and 25>) to determine if there is a significant difference.

Normal distribution was tested with D'Agostino and Pearson and Shapiro-Wilk tests. The Shapiro-Wilk test was preferred due to the fact it has been shown to be the most powerful test of asymmetric tests and most symmetric tests (Yap and Sim, 2011). The purpose of a normality test is to determine if data is drawn from a normally distributed population. An example of a normally distributed population would be a measure such as height in individuals – most of the data points will sit in the middle (the average height of a population), with data either side forming a bell curve of those shorter or taller than this average. With data looking at – for instance the relative expression of a gene, we do not know that the data fits into a bell curve. Factors such as outliers, low *n* numbers or the fact that gene expression follows an exponential distribution could mean the data does not fit normality. Whether data fits normality is important in determining the type of analysis used:

parametric for normal and non-parametric for non-normal generally. Data from this chapter passed normality tests (data not shown); t tests were therefore used. Outliers were removed based on IQR calculations (note: removal of outliers did not change patterns in expression nor significance).

2.3 Results

2.3.1 Optimising antibodies for GPR120, GPR43, GPR40 and GPCRc6a nutrient-sensing receptors

Several different antibodies for various GPCRs were optimised, at different concentrations and in different patient samples. None of the antibodies listed in Table 6 gave a positive/ above-background stain. The following figures show representative images from some of these optimisation experiments, illustrating the lack of staining. These antibodies were newly sourced or from existing lab stocks.

2.3.1.1 GPR120 and serotonin antibody staining

In Figure 21, three patient samples (BMI <25) were stained with antibodies directed against GPR120 (product code: SAB4501490, dilution: 1:200) and serotonin (20079, 1:200). I saw a strong signal for serotonin positive cells (green), but no specific positive signal for GPR120 (red). Serotonin-positive cells showing a stained "tail" that protrudes into the lumen (white arrow A) or a neuropod structure which can connect to vagal afferents on the basolateral surface (white arrow B) are typical of EECs.

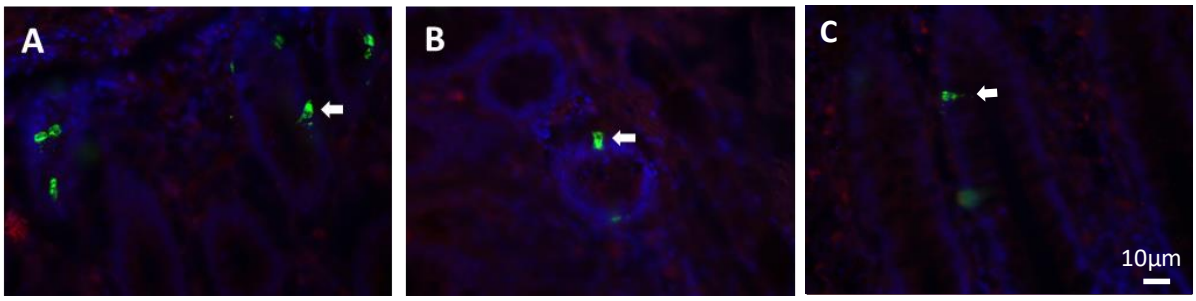


Figure 21: Immunolabelling of GPR120 and serotonin across three different patient colonic samples from the ascending colon. Images shown are representative fields of view from 3 different patients, focused on colonic crypts with a BMI of 20.76 (A), 24.84 (B) and 18.66 (C). Arrows denote stained cells: GPR120 stained in red, serotonin in green, DAPI in blue.

2.3.1.2 GPR120 labelling with three different antibodies showed no specific staining for any GPR120 antibody

In Figure 22, two different GPR120 antibodies were tested (AB97272, 1:200, and AB188954, 1:200) as well as the GPR120 antibody from Figure 14 (SAB4501490, 1:129, 1:400), at two different concentrations. No specific, positive signal for GPR120 staining at any concentration of any of the antibodies could be identified.

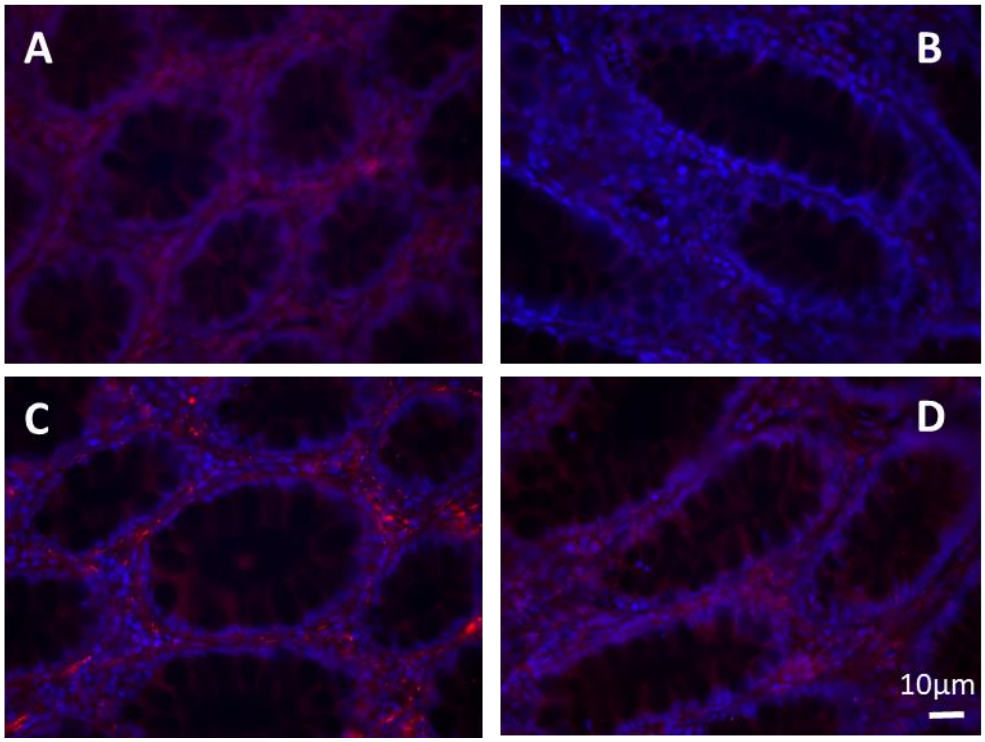


Figure 22: GPR120 immunolabelling optimisation with 3 different antibodies in a human patient colonic sample (BMI 30) from the ascending colon. Images shown are representative fields of view from each experimental condition: GPR120, SAB4501490, 1:129 (A); GPR120, SAB4501490, 1:400 (B); GPR120, AB97272, 1:200 (C); GPR120, AB188954, 1:200 (D). GPR120 stained in red (wavelength 568), DAPI in blue (405).

2.3.1.3 GPR119 antibody at three different concentrations lacked specific binding in human colonic tissue

An antibody for GPR119 (Abcam AB75312) was assayed at three different concentrations: 1:100, 1:200 and 1:500 in two patients (BMI 30 and 18), however, no specific staining of the receptor was observed when the primary antibody was incubated for either 24 or 48 hours in order to determine if changing incubation time resulted in a stronger stain (Figure 23). No difference in staining due to different BMI was observed.

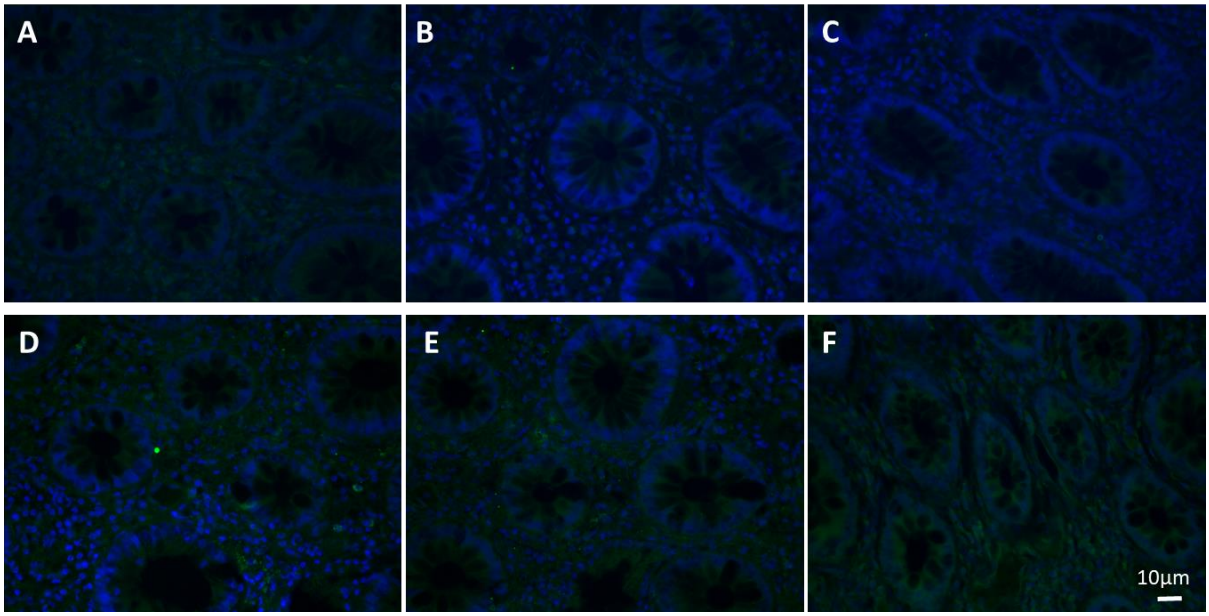


Figure 23: GPR119 (green, 488) optimisation immunolabelling in two human patient colonic samples (BMI 30 (A, B, C) and BMI 18 (D, E, F)) with three different antibody concentrations and two different primary antibody incubation duration. Images shown are representative fields of view from each condition. DAPI stain, blue (405). A: 1:100 antibody concentration, 24-hour incubation. B: 1:200, 24 hours. C: 1:500, 24 hours. D: 1:100, 48 hours. E: 1:200, 48 hours. F: 1:500, 48 hours.

2.3.1.4 GPR43 labelling showed no specific staining at any of the tested concentrations

An antibody for GPR43 (LifeSpan Biosciences LS-A6598-LSP) was assayed at three different concentrations: 1:100, 1:200 and 1:500, however, no specific staining of the receptor was observed (Figure 24).

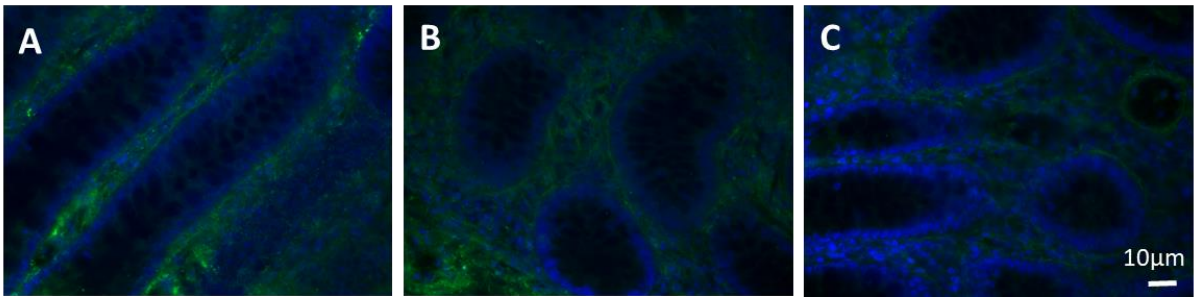


Figure 24: GPR43 (green, 488) optimisation immunolabelling of human patient colonic tissue (BMI 31.8) at three different antibody concentrations. A: 1:100, B 1:200, C: 1:500. Images shown are representative fields of view from each condition. DAPI in blue (405).

2.3.2 The mRNA expression of satiety mediators involved in appetite regulation is unchanged between healthy BMI (<25) and overweight/obese BMI (≥25) groups

In the proximal colon, there was no significant change in the expression of tryptophan hydroxylase 1 (TPH1), glucagon, PYY or somatostatin, between the healthy BMI (<25) or overweight/obese BMI group (≥25) (Figure 25 A–E). I also assessed the expression of the leptin receptor (found in colonic tissue (Hardwick et al., 2001)) to see if leptin resistance, characterised in obesity (242), could be due to a reduction in receptor expression. I observed no change in expression in tissues between BMI groups.

The sigmoid colon showed no change in the mRNA expression of the genes assessed, between the two BMI groups (Figure 25 F–J).

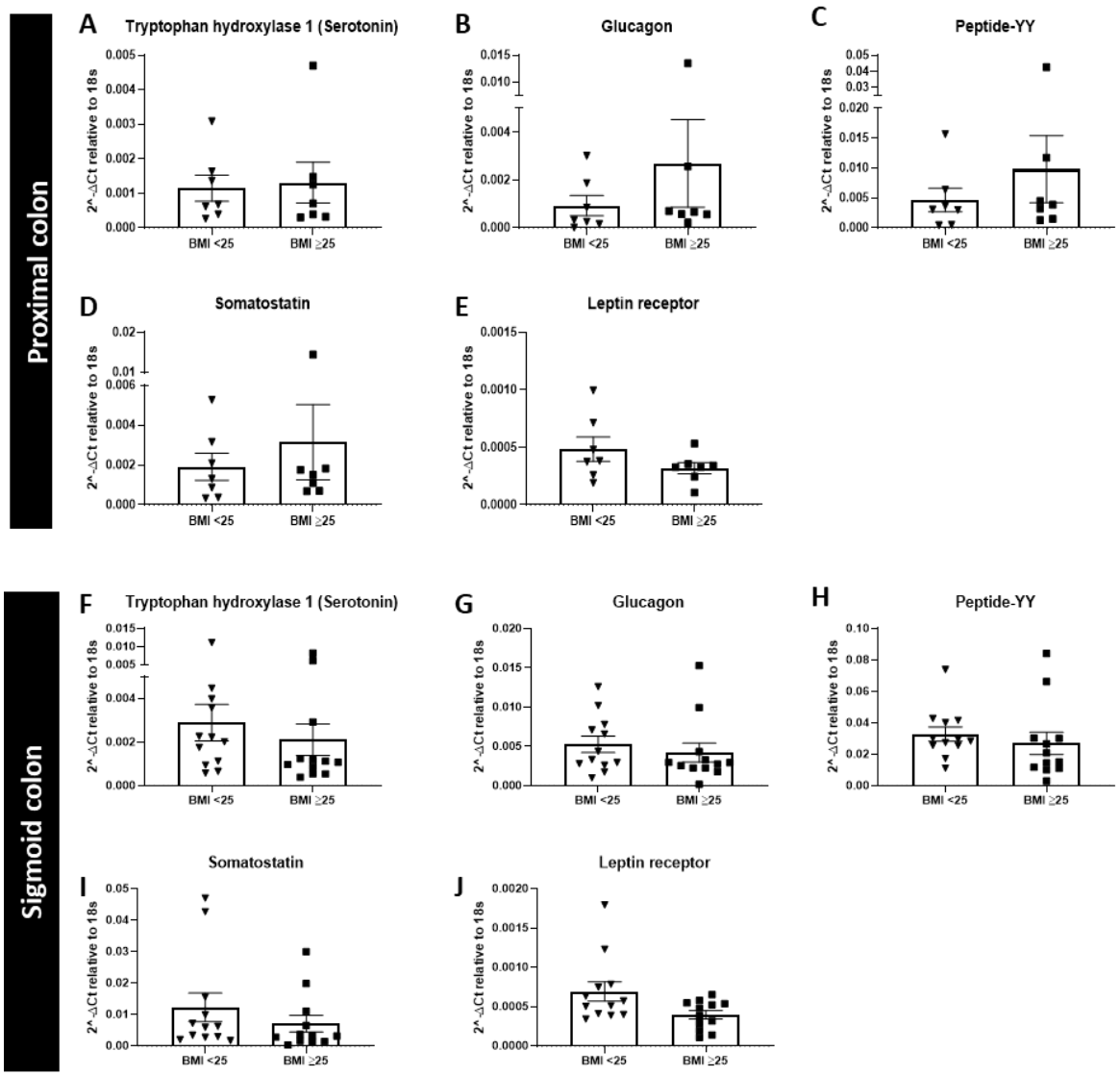


Figure 25: Relative mRNA expression of hormones and hormone receptors involved in satiety in the proximal and sigmoid colon. mRNA expression (relative to 18s) of hormone markers in the proximal (A–E), (body mass index (BMI) < 25 *n* = 7, BMI ≥ 25 *n* = 7), and sigmoid colon (F–J), (BMI < 25 *n* = 12, BMI ≥ 25 *n* = 12).

2.3.3 The expression of PYY and serotonin in enterochromaffin and L-Cells is unchanged between healthy BMI (<25) and overweight/obese BMI (≥25)

To understand how the expression of L and EC cells might change with obesity, I looked at the expression of PYY and serotonin in colonic tissue samples from the Royal London Hospital, U.K.

Serotonin containing EC cells were expressed in colonic crypts in both the <25 and ≥25 BMI groups (Figure 26 A and 2B, respectively). PYY-positive L-cells were also expressed in colonic crypts in both BMI groups (Figure 26 A, B). The quantification of EC and PYY cells showed no significant difference between BMI groups (Figure 26C).

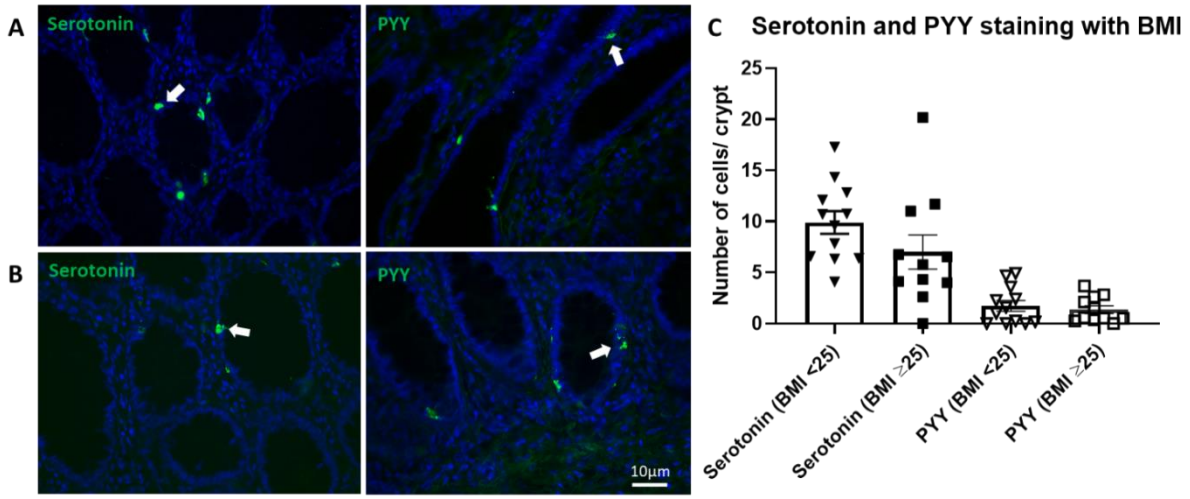


Figure 26: Serotonin and PYY immunohistochemistry expression in human colonic tissue of BMI < 25 and ≥25. (A, B): Representative images of serotonin and PYY expression in patients of BMI < 25 and BMI ≥ 25, respectively. Arrows denote serotonin and PYY positive cells. (C): Counts of cells per crypt stained positively for serotonin and PYY, grouped as BMI < 25 (*n* = 12) and BMI ≥ 25 (*n* = 11). Scale bars represent 10 µM.

The mRNA expression of the M/LCFA receptor GPR40 is significantly increased in the sigmoid colon of the ≥25 BMI group

In the proximal colon there were no significant differences in mRNA expression between BMI groups for the SCFA receptors GPR43, GPR41 and GPR109a (Figure 27 A–C). There were no significant differences in the mRNA expression of the LCFA receptor GPR120 (Figure 27 E) and the M/LCFA receptor GPR40 (Figure 27 D).

In the sigmoid colon, there were no significant differences in mRNA expression of GPR43, GPR41, GPR109a, GPR120, or CaSR (Figure 27 F–K). The mRNA expression of GPR40 was significantly increased in the BMI ≥ 25 compared to BMI < 25 group (p = 0.0464) (Figure 27 I).

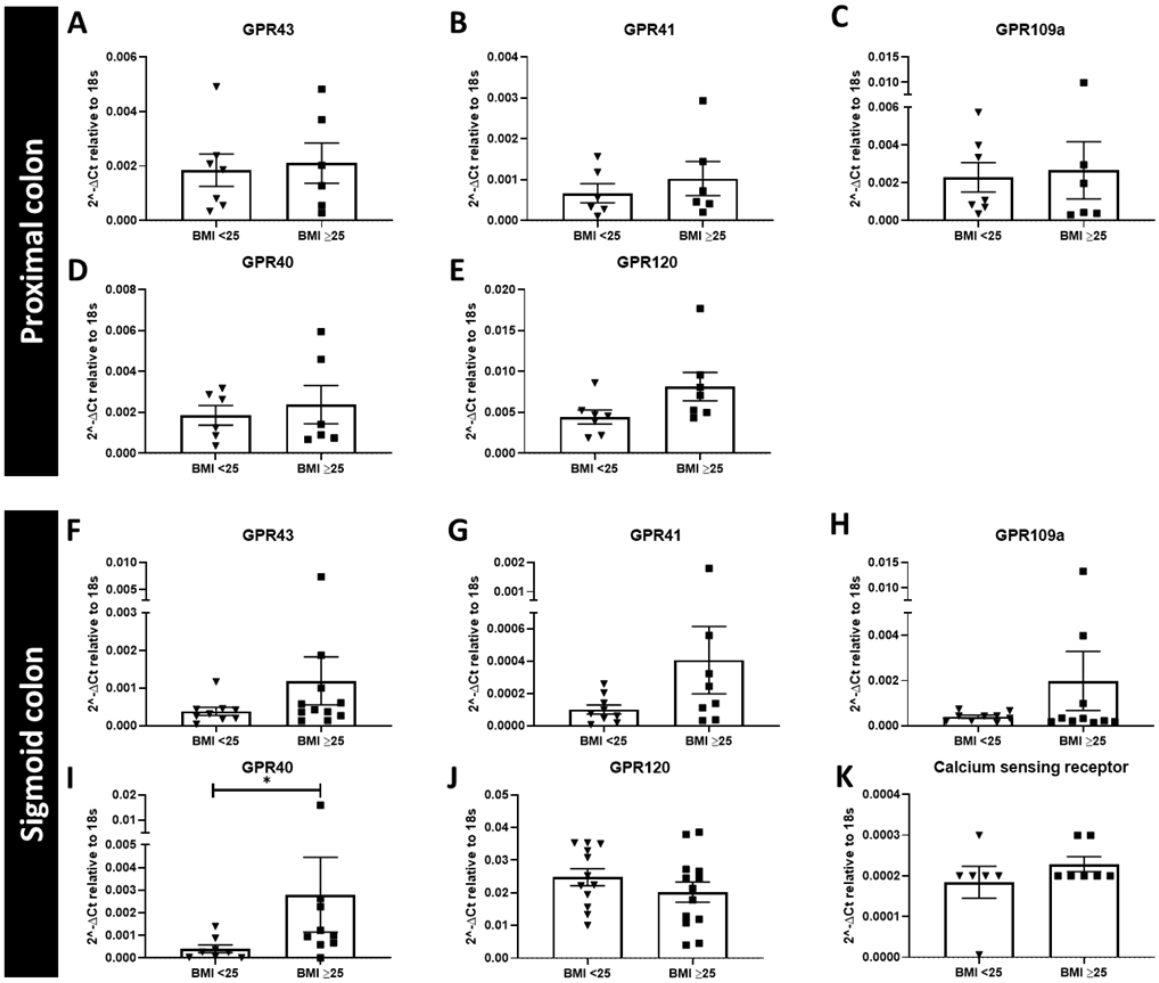


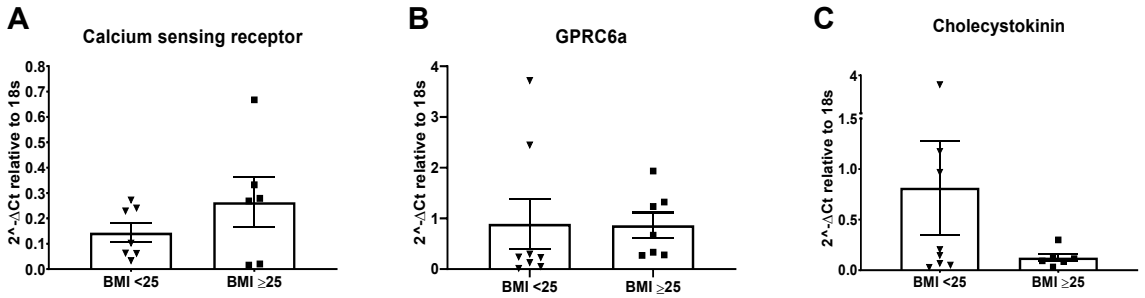
Figure 27: Relative mRNA expression of genes for nutrient receptors in the proximal and sigmoid colonic tissue from a Dutch cohort. Relative expression (against 18s) of nutrient receptors in the proximal (A–E, *n* = 6–7) and sigmoid colon (F–K, *n* = 6–13), with patients divided into those with a BMI < 25 and BMI ≥ 25, respectively.

Whilst all genes were assessed using TaqMan PCR plates, some genes were not amplified above the amplification threshold of the experiment. This made the N numbers for some genes too low for statistical analysis. This was exacerbated for proximal colon

samples, where expression was only detectable in under half the samples, compared to sigmoidal samples.

Individual qPCR expression experiments were conducted as previously described, on proximal colonic tissue obtained from patients attending the Royal London Hospital. There were no significant differences in expression of the amino acid sensing GPCRs, CaSR or GPRC6A in the BMI groups assessed (Figure 28 A, B). The expression of the anorectic hormone CCK was also not significantly changed between the two BMI groups (Figure 28 C).

Figure 28: Relative mRNA expression of nutrient receptors in colonic tissue from a UK



cohort. Relative expression (against 18s) of (A) CaSR (*n* = 7; *n* = 6), (B) GPRC6a (*n* = 8; *n* = 7) and (C) cholecystokinin (*n* = 8; *n* = 6) in a cohort of patients from the Royal London Hospital, divided into those with a BMI < 25 and BMI ≥ 25, respectively,

2.3.4 CaSR is highly expressed on serotonin positive ECs irrespective of BMI

Serotonin containing EC cells co-stained with CaSR in both BMI groups (Figure 29 A, B). Cell counting demonstrated no significant differences in individual CaSR or serotonin positive cells, or cells that were co-stained in the two BMI groups (Figure 29 A, B: white arrows). CaSR was expressed in 99% and 95% of serotonin containing EC cell in the normal BMI and obese/overweight groups, respectively (Figure 29 C).

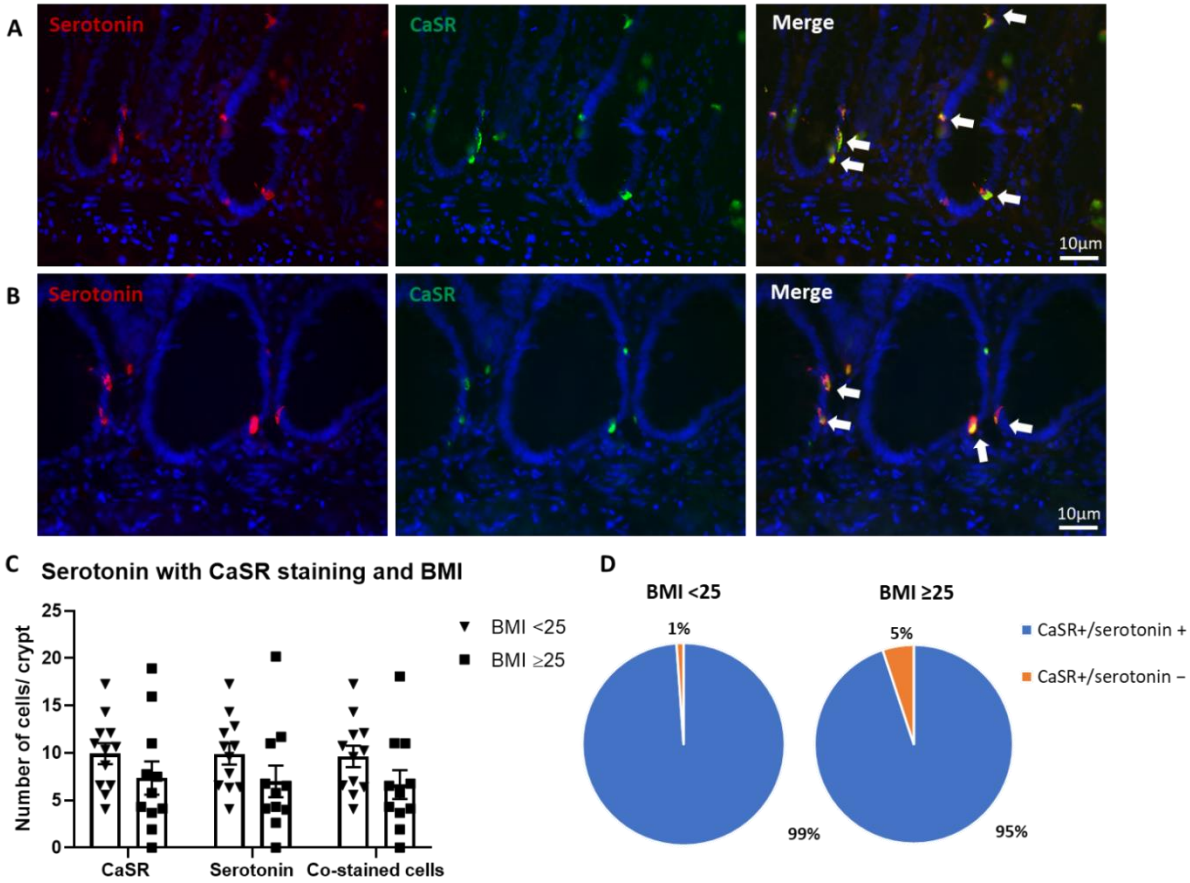


Figure 29: Serotonin and CaSR expression in human colonic samples according to BMI. (A): Representative image from BMI < 25 patient tissue staining for serotonin (red) and CaSR (green). (B): Representative image from BMI ≥ 25 patient tissue staining for serotonin (red) and CaSR (green). Arrows denote serotonin and CaSR co-stained cells. (C): Quantification of positive cells per crypt for CaSR, serotonin and co-stained cells in BMI < 25 (*n* = 12) and BMI of ≥25 (*n* = 11). (D): Proportion of cells per crypt CaSR+/serotonin+ or CaSR+/serotonin- in patients with a BMI < 25 and BMI ≥ 25. Scale bars represent 10 µm.

2.3.5 Limited expression ofCaSR on PYY expressing L-Cells

PYY and CaSR showed positive staining in our colonic tissue samples from both the healthy and overweight/obese groups (Figure 30 A, B, respectively: white arrows denote PYY stained (first panel), CaSR stained (middle panel) and co-stained cells (last panel)). Quantification of positively stained cells showed no significant changes in the expression of CaSR, PYY, or co-stained cells between healthy or overweight/obese BMI groups (Figure 30 C). Co-staining of PYY with CaSR was infrequent—5% and 6% of cells in the healthy BMI and obese/overweight BMI groups, respectively (Figure 30 D).

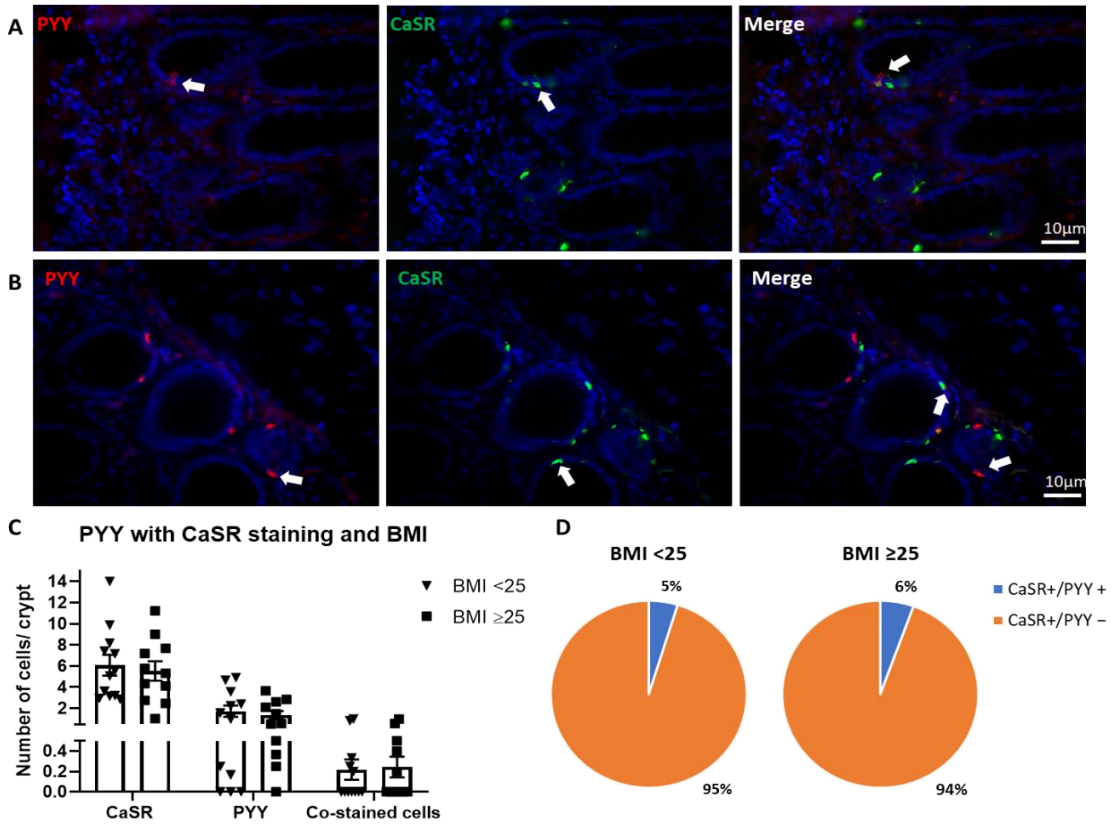


Figure 30: Serotonin and CaSR expression in human colon. (A): Representative image from a BMI < 25 patient tissue staining for PYY (red) and CaSR (green). (B): Representative image from a BMI ≥ 25 patient tissue staining for serotonin (red) and CaSR (green). Arrows denote PYY and CaSR co-stained cells. (C): Number of cells per crypt for CaSR, PYY and cells co-stained with both PYY and CaSR, samples differentiated as BMI < 25 (*n* = 12) and BMI ≥ 25 (*n* = 11). (D): Proportion of cells per crypt that are CaSR+/serotonin+ or CaSR+/serotonin- in patients with a BMI < 25 and BMI ≥ 25. Scale bars represent 10 µm.

2.4 Discussion

The main findings from this chapter demonstrate that a higher BMI (>25) does not appear to affect the expression of the nutrient receptors and hormones/ peptides commonly associated with peripherally mediated appetite-regulating mechanisms, regulated by EECs. However, mRNA expression of the nutrient-sensing receptor GPR40, which binds to MCFAs and LCFAs, was significantly higher in the sigmoid colonic tissue from individuals in the overweight/obese vs. normal BMI groups.

I show that cells of the human colon express, at the mRNA level, the nutrient receptors GPR43, -41, -40, -120, -119, -109a, -C6a and TGR5 and PTGER4 and, at both the mRNA and protein level, CaSR. I also show expression of the hormones/ peptides glucagon and somatostatin, TPH1 and the leptin receptor at the mRNA level, as well as PYY and serotonin at the protein level, in human colonic tissue. These findings concur with research in other studies (Symonds et al., 2015, France et al., 2016, Hardwick et al., 2001, Zhang et al., 1993) and serve to help validate our own findings.

CaSR positive cells co-expressed to a high degree (95-99% of total cells) Figure 29) with serotonin. CaSR positive cells co-expressed PYY with reduced frequency (5-6%). These co-expression ratios of EC or L cells did not alter significantly between the low and high BMI groups.

As human colonic tissue for mRNA analysis was sourced from patients with IBS and healthy controls in the Netherlands, while mRNA expression of CaSR, GPRC6a and CCK were determined from a cohort of patients from the UK without IBS, I assessed the influence of IBS on nutrient-sensing receptor gene expression. The presence of IBS, or sub-type influenced mRNA expression of some nutrient receptors or hormones/ peptides (see Appendix: Figure 52, Figure 53). However, the low number of healthy controls (N=1-2) means that it is not possible to draw any strong statistical inferences from the data. Further patient recruitment would be needed to categorically eliminate IBS as an extraneous variable. Though I have mRNA expression data for CaSR from the Dutch and UK cohort and they are from different colonic tissues, the fact that they both show no change in expression

between BMI groups increases our confidence that IBS status itself is not affecting mRNA expression.

The food frequency questionnaire used did not have sufficiently detailed information to demonstrate any possible relationship between food type/intake and BMI (Appendix, Table 22). For instance, there were no quantifiable and consistent measures of food intake, therefore it was not possible to relate the expression of a receptor to the relative intake of its (most relevant) ligand in a consistent manner and data points such as “Often” had to be employed - which were not validated (Appendix, Figure 51).

The main finding of this chapter, that a high BMI does not alter the mRNA or protein expression of GPCRs (except GPR40) or PYY and serotonin, was unexpected but may reflect the small sample size. Reduced expression of nutrient receptors and hormones (either caused by diet or other environmental changes) at the mRNA level, or protein level, could be a driver of obesity due to reduced satiety signalling. It has been previously shown that PYY circulating levels and post-prandial release (le Roux et al., 2006b) and colonic expression of TPH1 are lowered (Young et al., 2018) in obesity, in humans and mice, respectively. However, data on mRNA or protein expression may not be sufficient to elucidate the impact of weight change. Latter chapters will examine the ability of cells to become activated upon nutrient receptor binding and subsequently release hormones. Measuring the mRNA expression can give us an indication of the abundance of genes for hormones and receptors, however increases/ decreases in gene expression do not necessarily lead to a change in expression or release of translated proteins: Young et al. showed that in obese humans, while colonic mRNA levels of TPH1 did not change, levels of the plasma serotonin increased (Young et al., 2018). It is possible that mRNA expression of, for instance CaSR, might not change with obesity; however, cells may become resistant to its ligand, leading to decreased activation of the receptor, or reduced hormone release. This is due to post-translational modifications of a gene (proteolytic cleavage, phosphorylation, acetylation etc.) which can alter the structure and function of a protein. For this reason, measuring mRNA expression in tissue can describe the relative abundance of a gene, but not its protein.

Our data shows that GPCR expression varies between the sigmoid and proximal colon, both in terms of the relative expression of a gene, as well as any trends in expression variation between the BMI <25 and BMI ≥25 groups. Only GPR40 showed a significant change in expression between the two BMI groups, and only in the sigmoid colon. Research on the mRNA expression of GPR40 in the human colon, particularly any changes with changing BMI, is lacking. Previous work by the group has demonstrated that GPR40 mRNA expression is up-regulated in diet-induced obese mice vs. lean-fed mice, but not in a calorie-restricted weight-loss group (Peiris et al., 2018). Our work is one of the first to show increased GPR40 mRNA expression in obese humans.

GPR40 is a MCFA/LCFA receptor expressed centrally (Haynes et al., 2020, Freitas and Campos, 2021) and on human colonic EECs (Edfalk et al., 2008) (Briscoe et al., 2003). GPR40-positive EECs also express GLP-1, CCK, PYY and serotonin, whilst GPR40-null mice show reduced post-prandial GLP-1 secretion (Edfalk et al., 2008) - therefore it is possible that GPR40 is expressed on both PYY/ GLP-1-positive L cells and serotonin-positive EC cells. The difference between the proximal and sigmoid colon expression of GPR40 may be attributed to its expression on L-cells, as the density of these cells increases along the colon, with the highest levels expressed in the rectum (Gunawardene et al., 2011). EC are distributed equally throughout the colon (Ahlman and Nilsson, 2001). Therefore, it may be that greater numbers of L-cells that also express GPR40 are expressed in the sigmoid region, accounting for the small difference in expression observed.

GPR40 ligands include MCFAs (octanoic acid, decanoic acid and lauric acid) and LCFAs (docosahexaenoic acid, eicosapentaenoic acid and α-linolenic acid) (Briscoe et al., 2003). Ligand-receptor binding causes the release of GLP-1, GIP1 (Briscoe et al., 2003) and CCK (Liou et al., 2011b) and GPR40 has been shown to also be co-expressed with PYY and serotonin (Edfalk et al., 2008). Increased intake of dietary MCFAs, such as animal fats or dairy products is associated with obesity (Turner et al., 2009, Zacek et al., 2019, Marten et al., 2006). Changes to diet may be linked to increased GPR40 mRNA expression in obese individuals. It is therefore possible that a high-fat diet, irrespective of BMI, could drive changes in receptor expression, rather than any indirect effects of the obese state, and that there is no commensurate increase in satiety mechanisms, such as PYY release.

GPR120 expression was also unaltered in obesity, a surprising result, as absence of GPR120 expression induces an obese phenotype in humans (Ichimura et al., 2012), and mRNA expression is increased in the duodenum of obese and overweight humans (Little et al., 2014). Our data suggests that the colonic expression of GPR120 is stable and less likely to be influenced by increased caloric intake, as most nutrients are absorbed in the small intestine.

GRP43 and GPR41 knock-out mice have impaired L cell activity (Samuel et al., 2008, Tolhurst et al., 2012b), suggesting that these receptors are involved in peripheral mechanisms of satiety. Lu *et al.* further showed that the colonic mRNA expression of GPR43 was positively correlated with that of GLP-1 and PYY (Lu et al., 2016). I saw this same trend in our study, with no change in the expression of GPR43 or PYY (mRNA/ protein), GLP-1 (mRNA) between BMI groups. Both GPR41 and GPR43 showed no change in the mRNA expression in the proximal colon. Butyrate is a SCFA ligand for GPR43 (Page et al., 2012) and GPR41 (Page et al., 2012) and the butyrate-specific receptor GPR109a (Blad et al., 2012) but I show that despite increased SCFA availability in obese individuals (Schwiertz et al., 2010), there is no change in expression of these receptors in the proximal or sigmoid colon. SCFA fermenting bacteria are at their highest in the proximal colon, where nutrient availability is also at its highest (Macfarlane et al., 1992), with SCFA availability lowest in the distal colon (Topping and Clifton, 2001). I would therefore have most expected to see any changes in the proximal colon; however, I did not. Our findings suggest that neither an increased SCFA availability (Schwiertz et al., 2010), nor the obese state itself, induce a change in the expression of SCFA GPCRs at the mRNA level.

Our study also did not show a change in expression of the amino acid receptors GPRC6a or CaSR at the mRNA or protein level. GPRC6a and CaSR are both expressed on L cells and share structural similarities, differing only in their agonists (Rasoamanana et al., 2012): L-arginine, L-lysine (Górska-Warsewicz et al., 2018); phenylalanine, tryptophan, histidine, cysteine, respectively (Conigrave et al., 2000b). The role of GPRC6a in nutrient sensing is not well elucidated; GPRC6a activation induces release of GLP-1, and GLP-1-null mice have defects in glucose and fat metabolism and increased susceptibility to DIO (Pi et al., 2017). In humans, its role is even less clear, with single-nucleotide polymorphisms in the gene

associated with insulin resistance, cardiovascular disease and dementia (Pi et al., 2017). Our study showed no significant change in the receptor's mRNA expression in the colon.

CaSR is better studied than GPCR6a, though information on its role in the obese human colon is still lacking. In our food questionnaire data, I was unable to see any significant difference in the dietary intake of calcium (using milk/ buttermilk intake as a proxy (Conigrave et al., 2000b, Górska-Warsewicz et al., 2018)) in normal weight/ obese individuals. I also show no change in the mRNA expression of satiety hormones, and this may be driven by a commensurate lack of change in CaSR expression. CaSR was the only GPCR for which I was able to achieve positive antibody staining and it is the only GPCR for which I have both mRNA and cellular expression data. I saw no change in the expression between low/ high BMI groups at the mRNA or cellular levels. Due to a relatively number of recruited patients (due to the start of the SARS-CoV-2 pandemic), statistical power was lacking. CaSR is expressed in neuronal plexi throughout the GIT that are involved in regulating fluid movement (Tang et al., 2016), smooth muscle activity (Hebert et al., 2004), epithelial integrity and immune cell activity (Tang et al., 2016). Therefore, CaSR expression may be stable and unaltered by obesity. There are clear species differences, as mRNA expression of CaSR is increased in the stomach (antrum) in DIO, compared to standard diet, mice (Nunez-Salces et al., 2020).

CaSR expression or co-expression in either EEC subtype is not changed between BMI groups. It is possible therefore that serotonin release is driven more by CaSR agonism than PYY, and that a diet high in CaSR agonists/ calcium would favour a high release of serotonin (this will be examined in subsequent chapters). Obesity is associated with a low calcium intake, therefore CaSR expression and activity might be reduced in these patients. Tryptophan is present in dairy foods also high in calcium (Acevedo-Triana et al., 2017) and is also synthesised to serotonin by TPH1 and tryptophan. A reduction in calcium/ tryptophan intake may therefore drive reduced CaSR signalling and also serotonin production. In addition, gut-derived serotonin is unable to cross the blood-brain barrier and most of the effects of serotonin occur locally (Watanabe et al., 2010) - some of the anorectic effects of serotonin nay be driven by centrally-derived serotonin. In a study, rats gained weight when central serotonin synthesis was blocked (Namkung et al., 2015).

Our data shows that the mRNA expression of PYY and TPH1 were unchanged between BMI groups. Our group has previously shown that TPH1 and EC expression did not significantly change in obese mice (Peiris et al., 2018). Young *et al.* showed that plasma serotonin levels increased in obese vs. non-obese humans after intraduodenal glucose infusion, but that the colonic mRNA expression of TPH1 was unchanged (Young et al., 2018). This finding by Young and Peiris *et al.* mirrors our own. Our findings show that though neither CaSR nor serotonin expression changes with obesity, most CaSR-positive cells are also serotonin-positive. I was unable to measure CCK or GLP-1 co-staining with CaSR; these hormones might be more important than PYY or serotonin in mediating CaSR responses. I show no change in the mRNA expression of glucagon (GLP-1) or expression of PYY at mRNA and protein level. As previously discussed, PYY protein expression was not significantly different between the high and low BMI groups, and PYY rarely co-localised with CaSR positive cells. L cells are the major source of PYY (Batterham and Bloom, 2003), however PYY mRNA is found throughout the GIT (Broome et al., 1985) and there is a small population of immuno-stained PYY-positive and serotonin-positive EECs that might be ECs (Martins et al., 2017); PYY may be expressed on other types of EEC than just L cells. Without a secondary L cell marker, it is possible that the PYY positive cells I saw, especially those rare cells that co-stained with CaSR, are not in fact L cells, but another EEC sub-type.

Plasma levels of GLP-1 and PYY have been shown to be decreased post-prandially (le Roux et al., 2006b), but increased after a high fat meal (vs a high-carbohydrate meal) (Gibbons et al., 2013), in obese individuals mRNA expression of glucagon and PYY have been shown to be increased (non-significantly) in obese mice (Peiris et al., 2018). Although I did not assess changes to post-prandial expression (and indeed patients are fasted before surgery), I expected to see decreased mRNA expression of glucagon and PYY in obese individuals, to match a reduced satiety. GLP-1 and PYY have been shown to be co-expressed and co-released from the same secretory vesicles in L cells (Holst, 2007), therefore, it is expected that their release profile will be similar. However, our mRNA expression data did not demonstrate any changes to mRNA expression of PYY or glucagon, and our protein data showed no change in PYY protein expression. Our research seems to indicate that the cellular processes involved in the expression of PYY and glucagon genes, and the translation of the PYY gene are conserved in the obese state. Instead, it is likely that intracellular post-

translational modifications of the protein, including vesicular transport and release pathways are altered in the obese state, and this would explain the decreased post-prandial levels discussed. In addition, it is not possible to measure GLP-1 mRNA directly, as the hormone is created from the post-translational modifications of the glucagon gene. Though I examined glucagon mRNA expression, I did not examine the expression of other genes that might affect GLP-1 expression: for instance, PC1/3, the enzyme responsible for cleaving proglucagon mRNA in the colon. Neither was I able to measure the expression of enzymes like DPP-4/ NEP24.11, which are responsible for degrading GLP-1. A decreased expression or PC1/3 and an increased expression of DPP-4/ NEP24.11 could lead to an overall decrease in available GLP-1 and this may cause the decreased post-prandial levels of GLP-1 described in the literature.

Expression data on GLP-1 at the protein-level is also lacking. There are contradictory reports on post-prandial (circulating) GLP-1 expression in obesity, with studies showing reduced (Muscelli et al., 2008, Ranganath et al., 1996), increased (Fukase et al., 1995) or no change (Knop et al., 2012, Vilsboll et al., 2003) in expression. Peiris et. al have shown that the number of GLP-1 containing L-cells was not significantly different between lean and DIO mice, however, RYGB increased GLP-1 positive cells compared to the DIO group (Peiris et al., 2018). Obese individuals have been shown to have increased activity of DPP4, which inactivates GLP-1 (Baranowska et al., 2000), therefore this might be the driver of reduced satiety, rather than a reduced production of GLP-1, driven by reduced genetic expression or translation. I also did not assess GLP-1 receptor expression, which may also be altered in obesity.

Collectively, the data presented here suggest that while the expression of L and EC cells as well as that of nutrient receptors and satiety mediators are unchanged in obesity, the intra-cellular mechanisms leading to hormone release may be altered.

As previously mentioned, neither TPH1 mRNA expression or the number of serotonin containing EC cells differed between the high and low BMI groups, suggesting that expression of TPH1 mRNA and serotonin-containing EC cells in humans remains stable despite weight changes, concurring with previous studies in both mice and humans. I report

that serotonin-expressing EC cells commonly expressed CaSR, however, information in the literature is lacking, particularly in humans. CaSR has been shown to be expressed on chromogranin A-positive cells in the colon (Sheinin et al., 2000); chromogranin is a generic, non-specific marker of EECs, including ECs (Buffa et al., 1988). A study by Lund *et al.* demonstrated low CaSR transcript expression in FACS purified serotonin cells from the murine colon (Lund et al., 2018) and studies in goat mammary glands have shown that in epithelial cells, serotonin increased mRNA abundance of CaSR (Zang et al., 2018). In mice, serotonin deficiency decreases mRNA expression of intracellular calcium transporters and 5-HT_{2b}R (Liu et al., 2018). A study investigating the role of CaSR-sensing of emesis-inducing agent vomitoxin, might provide tangential (and unique) evidence of colonic serotonin sensing. Vomitoxin was shown to activate EEC CaSR in mice, and a CaSR antagonist given to minks prevented emesis and reduced vomitoxin-induced increases in plasma PYY and serotonin levels (Wu et al., 2017). This study may therefore provide evidence of CaSR activation inducing serotonin and PYY release in colonic ECs and L cells, respectively. As discussed previously, tryptophan, is metabolised to serotonin and is also an agonist for CaSR (Conigrave et al., 2000b). Serotonin is also released by cells by the activity of Piezo2 mechanosensitive ion channels which are dependent on intracellular Ca^{2+} increase (Alcaino et al., 2018), a physiological condition regulated by CaSR (Reimann et al., 2012). Serotonin and CaSR activity may therefore be closely linked by dietal availability of tryptophan and calcium.

The mRNA expression of the anorexigenic hormone CCK and the leptin receptor, were unchanged between the low and high BMI groups in colonic tissue. I posit that leptin resistance could be driven by an altered expression of the leptin receptor in the colon: reduced receptor expression may abrogate leptin-satiety signalling. However, I see no change in the expression of the receptor. Leptin resistance could be driven by neural instead of peripheral mechanisms; with altered transport into the brain or signalling within hypothalamic neurons (73). Hypothalamic nuclei expressing LEP-R upregulate the protein suppressor of cytokine signalling 3 (SOCS3), which is a negative regulator of leptin, therefore chronic leptin expression may lead to leptin resistance (73).

Unchanged levels of CCK seen in this study may reflect the small population of I-cells in the colon, as these cells are primarily expressed in the duodenum and SI (Ritter, 2004). I also showed no change in mRNA expression of SST. SST is released by the EEC subtype, D cells in the GIT, and these cells are most highly expressed in the duodenum and pancreas (Buffa et al., 1978) therefore, as for CCK, changes to SST expression may occur primarily in these tissues.

To conclude, the data from this chapter highlights the MCFA/LCFA receptor GPR40 as an important target for study, being the only examined GPCR significantly altered (increased) in expression in the high BMI group. No hormones, hormone receptors/ analogues were significantly altered in the overweight/obese state. However, the ability of cells to be activated upon GPCR-ligand binding, and subsequently release hormones and peptides, might be altered with increasing BMI and this will be the focus of subsequent chapters.

3 Chapter 3: Elucidating molecular pathways of cellular activation in response to nutrient receptor activation in murine colonic tissue

3.1 Introduction

In this chapter I will assess potential changes in the number of PYY containing L-cells and serotonin-containing EC cells and their co-expression of cell activation markers CaMKII and pERK following agonist action of the GPCRs: GPR119, GPR40, GPR120, GPRC6a and CaSR. These GPCRs were examined due to their role in mediating satiety through the release of hormones and peptides in the colon (as described in Chapter 1). Understanding the cell activation state in response to receptor stimulation will provide insight into the ability of these GPCRs to activate L and EC cells and colonocytes to activate peripheral satiety pathways. In this chapter mouse colonic tissue was used, due to inability to obtain human colonic tissue, because of the SARS-CoV-2 pandemic.

3.1.1 ERK and CaMKII pathways of cellular activation

Extracellular signal-regulated kinase (ERK) and CaMKII are two intracellular protein response pathways (Smedley et al., 2021) also used as cell activation markers in EECs (Liou et al., 2011a, Symonds et al., 2015). They become phosphorylated (pERK, phosphorylated-CaMKII) by downstream effects of GPCR activation, causing changes in secretion, gene expression and cell metabolism (Belcheva and Coscia, 2002). Activated ERK can operate in different intra-cellular regions (cytoplasm, nucleus) depending on the activation pathway (β-arrestin, G-protein-coupled receptor, respectively) (Eishingdrelo and Kongsamut, 2013). This phenomenon is termed the “barcode” hypothesis: nutrient receptors can have differing downstream activation pathways to different ligands for instance (driven by different receptor phosphorylation patterns), leading to the binding of different intracellular effectors. This would lead to changes in cellular activation and transcription, translation and release of mediators such as hormones would all be affected. The bar code hypothesis is summarised in Figure 31.

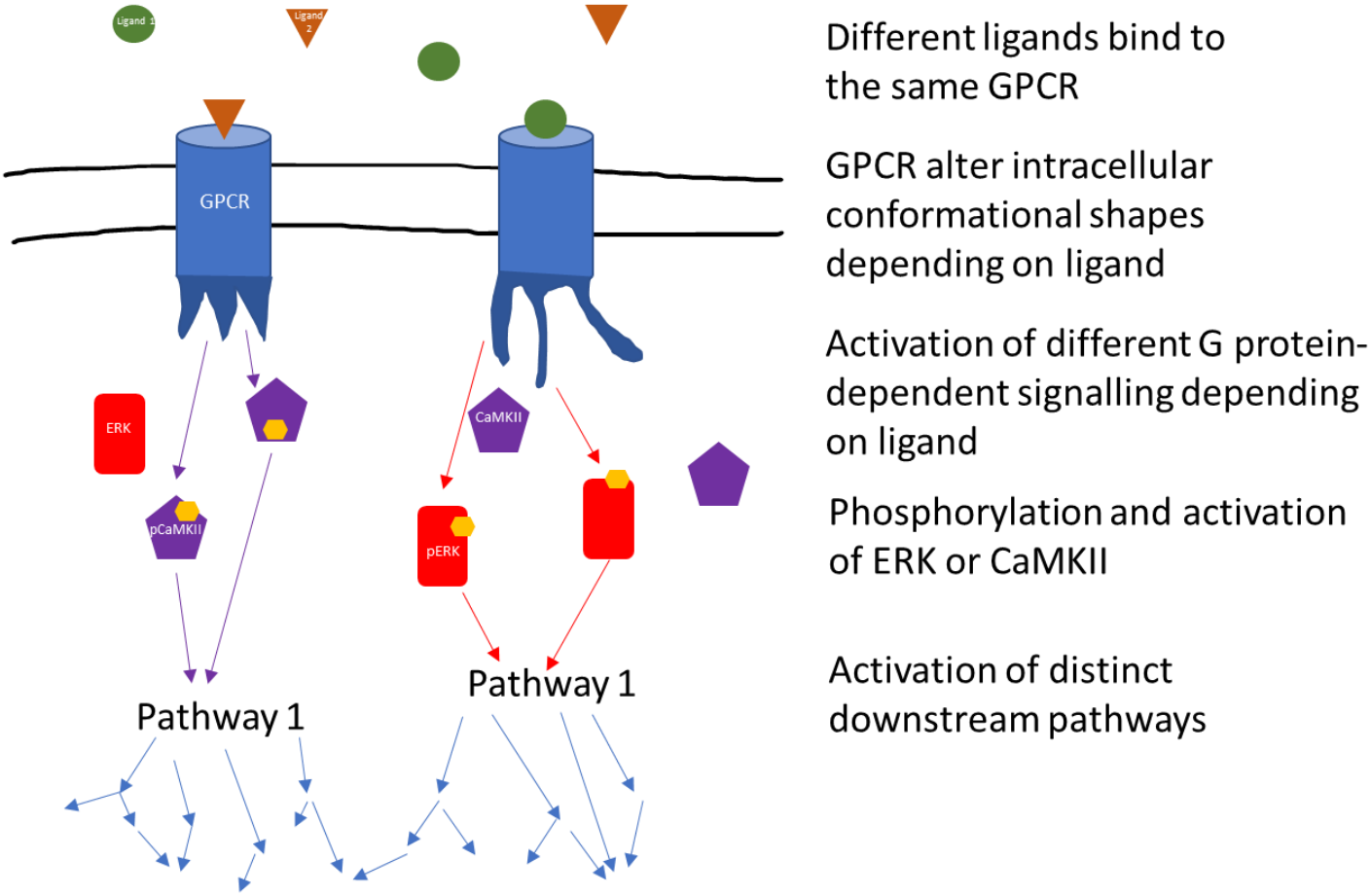


Figure 31: The bar code hypothesis. Different ligands (circles, triangles), can bind to the same GPCR on the cell membrane and differing activation of eRK, CaMKII which activate alternate pathways leading to alterations in cell activation, translation, transcription etc.

CaMKII is activated by increases in intracellular calcium, caused by its release from intracellular stores upon activation of phospholipase C and inositol trisphosphate (Figure 10) (Pfleger et al., 2019). These can be caused by activation of GPCRs on EECs (Figure 32) (Pfleger et al., 2019): GPCR-ligand binding can indirectly activate CaMKII. GPCRs can activate ERK via G-protein α subunits' activation of Ras, Rap, PKC and tyrosine kinases which can phosphorylate ERK, releasing it from the kinase complex and allowing it to phosphorylate and activate other cellular proteins (Eishingdrelo and Kongsamut, 2013).

Increased numbers of CaMKII and pERK immunoreactive cells (markers of cell activation) are observed in the mouse colonic mucosa after stimulation with CaSR agonists (phenylalanine and tryptophan) (Symonds et al., 2015). Human colonic tissue stimulated

with lauric acid (shown to stimulate PYY, GLP-1, serotonin release) led to an increase in immunoreactive pERK cells compared to a control (Symonds et al., 2015). pERK and pCaMKII activity have been shown to be cell-specific, with serotonin containing cells (ECs) activating the pCaMKII pathway, and PYY/ GLP-1 containing cells (L cells) activating the pERK pathway (Peiris et al., 2021). Peiris *et al.* demonstrated that human EECs stimulated with the GPR120 agonist TUG891 did not change levels of pERK but did increase CaMKII – this same effect could not be replicated with lauric acid (GPR85 agonist) (Peiris et al., 2021). In colonic biopsies, Peiris *et al.* demonstrated that: lauric acid activated PYY, GLP-1, and serotonin release; TUG891 increased PYY and GLP-1; lauric acid and TUG891 together increased PYY and GLP-1 release 2-4 times more than either nutrient alone, but not serotonin release (Peiris et al., 2021). GPCRs are therefore able to cause distinct and parallel intracellular signalling cascades in response to different, specific stimuli and this may aid in the translation of the composition of a nutrient load in the colon into a GLP-1, PYY or serotonin response.

3.1.2 G-protein-coupled receptor activation

CaSR, a receptor for the amino acid tryptophan, is highly expressed within the colon (Symonds et al., 2015): on L cells (Reimann et al., 2012) and, at a lower level, on ECs (Lund et al., 2018). L-tryptophan-mediated GLP-1 release is blocked by the CaSR antagonist NPS2143 (Acar et al., 2020). CaSR-dependent increases in $[Ca^{2+}]_i$ are also dependent on phosphoinositide phospholipase (C-IP3): they can be blocked by its blockade (Hebert et al., 2004). CaSR has been shown to activate multiple signalling pathways, involving $G_{q/11}$, $G_{i/o}$, $G_{12/13}$ and G_s proteins (Geng et al., 2016).

GPRC6a is a receptor for calcium and L-α-amino acids: lysine, alanine, ornithine and L-arginine (Clemmensen et al., 2014), and is expressed on L cells (Oya et al., 2013). L-arginine activation of fibroblasts is GPRC6a-dependent and involves activation of the ERK1/2 pathways (Fujiwara et al., 2014) whilst in murine β-cells activation causes insulin release via GPRC6a activation of cAMP pathways (Pi et al., 2012). GPCRC6a is likely to signal by different pathways depending on the tissue, cell-type and agonist: via G_q coupling, but not G_i or G_s in HEK293 (as a chimeric receptor) and CHO cell lines (increased $[Ca^{2+}]_{i,}$) (Clemmensen et al.,

2014) or G_s coupling in a GPRC6a-HEK293 line (cAMP accumulation) (Pi et al., 2012) after receptor activation. However, the exact role of GPRC6a in the colon and the pathway for EEC activation remains undetermined.

GPR40 couples with the G_q protein and activates PLC, increasing intracellular calcium via IP3 or DAG phosphorylation of PKC (Kimura et al., 2020) and activating ERK1/2 (Figure 32) (Itoh et al., 2003a). Ligand binding and activation of G_i or G_s proteins leads to reduced or increased cAMP production, respectively (Itoh et al., 2003a). LCFAs such as arachidonic, eicosapentaenoic, oleic, linoleic, linolenic and docosahexaenoic acids have a high affinity for GPR40 in CHO–hGPR40 cells (Itoh et al., 2003a). GPR40 activation with dietary fat or LCFAs (isolated I cells) in mice has been shown to lead to release of GLP-1, GIP and CCK, respectively (Edfalk et al., 2008).

GPR120 is highly expressed in the human and mouse colon (Hirasawa et al., 2005) and couples with G_q protein and mediates increases in intracellular Ca^{2+} levels (but not cAMP levels) via G_i or G_s upon ligand-binding (Figure 32) (Kimura et al., 2020). The receptor is likely to signal via the ERK pathways: α-linolenic acid has been shown to strongly activate the ERK pathway in GPR120-, GPR40-expressing STC-1 cells: RNA-interference of GPR120 expression, not GPR40, reduced ERK activation (Katsuma et al., 2005a). GPR120 activation by LCFAs (saturated C_{14-18}, unsaturated C_{16-22}) has been shown to promote GLP-1 release, increase $[Ca^{2+}]_i$ and induce ERK activation in STC-1 cells, as well as increasing pERK expression in GPR120-expressing HEK293 cells (Hirasawa et al., 2005). GPR120 seems to not signal via the ERK pathway in some cell types: whilst TUG891 increased concentration-dependent $[Ca^{2+}]_i$ more than other ligands, TUG891 showed a lower affinity for GPR120 in a pERK assay in HEK293T cells (Hudson et al., 2013).

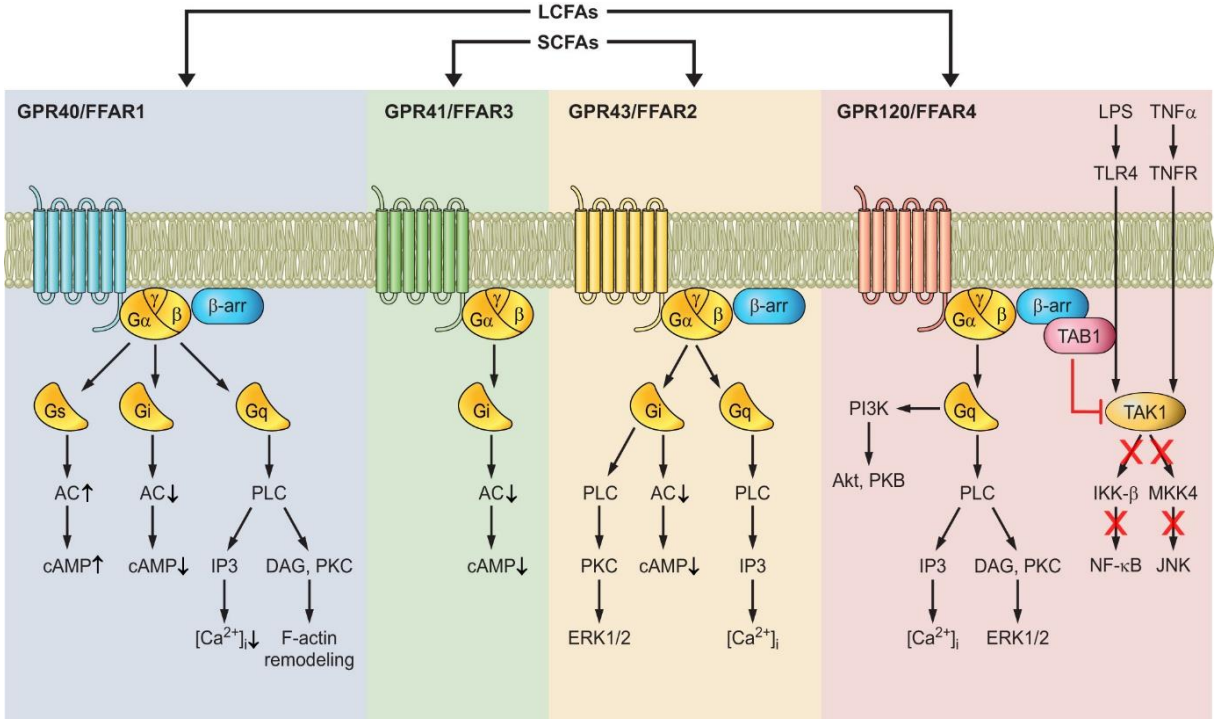


Figure 32: Ligand specificity and signalling pathways of the GPCRs GPR40, -41, -43 and -120. LCFAs activate GPR40 and GPR120, SCFAs activate GPR41 and GPR43. Downstream pathways of G protein activation include the activation or inhibition of enzymes such as AC or PLC which in turn can alter ERK phosphorylation, changes to intracellular calcium levels, and activation of transcription factors like NF-κB.
AC, adenylate cyclase; DAG, diacylglycerol; IP3, inositol trisphosphate; LPS, lipopolysaccharide; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PKD, protein kinase D; PLC, phospholipase C; TNF, tumour necrosis factor. Figure reprinted from (Kimura et al., 2020).

GPR119 is activated by endogenous oleoylethanolamide (OEA) produced in the SI, this has been shown to reduce food intake, adiposity and body weight in rats (Overton et al., 2006a). PSN632408 is a small-molecule GPR119 agonist with a similar efficacy and potency and shown to produce the same effect as OEA in rats (Overton et al., 2006b). In the murine SI, RNA expression of GPR119 is associated with cells expressing glucagon and CCK (Haber et al., 2017). However, the molecular pathway for GPR119 expression and activation remains to be fully elucidated in colonic EECs. GPR119 agonists have been shown to increase cAMP

levels in a HEK-293 line and the study authors postulated this was most likely via $G_{\alpha s}$ coupling (Overton et al., 2006b).

The aim of this chapter was to characterise the intracellular activation pathways relevant to specific GPCRs, by stimulating with specific ligands and determining if these pathways are specific to, or shared by, several GPCRs.

3.2 Methods

3.2.1 Mouse tissue experiments

Experiments were performed using adult (12 weeks) female C57BL/6 mice maintained on standard laboratory chow and water in a controlled environment (12 h light/dark cycle, 22±0.5°C, 40–60% humidity), within a pathogen-free facility. Mice were sacrificed by CO_2 asphyxiation.

Sacrificed mice were pinned to a gel block placed on ice, with pins in each limb. Mice were sprayed with ethanol prior to dissection. The area above the urethral opening was pinched with tweezers and scissors were used to cut up the navel to the neck, cutting through the skin and muscle to expose organs. Cold PBS was poured onto the exposed organs to prolong tissue viability. The intestines were exposed and the region from below caecum to above the rectum was excised and placed into a dish filled with cold Krebs solution, on ice (see Nutrient Solutions section, Table 11). The fat surrounding the colon was trimmed off and the colon cleaned and flushed with clean Krebs, by pipette. The colon was opened by cutting ventrally down its length and pinned out with the mucosa facing up, in Krebs solution, for 30 minutes (carbogenated (95% O_2/ 5% CO_2) on ice, on a Sylgard™-coated plate, keeping care to only pin the edges of the tissue. Tissue from the ascending colon region was then cut by scalpel into squares of approximately 1cm^2 and mounted into Ussing chambers. Care was taken that the centre of colon sections was undamaged/ punctured and therefore most likely to be viable, as this is the region of stimulation.

3.2.2 Ussing chamber experiments

Ussing chambers were used to stimulate live tissue *in vitro* with agonists of nutrient-sensing GPCRs. This method allows the luminal mucosa to be stimulated in such a way to mimic physiological conditions. An Ussing chamber (CHM4 (Extra Small), World Precision Instruments) is formed of 2 glass cylinders with a channel for liquid running through the centres (Figure 33). These cylinders connect and allow tissue to be pinned in-between, separating tissue into 2 distinct surfaces. These cylinders are connected to compartments which can contain solution, and which are surrounded by circulating water warmed by a

water bath (SUB Aqua Plus Series, Grant). These compartments are connected to gas lines which allows for the solutions within to be constantly carbogenated and warmed (42°C). This solution is then cycled over the tissue and out of the chamber and because the solutions never mix, the tissue can be exposed to receive a specific solution to only one surface. An image of a running Ussing chamber can be seen in Figure 34.

Figure 33: Ussing chamber cylinders (detached). Image sourced from World Precision Instruments: https://www.wpiinc.com/var-2567-chambers-for-ussing-system.

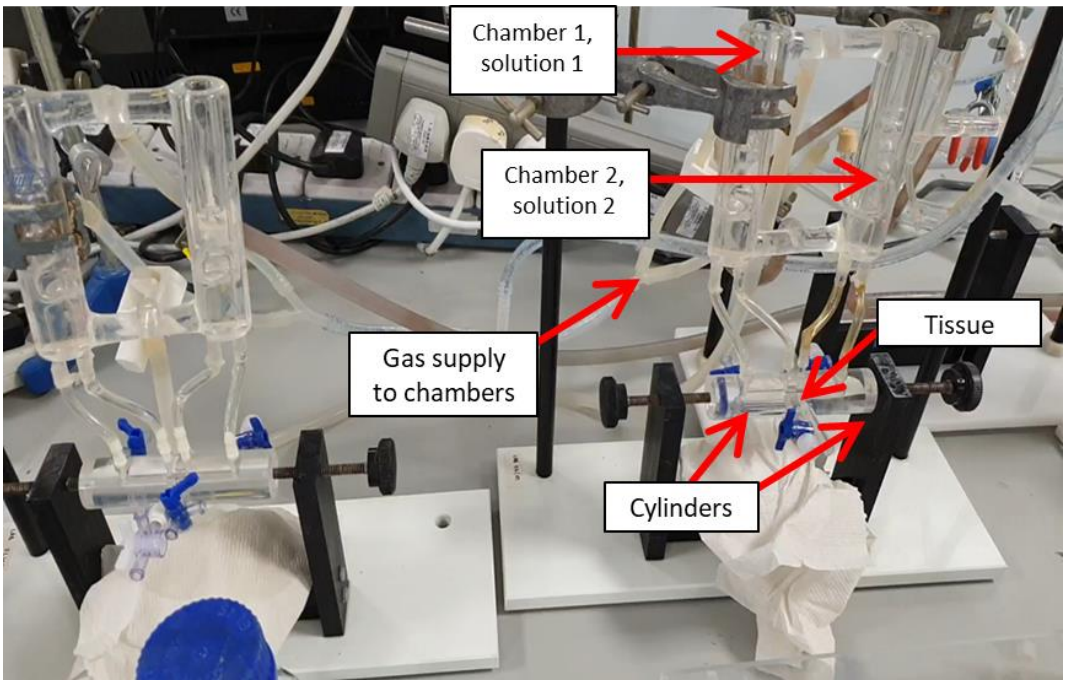


Figure 34: Ussing chamber set up. Tissue is clamped between two Ussing chamber cylinders, separating the tissue into basolateral and luminal surfaces. Each surface is directly stimulated with warmed, oxygenated solution, the basolateral surface receiving physiological Krebs solution, and the luminal surface the experimental/ control solution.

25 mice were divided into 5 different experimental groups; each of these groups were further divided into 3 separate experimental subgroups as shown in Table 10.

Table 10: Experimental plan of mice used in Ussing chamber experiments.

Mouse group	Agonist	Condition 1	Condition 2	Condition 3
Mice 1-5	L-Tryptophan	Negative control	Drug [c] 1	Drug [c] 2
Mice 5-10	L-Arginine	Negative control	Drug [c] 1	Drug [c] 2
Mice 10-15	TUG891	Negative control	Drug [c] 1	Drug [c] 2
Mice 15-20	PSN375963	Negative control	Drug [c] 1	Drug [c] 2
Mice 20-25	AS2034178	Negative control	Drug [c] 1	Drug [c] 2

The luminal surface was exposed to 10 mL of two different concentrations of nutrient solutions in Krebs containing a condition listed in Table 11, and the basolateral surface was exposed to 10 mL Krebs solution to maintain tissue viability, for 20 min. All solutions were carbogenated and maintained at a temperature of 42°C. Following completion of stimulation, the mucosa was fixed in 4% PFA in PBS overnight.

3.2.3 Nutrient solutions

All solutions contained 124.05 mM NaCl, 4.78 mM KCl, 1.33 mM NaH_2PO_4, 2.44 mM $MgSO_4$, 5.50 mM D-glucose, 2.5mM CaCl and 25.00 mM $NaHCO_3$ (Sigma-Aldrich) and were carbogenated with 95% O2 and 5% CO_2. For each agonist used, the luminal surface of the murine tissue was exposed to two different concentrations (Table 11), as well as a negative control solution consisting of only Krebs. These drug concentrations were chosen based on previous research by the group (Symonds et al., 2015).

The receptor agonists for GPCRs were selected based on their EC50 and receptor specificity, based on the following studies:

- CaSR: Tryptophan has the lowest EC50 for CaSR compared to other ligands like calcium, phenylalanine, histidine (Conigrave et al., 2000b).

- GPRC6a: L-arginine has been shown to be a high affinity ligand for GPRC6a (Clemmensen et al., 2014)
- GPR40: The GPR40 agonist AS2034178 has been shown to induce glucose-dependent insulin secretion in mice and is able to induce a higher $[Ca^{2+}]_i$ level than linolenic acid at 1 µM and similar levels at 10 µM in a CHO cell line overexpressing the receptor (Tanaka et al., 2013).
- GPR120: TUG891 is a synthetic ligand shown to activate GPR120 in a manner similar to α-linolenic acid in humans (ERK phosphorylation, Ca^{2+} mobilisation) (Hudson et al., 2013). TUG891 has a high affinity for human and mouse GPR120 and GPR40, showing strong selectivity for human GPR120, but only a mild selectivity for mouse GPR120 over GPR40, stimulating GLP-1 release (Hudson et al., 2013).
- GPR119: PSN632408 was shown to be highly selective for GPR119: when tested against 107 other targets (including other GPCRs, hormone receptors), none showed higher than 30% selectivity (Overton et al., 2006b).

Table 11: GPCR agonists and concentrations used for Ussing chamber experiments

Agonist	Agonist target	Concentrations of agonist used		Company	Catalogue #
L-Tryptophan	Calcium sensing receptor	1mM	3mM	Sigma	M6250
L-Arginine	GPRC6a	1mM	5mM	Sigma	A5006
TUG891	GPR120	1µM	10µM	Tocris	4601
PSN375963	GPR119	1µM	10µM	Tocris	3353
AS2034178	GPR40	1µM	10µM	Tocris	5035

3.2.4 Immunohistochemistry

Immunohistochemistry was used to measure cell activation after exposure to agonists, via the cell activation markers CaMKII or pERK. Cells were identified as L cells or EC by staining of PYY or serotonin, respectively. Fixed colonic tissue was cryoprotected in OCT with the mucosa facing down so sections would be across the top of the tissue. All tissue was mounted in this same orientation to ensure the luminal side of the mucosa was cut laterally. After cryoprotection tissue was frozen in liquid nitrogen. Sections of 10 µm were cut on a cryostat and collected on Superfrost Plus™ microscope slides (631-0108, VWR) for

sectioning. Frozen sections were brought to room temperature and rehydrated with PBS for 5 minutes, blocked with Trident Universal Protein Blocking Reagent (animal serum free) (GTX30963, Insight Biotechnology limited) for 1 hour (at RT) and primary antibody applied (Table 12) for 18 hours at 4 °C. Tissue was then washed 3x 5 minutes in PBS and incubated for 1 hour with species-specific Alexa Fluor conjugated secondary antibodies (Table 13). Slides were co-stained with the following combinations of antibodies: pERK and serotonin; pERK and PYY; CaMKII and serotonin; CaMKII and PYY. Slides were cover-slipped with a mounting media containing DAPI (VECTASHIELD – Vector laboratories H-1500). Negative controls were obtained by omitting the primary antibody. Sections were visualised and imaged on an epifluorescence microscope (Leica DM4000 Epi-Fluorescence Microscope) and images acquired on a monochrome CCD digital camera system (Leica DFC365) using Metamorph imaging system software. Images were analysed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018).

Table 12: Primary antibodies used for immunohistochemistry.

Protein	Species	clonality	Company	Product ID	Dilution
CaMKII kinase	Rabbit	poly	Abcam	ab5683	1:100
pERK	Rabbit	mono	Cell signalling	4370T	1:400
PYY	Rabbit	poly	US Biological	P3285-10	1:400
5-HT	Goat	poly	Immunostar	20079	1:400

Table 13: Secondary antibodies used for immunohistochemistry.

Species	Species reactivity	Wavelength	Company	Product ID	Dilution
Donkey	Rabbit	488	Invitrogen	A21206	1:400
Donkey	Goat	568	Invitrogen	A11057	1:400
Donkey	Mouse	568	Invitrogen	A11037	1:400

3.2.5 Immunohistochemistry image analysis

Images were taken of at least five fields of view (FOV), keeping the luminal surface in view. pERK and CaMKII immunoreactive cells have been shown to be stained uncommonly within crypts and more commonly expressed towards the apical surface where mature cells might be more likely to be exposed to nutrients (Symonds et al., 2015, Peiris et al., 2018).

Due to the large number of positively stained cells, as well as greatly increased number of crypts in each FOV (representative image: Figure 35), the cell counting process was automated. Using ImageJ, for each FOV, the area of crypts was selected at a maximum 3 crypts deep from the luminal surface. This area was measured and recorded as mm^2 (Figure 36). The 'threshold' tool was then used to detect positively stained cells by adjusting the parameters on the "dark background" setting – this meant that cells brighter than background staining could be more easily distinguished. The number of CaMKII or pERK positive cells was then counted by the 'analyse particles' tool (Figure 37) giving a count of the number of stained cells (Figure 38).

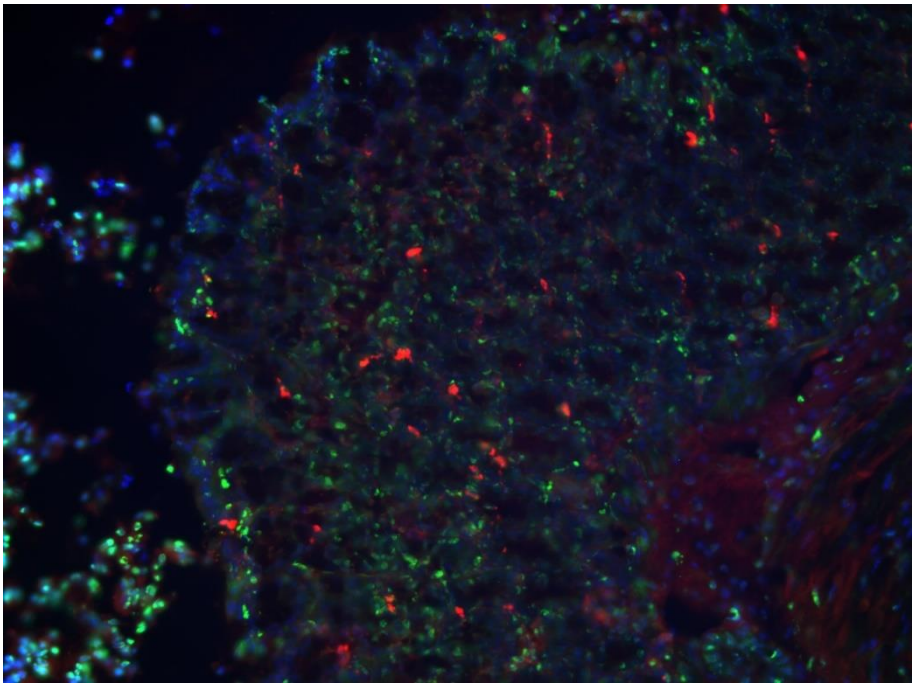


Figure 35: Field of view of immunohistochemistry staining from unstimulated mouse colonic tissue. CaMKII positive signals are denoted in green, serotonin positive signals in red and DAPI signal in blue.

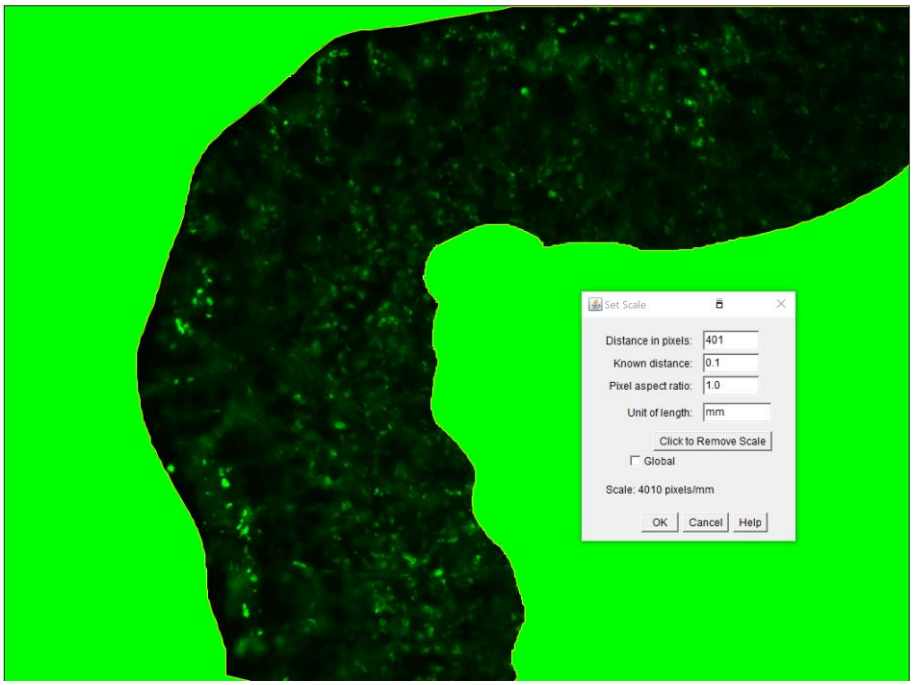


Figure 36: Field of view from Figure 35, showing only staining in the GFP channel, with colonic crypts, three-deep from the luminal surface (seen towards the left and top of the image) selected and measured (0.408 mm^2). Image created and processed in ImageJ.

Figure 37: Field of view from Figure 36 thresholded, to show only positively stained cells (black "dots"). Analyse particles settings in ImageJ also shown.

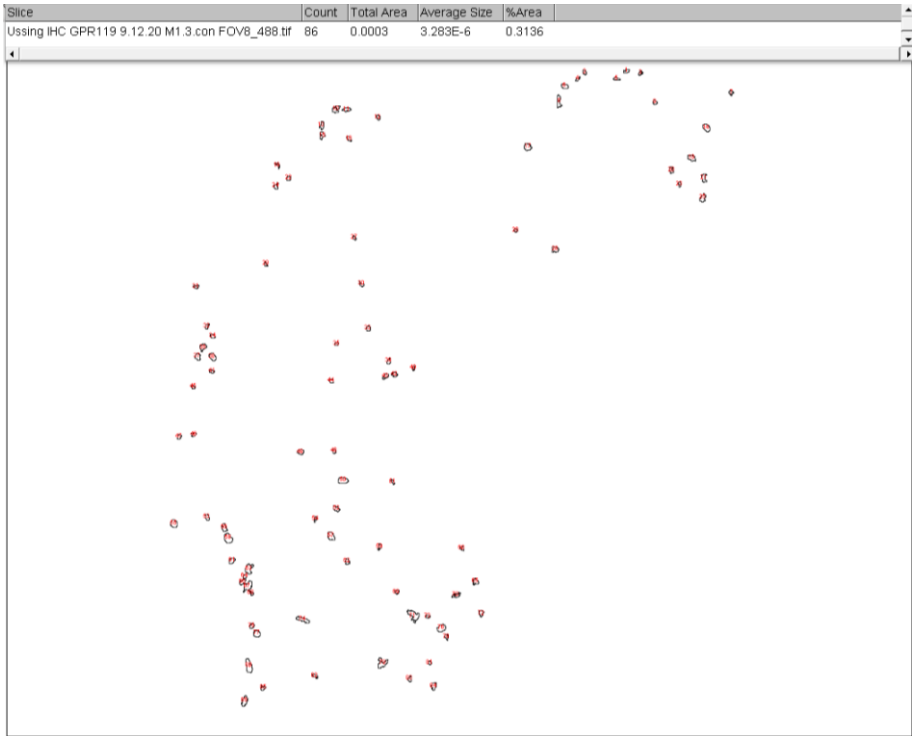


Figure 38: Counted cells as determined by the 'analyse particles' tool shown in Figure 37. Counted cells denoted by red ring. 86 CaMKII-positive cells were counted within this FOV. Image created and processed in ImageJ.

PYY or serotonin positive cells were, for the most part, much fewer and could be counted manually based on the inclusion and exclusion criteria, as previously described in Chapter 2 (Table 9). However, for pERK and CaMKII staining, the number of positively stained cells were much higher (in the 100s), therefore the automated counting method was used. The number of cells/ mm^2 was calculated for the GFP stain (CAMKII/ pERK) and RFP stain (serotonin/ PYY) stained cells individually, as well as the cells/mm^2 of GFP+RFP co-stained cells. The average number of cells/mm^2 was calculated for 5 FOVs for each condition (negative control, agonist concentration 1, agonist concentration 2). The average cells/ mm^2 for each mouse, agonist and condition (see Table 10) were then plotted by cell activation marker and hormone/ signalling peptide.

In effect, the number of CaMKII/ pERK positive cells were recorded twice: once for slides stained with CaMKII/pERK and serotonin antibodies, and again with CaMKII/ pERK and PYY antibodies. The count that was used for further calculations was the condition that had the highest number of observations. For instance, for CaMKII and serotonin (1 mM GPRC6a

agonist), a total of 19 FOVs were counted across 5 mice, whereas the same condition for CaMKII and PYY had 20 FOVs (25 would be the “maximum” observation count for each condition – 5 mice, 5 FOVs). Therefore, the latter CaMKII cell count would be used. This only affected graphs/ calculations of CaMKII/pERK cell count alone. Images/ mice were discounted from the final counts where there was poor staining/ poor tissue condition. Therefore, not all conditions have n=5 counts for each of the 5 average field of views or 5 mice per group.

Insets for figures created with code by Gilles Carpentier, Faculté des Sciences et Technologie, Université Paris Est Créteil, Val de Marne, France, in ImageJ.

3.2.6 Statistical and data analysis

Statistical analysis for cell counts were performed using one-way ANOVA (Kruskal-Wallis test, Dunn’s multiple comparisons test) (GraphPad Prism, V9, GraphPad Software, Inc), with $p < 0.05$ considered statistically significant. Data is expressed as mean ± SEM. ANOVA was used because we compared more than 2 internal data sets representing a control solution and two different concentrations. A t test can therefore not be used in this scenario.

Tests for normality were performed using the Shapiro-Wilk test as previously described. Often there were not enough data points to assess normality (N=3-4). Where N=5, data passed normality (not shown).

3.3 Results

3.3.1 Optimising antibodies for the cell activation markers CaMKII and pERK

3.3.1.1 CaMKII antibody optimisations

Optimisation experiments were initially performed with the CaMKII antibody V1111 (Promega) that had been published in Symonds, Peiris *et al.* 2015 (Symonds et al., 2015). Despite staining well, it was discontinued by the manufacturer, therefore a new CaMKII antibody was obtained (ab5683, Abcam).

Mouse colon tissue stimulated with a low and high concentration (5.5, 10 mM respectively) of glucose demonstrated robust staining and expression of CaMKII, without high levels of background. The 5.5 mM concentration is the same used in the Krebs solution: to be low enough to not cause background activation of colonocytes, yet still prevent the cells from undergoing glucose-starvation. This ensures the cellular activation seen is due to GPCR stimulation, not glucose. The 1:200 and 1:400 concentrations of CaMKII antibody showed weak staining, therefore the 1:100 concentration was used, as it provided a bright signal in stimulated and unstimulated tissue (Figure 39 B, F). Insets of Figure 39 B and C show classic EEC flask-like shape, with a luminal tail and thicker base.

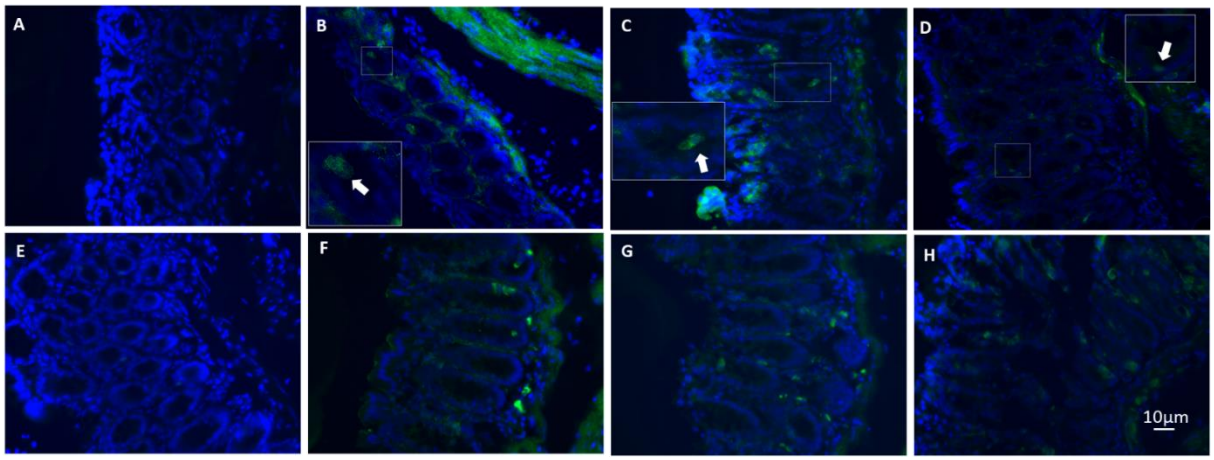


Figure 39: Immunolabelling of CaMKII (denoted with white arrow) at three different concentrations (1:100 – B, F, 1:200 – C, G, 1:400 – D, H) in murine colonic tissue stimulated in an Ussing chamber with a low (5.5 mM, A-D) and high (10 mM, E-H) glucose concentration, fixed and stained by IHC. CaMKII stained in green, DAPI blue. No primary antibody control condition (A, E) had no CaMKII antibody added to slides. Insets created with code by Gilles Carpentier, Faculté des Sciences et Technologie, Université Paris Est Créteil, Val de Marne, France, in ImageJ.

3.3.1.2 pERK antibody optimisation

Figure 40 and Figure 41 show staining with a pERK antibody of murine tissue from 3 mice, stimulated with a high and a low/ high concentration of glucose respectively.

In Figure 40 I see positive staining with the pERK antibody at 1:100 and 1:400 in tissue from 3 mice stimulated with a high concentration of glucose (10 mM). The tissue stained with a 1:100 concentration of pERK was oversaturated, whereas the 1:400 concentration demonstrated acceptable signal to noise ratio.

In Figure 41 a similar experiment was run, except it also included tissue stimulated with the low (5.5 mM) concentration of glucose, as well as high, with tissue from the same mice, using the 1:400 concentration of pERK antibody. We saw no background but robust staining of pERK, in both the stimulated and unstimulated tissue.

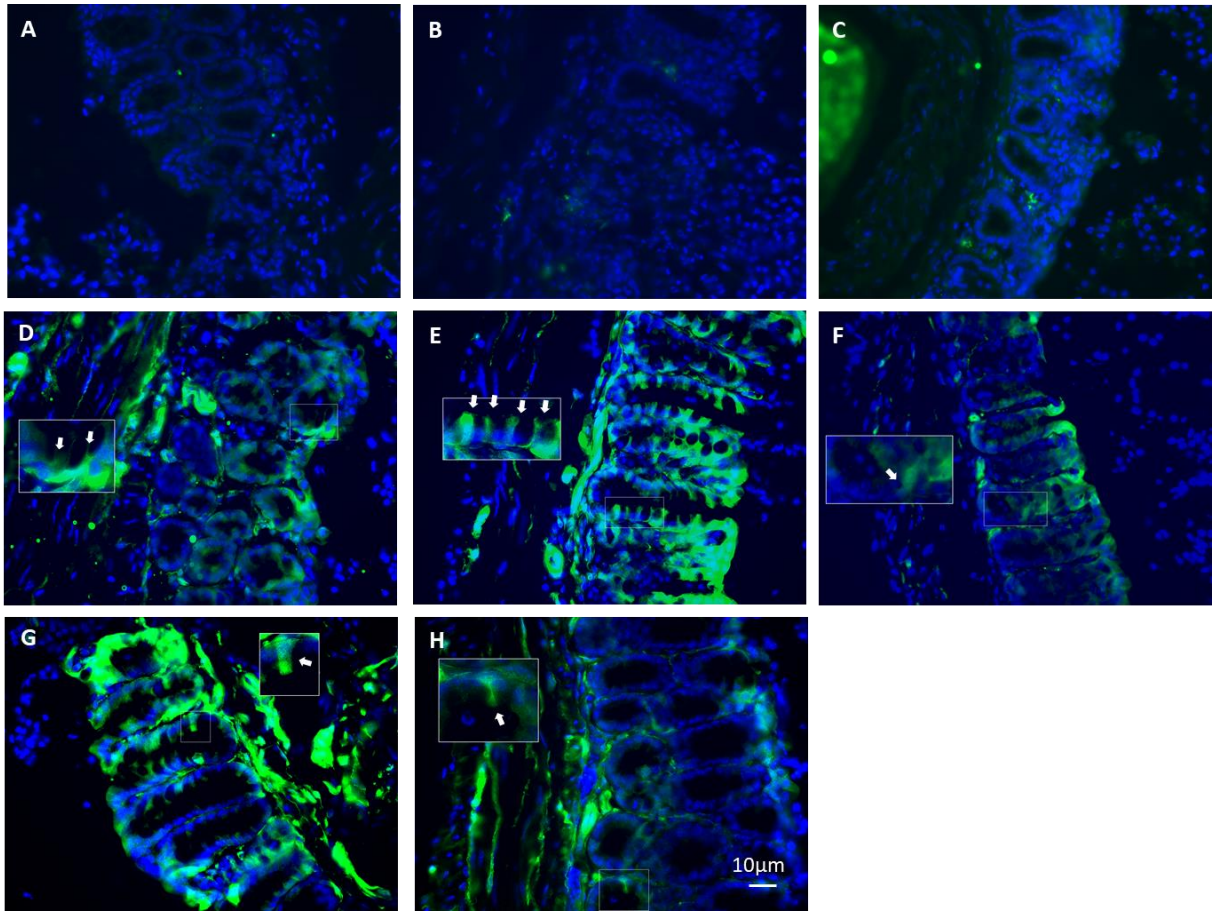


Figure 40: Staining of murine colonic tissue stimulated with a 10 mM glucose Krebs solution in an Ussing chamber then fixed for IHC. pERK (at 1:100 (D-F) and 1:400 (G, H) concentration) stained in green, DAPI in blue. Representative images of 3 mice: M1 (A, D, G), M2 (B, E, H), M3 (C, F). There is no image for M3 1:400 due to an experimental issue. The no primary antibody control condition (A-C) had no pERK antibody added to slides. Insets show pERK-positive EECs (white arrows). Of note is the flask-like shape, with the luminal tail and thicker base.

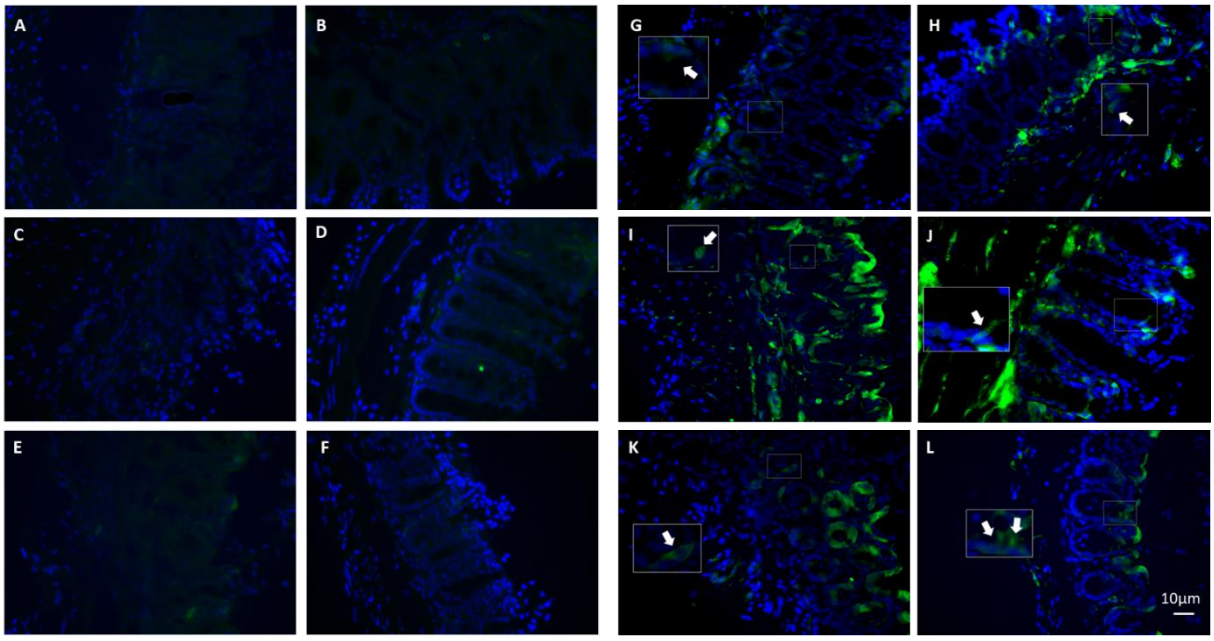


Figure 41: Staining of murine colonic tissue stimulated with a 5.5 mM (A, C, E, G, I, K) or 10 mM (B, D, F, H, J, L) glucose Krebs solution in an Ussing chamber, then fixed for IHC. Representative images of 3 mice: M1 (A, B, G, H), M2 (C, D, I, J), M3 (E, F, K, L). pERK at 1:400 concentration (G-L) is stained in green, DAPI in blue. No primary antibody control condition (A-F) had no pERK antibody added to slides.

3.3.2 Stimulation with a GPR119 agonist did not alter the activation or expression of EECs

Colonic tissue from 5 mice was stimulated with 1 µM or 10 µM of the GPR119 receptor agonist PSN375963 (in Krebs) or buffer control (Krebs only). Cells were stained for CaMKII or pERK (Figure 42A, B) as well as co-stained with CaMKII or pERK with serotonin or PYY (Figure 42C-D). Cells were co-stained to delineate activated PYY or serotonin-positive cells (via the pERK or CaMKII pathway), in response to the GPR119 agonist.

I observed no significant change in the expression of CaMKII or pERK positive cells (Figure 42 G, H). Furthermore, I observed no significant change in the expression of CaMKII or pERK positive cells co-stained with serotonin (Figure 42 I, J) or PYY (Figure 42 K, L) with stimulation of either concentration of GPR119 agonist. Serotonin positive cells co-stained with CaMKII or pERK were more common than PYY co-stained cells. I saw the lowest expression of pERK/ PYY cells, with PYY co-stained cells in general being the rarest (Figure 42

L). I also saw the highest expression of CaMKII/ serotonin co-stained cells (Figure 42 I). Generally pERK staining occurred on the luminal surfaces of crypts, whereas staining for serotonin and PYY occurred towards the submucosa, as described in Chapter 1. CaMKII staining was distributed evenly throughout the colonic tissue.

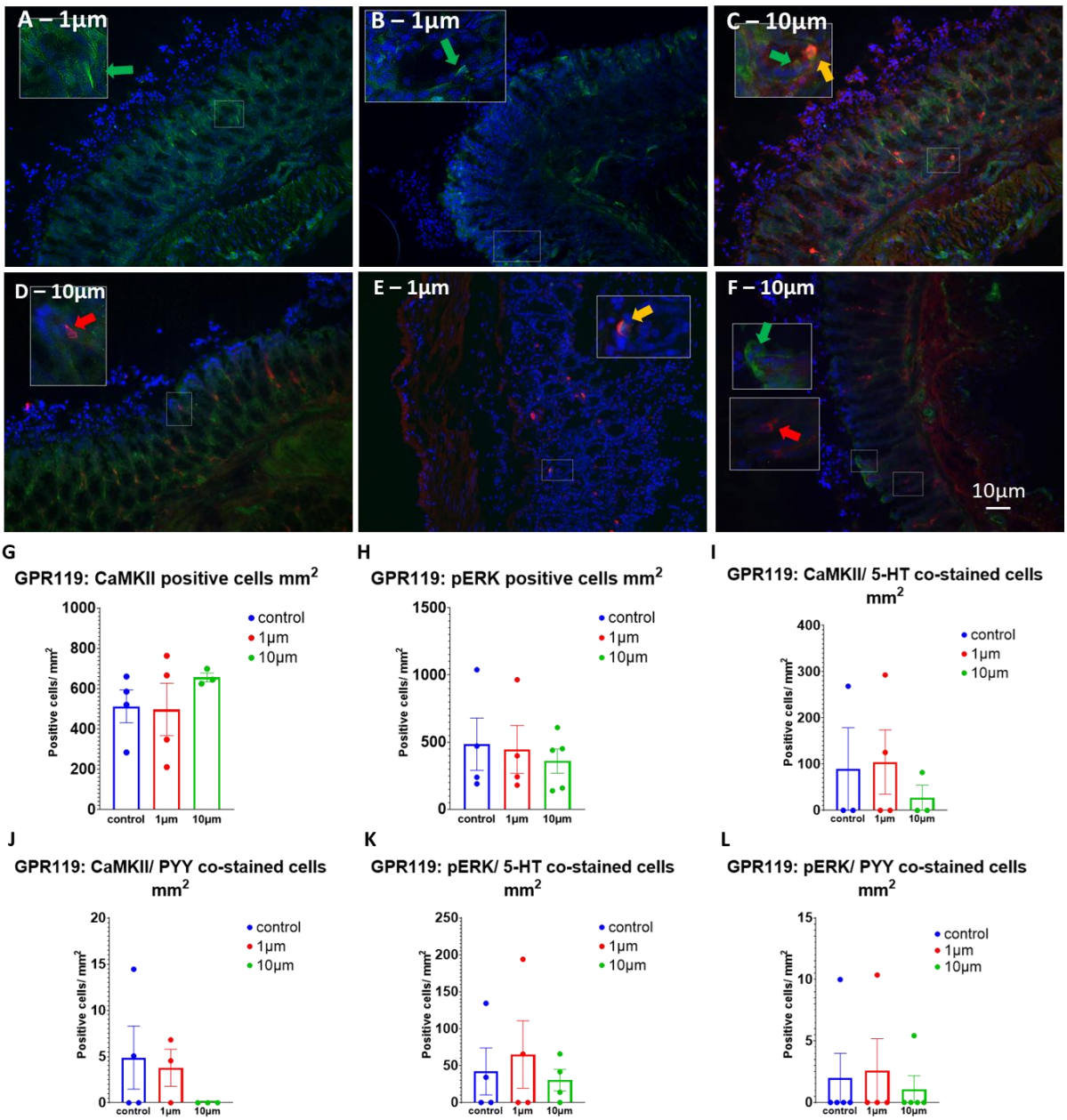


Figure 42: Expression of CaMKII, pERK, serotonin, PYY in cells from murine colonic tissue, stimulated with a GPR119 agonist. A-F = representative images of IHC staining (mixed concentration (blue stain = DAPI)). Images showing: CaMKII-positive cells (A, green); pERK-positive cells (B, green), CaMKII/ serotonin co-stained cells (C, green = CaMKII, red = serotonin); CaMKII/ PYY co-stained cells (D, green = CaMKII, red = PYY); pERK/ serotonin co-stained cells (E, green = pERK, red = serotonin); pERK/ PYY co-stained cells (F, green = pERK, red = PYY). Arrows denote singly stained (red, green) and co-stained (orange) cells. G-L = count of cells/mm2 stained with antibodies for the previous conditions, at two different concentrations (1/ 10µM in Krebs) and control (Krebs solution alone).

3.3.3 The expression of pERK/ serotonin co-stained cells was significantly increased in tissue stimulated with the GPR40 agonist AS2034178

Colonic tissue from 5 mice was stimulated with 1 µM or 10 µM of the GPR40 receptor agonist AS2034178 (Tocris) in Krebs or a control condition (just Krebs). Cells were stained for CaMKII or pERK (Figure 43 A, B) as well as co-stained with CaMKII or peRK with serotonin or PYY (Figure 43C-D).

I observed a significant increase (p = 0.0482) in the number of pERK/ serotonin co-stained cells/mm2 following stimulation with 1 µm AS2034178 compared to buffer control (Figure 43 K). I saw no significant change in the expression of cells stained with other markers after stimulation with the GPR40 agonist (Figure 43 G-J, L). Serotonin positive cells co-stained with CaMKII or pERK were more common than PYY co-stained cells. I observed the lowest expression of pERK/ PYY cells (Figure 43 L), with PYY co-stained cells in general being the rarest. The highest expression was observed in pERK/ serotonin co-stained cells (Figure 13K). In general staining occurred within colonic crypts in the centre of the tissue, rather than at the luminal or submucosal regions.

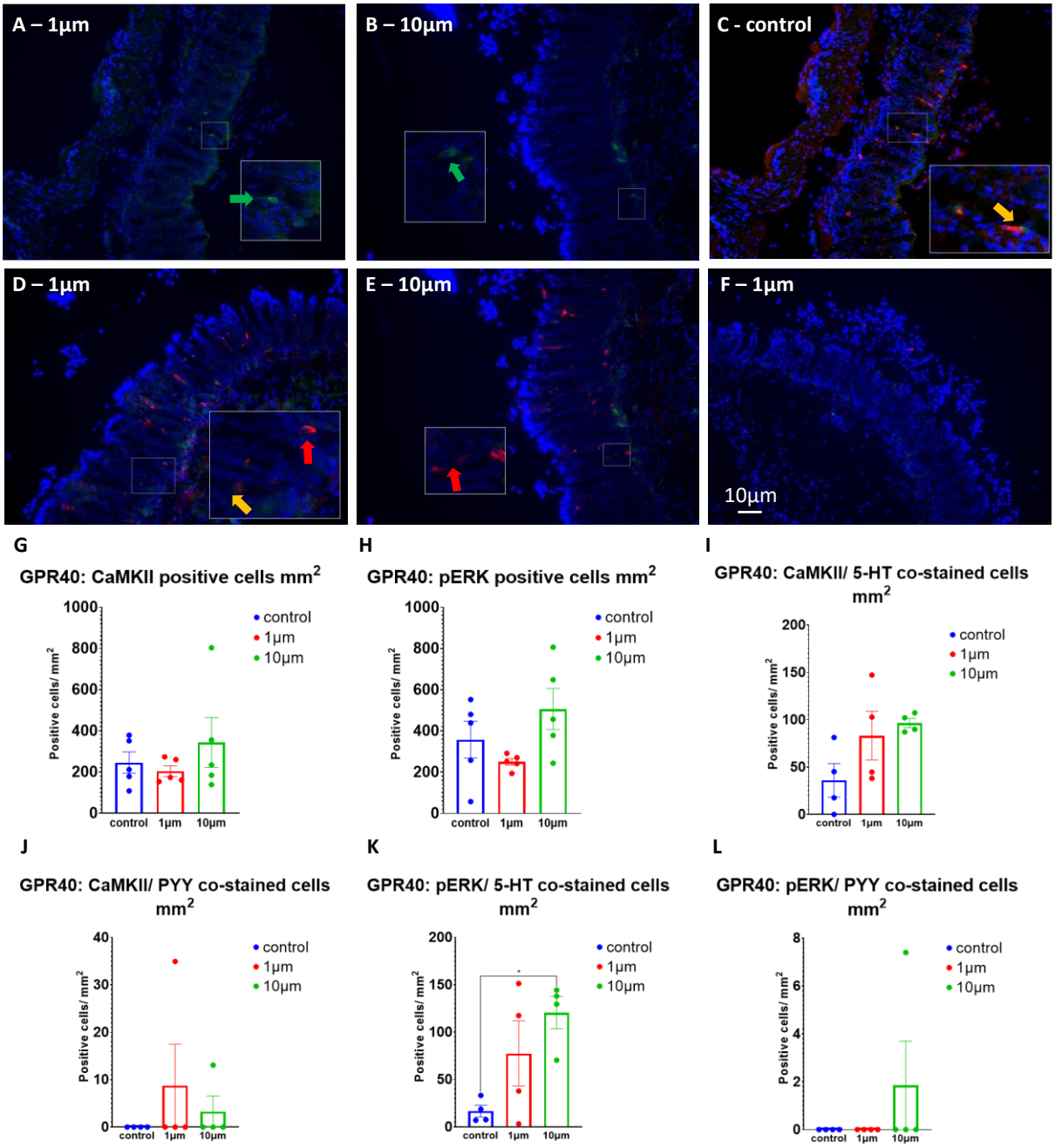


Figure 43: Expression of CaMKII, pERK, serotonin, PYY in cells from murine colonic tissue, stimulated with the GPR40 agonist AS2034178. A-F = representative images of IHC staining (mixed concentration, (blue stain = DAPI). Images showing: CaMKII-positive cells (A, green); pERK-positive cells (B, green), CaMKII/ serotonin co-stained cells (C, green = CaMKII, red = serotonin); CaMKII/ PYY co-stained cells (D, green = CaMKII, red = PYY); pERK/ serotonin co-stained cells (E, green = pERK, red = serotonin); pERK/ PYY co-stained cells (F, green = pERK, red = PYY). Arrows denote singly stained (red, green) and co-stained (orange) cells.

3.3.4 Stimulation with a GPR120 agonist did not alter the activation or expression of EECs

Colonic tissue from 5 mice was stimulated with 1 µM or 10 µM of a GPR120 receptor agonist TUG891 (Tocris) in Krebs or a control condition (just Krebs). Stimulated tissue was fixed, stained and the numbers of cells/ mm^2 calculated.

In Figure 44 I saw no significant change in expression of CaMKII, pERK positive cells co-stained with serotonin and PYY (Figure 44 I-L), with stimulation of either concentration of GPR120 agonist. Serotonin positive cells co-stained with CaMKII or pERK were more common than PYY co-stained cells. I observed the lowest expression of pERK/ PYY cells, with PYY co-stained cells in general being the rarest, and the highest expression being of CaMKII/ serotonin co-stained cells.

In general, co-staining of activation marker and hormone/ mediator marker was rare, despite similar numbers of CaMKII/ pERK single-stained cells compared to other agonist experiments. When co-staining did occur, it was observed mostly in the upper half of the tissue facing the lumen.

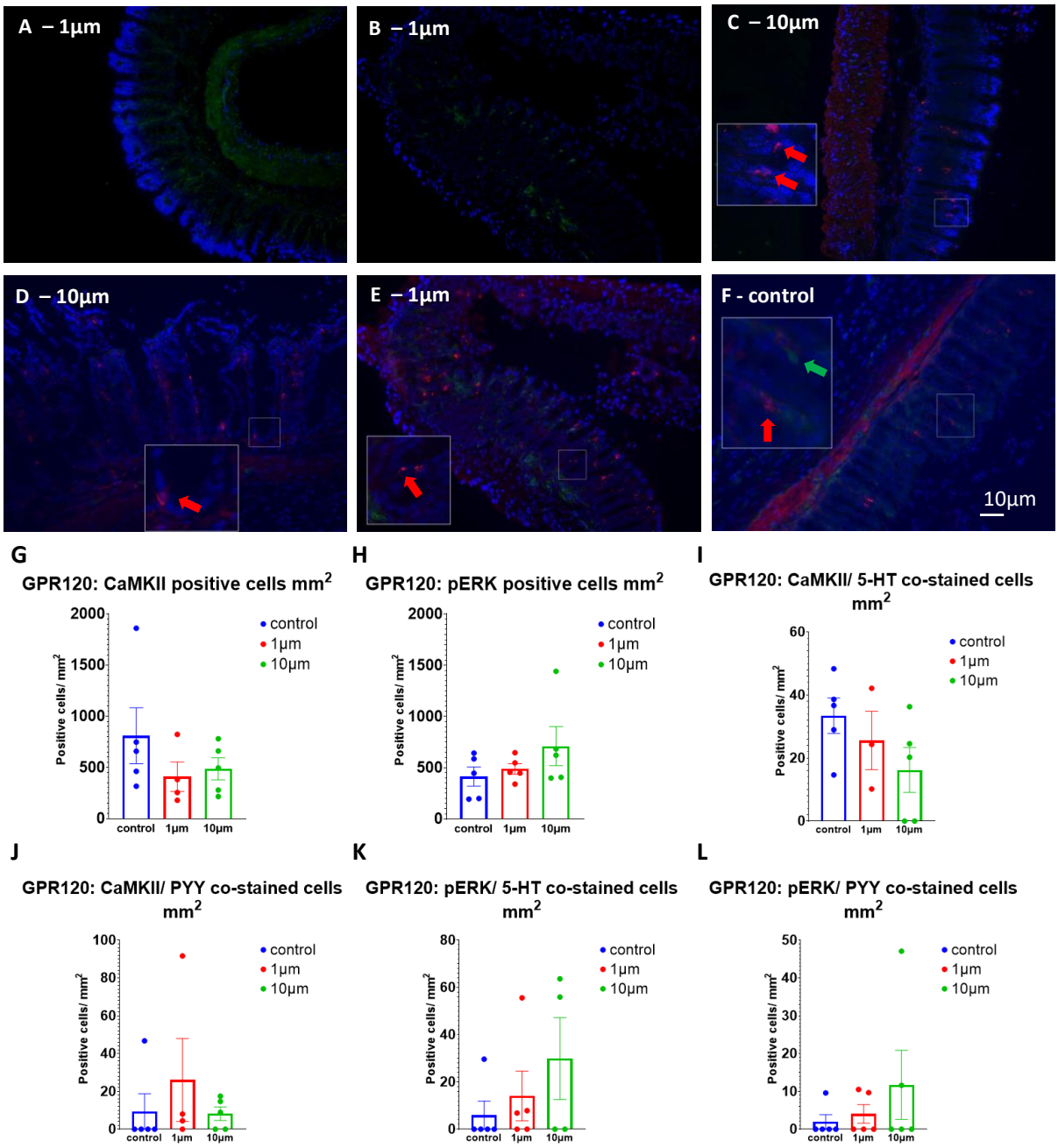


Figure 44: Expression of CaMKII, pERK, serotonin, PYY in cells from murine colonic tissue, stimulated with the GPR120 agonist TUG891. A-F = representative images of IHC staining (mixed concentration, (blue stain = DAPI). Images showing: CaMKII-positive cells (A, green); pERK-positive cells (B, green), CaMKII/ serotonin co-stained cells (C, green = CaMKII, red = serotonin); CaMKII/ PYY co-stained cells (D, green = CaMKII, red = PYY); pERK/ serotonin co-stained cells (E, green = pERK, red = serotonin); pERK/ PYY co-stained cells (F, green = pERK, red = PYY). Arrows denote singly stained (red, green) cells.

3.3.5 Stimulation with a GPRC6a agonist did not alter the activation or expression of EECs

Colonic tissue from 5 mice was stimulated with 1 mM or 5 mM of a GPRC6a receptor agonist L-arginine (Sigma) in Krebs or a control condition (just Krebs). Stimulated tissue was fixed, stained and the numbers of cells/ mm^2 calculated.

In Figure 45 I saw no significant change in expression of CaMKII, pERK positive cells co-stained with serotonin and PYY (Figure 45 I-L), with stimulation of either concentration of GPR120 agonist. Serotonin positive cells co-stained with CaMKII or pERK (Figure 45 I, K) were more common than PYY co-stained cells (Figure 45 J,L). I observed the lowest expression of pERK/ PYY cells (Figure 45 L), with PYY co-stained cells in general being the rarest. CaMKII/ serotonin co-stained cells were most highly expressed (Figure 45K).

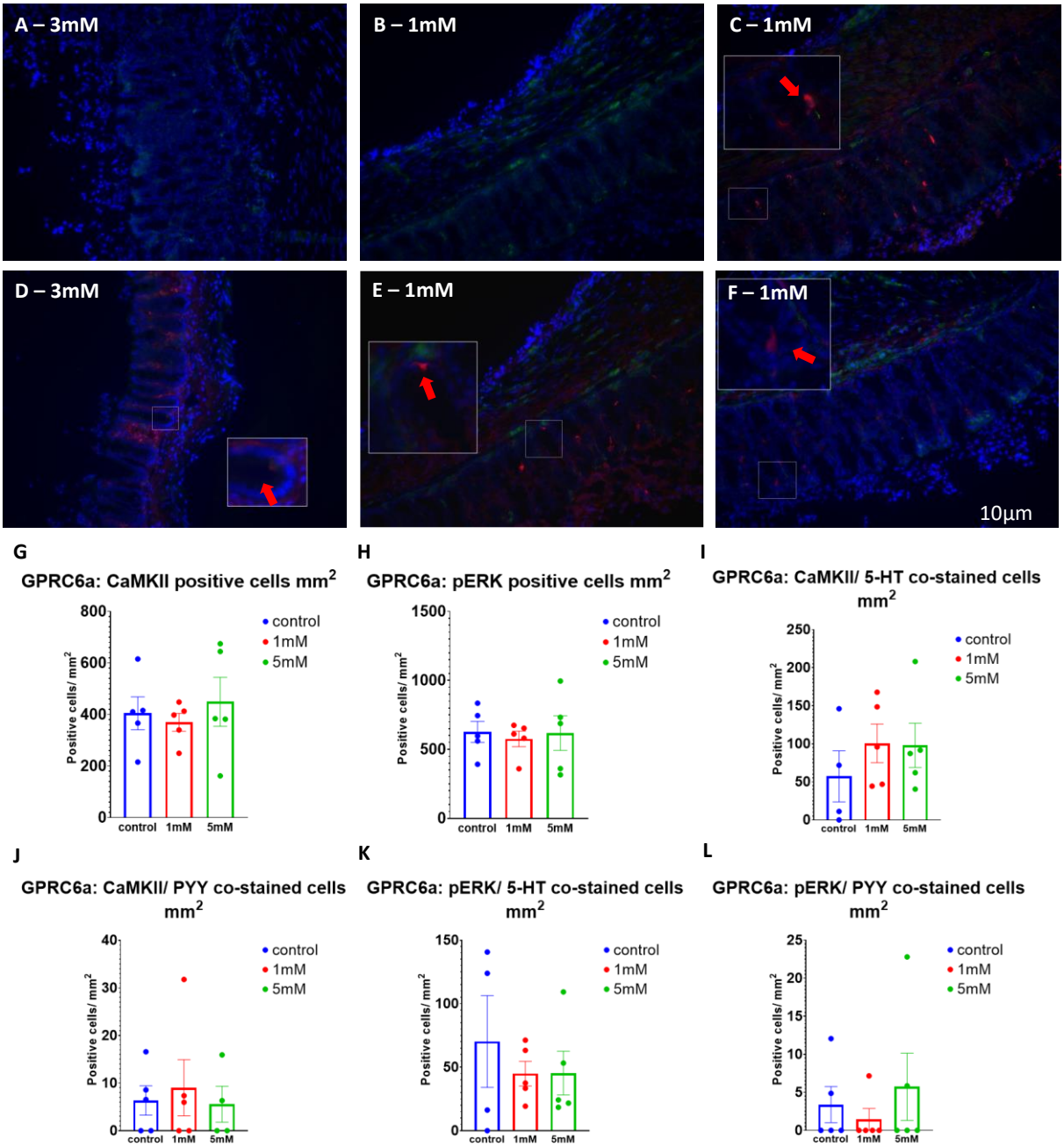


Figure 45: Expression of CaMKII, pERK, serotonin, PYY in cells from murine colonic tissue, stimulated with the GPCR6a agonist L-arginine. A-F = representative images of IHC staining (mixed concentration, (blue stain = DAPI). Images showing: CaMKII-positive cells (A, green); pERK-positive cells (B, green), CaMKII/ serotonin co-stained cells (C, green = CaMKII, red = serotonin); CaMKII/ PYY co-stained cells (D, green = CaMKII, red = PYY); pERK/ serotonin co-stained cells (E, green = pERK, red = serotonin); pERK/ PYY co-stained cells (F, green = pERK, red = PYY). Arrows denote singly stained (red, green) cells.

3.3.6 The expression of CaMKII/ PYY co-stained cells was significantly decreased in tissue stimulated with the CaSR agonist L-tryptophan.

Colonic tissue from 5 mice was stimulated with 1 mM or 3mM of a CaSR receptor agonist L-tryptophan (Sigma) in Krebs or a control condition (just Krebs). Stimulated tissue was fixed, stained and the numbers of cells/mm^2 calculated.

In Figure 46J, I observed a significant decrease in the number of CaMKII/ PYY co-stained cells/ mm^2 between the control condition and the 3 mM concentration (p = 0.0384). There was no significant change in the expression of cells stained with other markers after stimulation with the CaSR agonist. Serotonin positive cells co-stained with CaMKII or pERK (Figure 46I, K) were more common than PYY co-stained cells (Figure 46 J, L). I saw the lowest expression of pERK/ PYY cells (Figure 46 L), with PYY co-stained cells in general being the rarest. CaMKII/ serotonin and CaMKII/ PYY co-stained cells were most highly expressed (Figure 46 I, J).

Overall, co-staining of activated cells and PYY or serotonin was uncommon. Of note is the neuropod structure and “open” shape of the PYY-positive cell inset in Figure 46 F; this has been previously demonstrated in EECs and specifically L cells (Liddle, 2019).

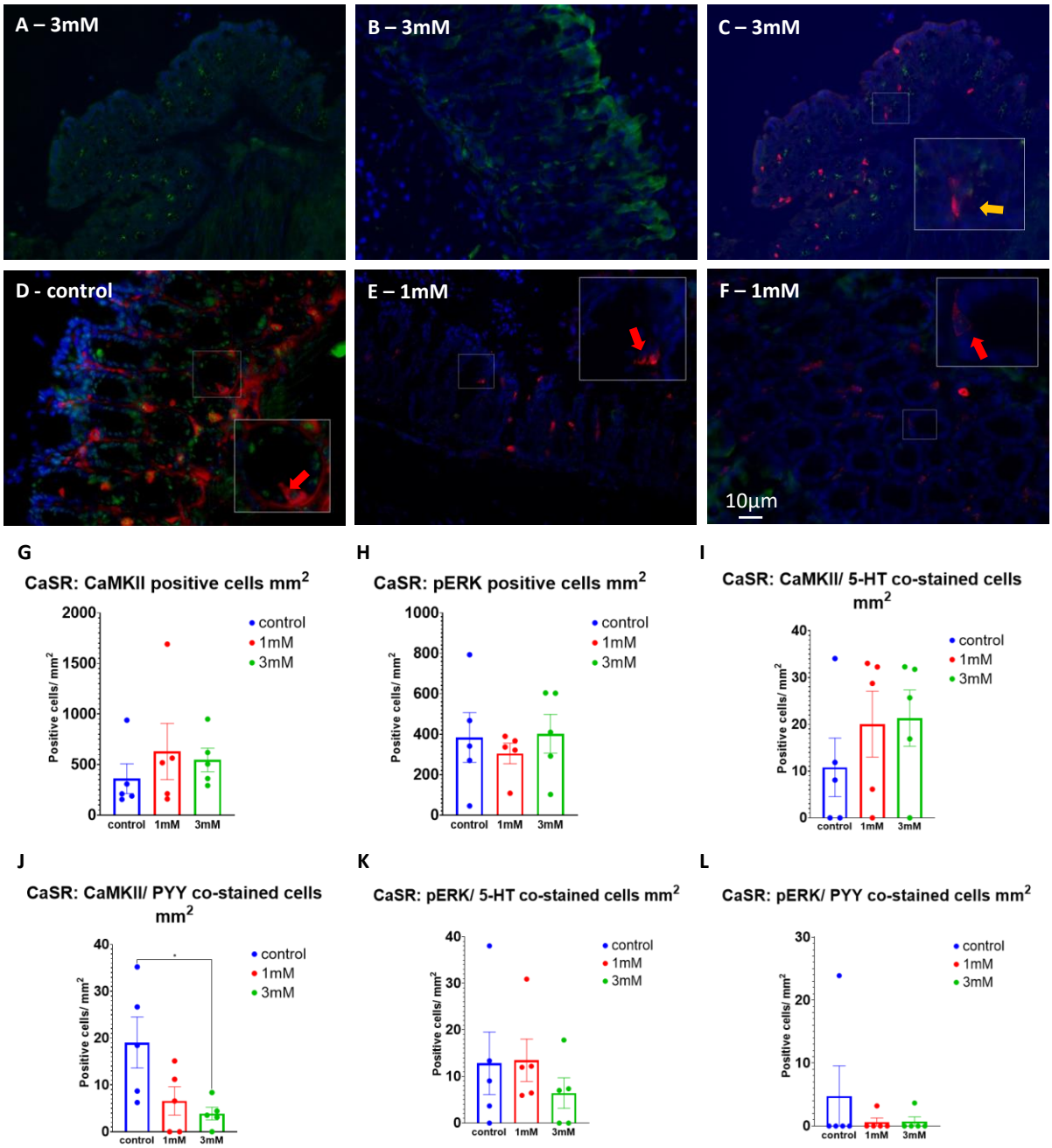


Figure 46: Expression of CaMKII, pERK, serotonin, PYY in cells from murine colonic tissue, stimulated with the CaSR agonist L-tryptophan. A-F = representative images of IHC staining (mixed concentration, (blue stain = DAPI). Images showing: CaMKII-positive cells (A, green); pERK-positive cells (B, green), CaMKII/ serotonin co-stained cells (C, green = CaMKII, red = serotonin); CaMKII/ PYY co-stained cells (D, green = CaMKII, red = PYY); pERK/ serotonin co-stained cells (E, green = pERK, red = serotonin); pERK/ PYY co-stained cells (F, green = pERK, red = PYY). Arrows denote singly stained (red, green) cells.

Table 14: Summary of the change in number of stained cells across agonist conditions in Chapter 3

Stained/ co-stained cells	GPR119	GPR40	GPR120	GPRC6a	CaSR
CaMKII	*No change*	*No change*	*No change*	*No change*	*No change*
pERK	*No change*	*No change*	*No change*	*No change*	*No change*
CaMKII/ 5-HT	*No change*	*No change*	*No change*	*No change*	*No change*
CaMKII/ PYY	*No change*	*No change*	*No change*	*No change*	Significant decrease in 3mM vs control
pERK/ 5-HT	*No change*	Significant increase in 10µM vs control	*No change*	*No change*	*No change*
pERK/ PYY	*No change*	*No change*	*No change*	*No change*	*No change*

3.4 Discussion

The aim of this chapter was to examine how agonist stimulation of specific GPCRs modulates intracellular pathways in PYY-expressing L cells and serotonin-expressing ECs, to understand how they might be able to regulate satiety. Table 14 gives a summary of the results of this chapter.

The highly selective GPR119 agonist PSN375963 (EC_{50} 7.9 M for murine GPR119) (Overton et al., 2006b) did not significantly alter cell activation, or PYY or serotonin expression in these cells. Research by others on the role of PYY in GPR119-expressing cells has led to contradictory observations. One study demonstrated that agonist stimulation of GPR119 in non-obese individuals did not increase PYY or CCK plasma levels, instead increasing GLP-1 levels (Hansen et al., 2011). While Gribble *et al.* showed that agonist stimulation of GPR119 (agonist AR231453) did not activate human colonic cells to release GLP-1 or PYY but did so in murine cells (Habib et al., 2013). Tough *et al.* demonstrated GPR119 activity in human and murine colonic tissue is PYY-dependent: OEA depolarised L cells and slowed colonic transit - both could be abolished by treating tissue with PYY receptor antagonists (Tough et al., 2018b). Chu *et al.* showed that GPR119 expression in the colon was most highly co-expressed with GLP-1 in an L cell line and that activation of the

receptor in GLUTag cells expressing GPR119 increased GLP-1 release and cAMP stimulation (Chu et al., 2008).

Our data suggests that serotonin positive cells are not activated by GPR119 stimulation with PSN375963. GPR119 has not been shown to be expressed on serotonin-positive, FACS-purified murine SI cells and in general is expressed at low levels in the colon (Lund et al., 2018), being more highly expressed on GLP-1 positive cells of the SI (Ekberg et al., 2016) which reflects the data presented here.

The disparate results from studies on GPR119 activation, could be explained by the focusing of studies in the proximal jejunum and the colon as a whole, as well as the choice of agonists. GPR119 is most highly expressed in the distal and ascending colon (Tough et al., 2018b), though to a lesser extent than in the jejunum, duodenum and stomach (Chu et al., 2008) in mice and humans. Different agonists could induce a differential GLP-1/ PYY release: colonic EC and L cells that express GPR119 are not activated by the OEA analogue PSN375963 but by 2-monoacyl-glycerol, which is formed by the hydrolysis of triacylglycerol (among other products) by the pancreas (Janssen and Depoortere, 2013) and which has been shown to promote GLP-1 release, but not PYY (Hansen et al., 2011). Alternatively, GPR119 activation could drive activation of cellular pathways other than ERK. Chu *et al.* demonstrated that the GPR119 agonist AR231453 significantly increases cAMP accumulation (Chu et al., 2008), which could activate PKA or other cAMP effectors which could lead to downstream effects including transcriptional regulation and changes in calcium and ion channels (Sassone-Corsi, 2012). Therefore, this modulation of cAMP could lead to hormone release and regulation of satiety, independent of the CaMKII or pERK pathways, upon GPR119 activation.

The number of cells expressing both pERK and serotonin in colonic tissue stimulated with 10 µM (but not 1 µM) of the GPR40-specific agonist AS2034178 significantly increased, compared to the control. Information on hormone and receptor co-expression in the colon is limited: EECs in the murine duodenum and SI have been shown to co-express GPR40 with PYY and serotonin (Edfalk et al., 2008). My data suggests that GPR40 activation may target preferentially the pERK pathway in EC cells. However, other studies have shown that stimulation of mouse colonic mucosa with a GPR40 agonist increased the numbers of both

pERK and CaMKII immunoreactive cells (Peiris et al., 2018). In addition, serotonin-containing colonic cells have been shown to be activated via the CaMKII pathway, whilst PYY, GLP-1 containing cells were activated via the pERK pathway (Peiris et al., 2021).

Research is currently lacking on the role of serotonin in GPR40-positive colonic cells. It is known these cells express serotonin: 21% and 19% of murine, GPR40 expressing cells express PYY and serotonin, respectively (Edfalk et al., 2008). This small population of cells might be the same population which increased expression of serotonin in response to GPR40 and pERK activation. AS2034178 activation of GPR40 has been shown to decrease weight and improve whole-body glucose metabolism in mice, decrease plasma levels and production of glucose and decrease blood HbA1C levels (Tanaka et al., 2013). The study authors were unable to explain these effects; our data would seem to indicate that increased expression of serotonin in a population of colonic EECs, activated via the pERK pathway, could be the causative factor. In the following chapter, I will examine the release of serotonin from these cells, after GPR40 activation. I saw very little PYY staining/ co-staining in our cells and unaltered by GPR40 stimulation, suggesting activated cells are not PYY-positive. Though GPR40-positive cells have been shown to express PYY (Habib et al., 2013), I consistently see rare PYY staining throughout our experimental conditions, therefore it is possible that the sections may not contain this cell type.

TUG891 (GP120 agonist) did not change the number of cells expressing CaMKII, pERK or cells co-stained with serotonin or PYYTUG891. Data on GPR120 activation specifically with TUG891 is conflicting. TUG891 has been shown to activate GPR120 in a manner similar to *α*-linolenic acid in humans (Hudson et al., 2013); GPR120 activation is associated with increased $[Ca^{2+}]_i$, ERK activation and increased release of GLP-1 and CCK in STC-1 cells (Hirasawa et al., 2005, Tanaka et al., 2008). Peiris *et al.* did not see activation of pERK by TUG891 stimulation (Ussing) of tissue; instead, they reported CaMKII activation (Peiris et al., 2021). In our study I did not see an increase in pERK or CaMKII activation in the murine colon in L or EC cell populations. The most likely reason is that these studies assess GPR120 activation in human cell lines/ tissue, whereas the present study used mice, and intracellular signalling pathways may be species specific.

The numbers of cells expressing the activation markers CaMKII or pERK and co-expressing serotonin, PYY, in colonic tissue stimulated with the GPRC6a agonist L-arginine were unchanged, compared to control.

In the colon GPRC6a is expressed on L cells (Oya et al., 2013) and is a receptor for lysine, alanine, ornithine and L-arginine (Clemmensen et al., 2014). However, there are currently no studies that examine the expression or role of GPRC6a activation in serotonin positive enteroendocrine cells: in fibroblasts, L-arginine stimulation leads to activation of ERK pathways in a GPRC6a-dependent manner (Fujiwara et al., 2014), while in HEK-293T cells cAMP pathways are activated (Pi et al., 2012). It is possible that GPRC6a activation induces cellular activation in the colon, not via pERK or CaMKII pathways, but instead via cAMP pathways. Alternatively, GPRC6a activation may not affect the release of PYY or serotonin, but instead regulate release of other anorectic hormones like CCK.

In general, the numbers of all colonic cells expressing CaMKII or pERK were unaffected by the CASR agonist, L-tryptophan. Acar *et al.* have shown that CaSR activation with L-tryptophan on EECs promotes the release of CCK and GLP-1, but not PYY (Acar et al., 2020). I therefore expected to see a change in activated serotonin-positive, rather than PYY-positive cells, however, I saw no change in the former. However, the significant reduction in the number of CaMKII/ PYY co-expressing cells at the 3 mM concentration, compared to the control matches data by Acar *et al.*: stimulation with L-tryptophan inhibits and reduces PYY release (Acar et al., 2020). L-tryptophan stimulation of colonic tissue might therefore reduce the number of activated L cells, despite increased GLP-1 release (Tang et al., 2016, Reimann et al., 2012). CaSR activation is associated with anorectic effects: increased post-prandial release of GLP-1, GIP, insulin and the regulation of fluid movement in the colon (Tang et al., 2016) so I expected to see an increase in the activation of PYY-positive (L cells). It is possible that GLP-1 release occurs via mechanisms independent of pERK or CaMKII pathways, instead perhaps activating cAMP pathways (Hebert et al., 2004) or PI-PLC-IP3-driven increases in intracellular Ca^{2+} (Hebert et al., 2004). Alternatively, L-tryptophan may drive specific cellular pathways and other amino-acid CaSR agonists may drive others: L-glutamine has been shown to stimulate the co-release of GLP-1 and PYY from murine colonic EECs, with a CaSR agonist/ antagonist enhancing and inhibiting these responses, respectively

(Joshi et al., 2013). Additionally, just because one particular activation pathway shows reduced expression, it does not mean that hormone release, or overall cell activation is reduced.

CaSR has been shown to be co-expressed with serotonin (though at a low level) in the murine colon (Lund et al., 2018) and CaSR agonist-dependent reductions in the glycaemic response to an oral glucose tolerance test is abrogated by a serotonin receptor antagonist in rats (Muramatsu et al., 2014). These tests were carried out after oral and duodenal CaSR agonist administration (not L-tryptophan): it is not possible to determine the exact role of colonic CaSR stimulation in this study. This may explain why there is no change in the activation of serotonin-positive cells. L-tryptophan is a ligand for CaSR, tryptophan hydroxylase and the substrate for the biosynthesis for serotonin (Conigrave et al., 2000b): CaSR might compete with tryptophan hydroxylase for tryptophan, reducing CaSR activation or serotonin expression. A tryptophan hydroxylase inhibitor could have been used to eliminate any competition with the enzyme. CaSR has also been shown to have separate receptor functions: at low/ high concentrations of extracellular calcium acting as a calcium/ aromatic l-amino acid respectively (Conigrave and Brown, 2006). Extracellular calcium and amino acids can also act as co-agonists of CaSR (Conigrave et al., 2000a, Young and Rozengurt, 2002) and extracellular calcium has been shown to enhance L-tryptophan binding (EC50 0.5 mM to 0.12 mM) (Geng et al., 2016). Krebs solution, in which both the control and experimental solutions were prepared, contains 2.5 mM $CaCl_2$ - (high concentration) and CaSR should have not only functioned as an amino acid receptor, but also enhanced tryptophan binding.

To conclude, agonist stimulation of nutrient receptors significantly altered the activation of cells expressing PYY, serotonin via the CaMKII/ pERK intracellular pathways. In Chapter 1, I demonstrated increased mRNA expression of GPR40 in the human sigmoid colon and in this Chapter I showed that stimulation of this receptor in (normal weight) mice increases the number of serotonin, pERK expressing cells. This might indicate that stimulation of an increased population of GPR40 expressing cells might increase cellular activation, especially in EC. Whether there is a subsequent increased serotonin release remains to be investigated and will form part of Chapter 3. I saw a significant decrease in the expression of serotonin

and PYY in CaMKII positive cells in tissue stimulated with amino acids. This might reflect an orexigenic effect of amino acids, however, it is more likely that high levels of amino acids signal via either different pathways (cAMP) or different peptides/ hormones (CCK, GLP-1). I chose CaMKII and pERK activation pathways to study as part of this chapter, however there are many other pathways that can be activated downstream of GPCRs, including the cAMP, PI3 and PKC pathways (Kimura et al., 2020). Therefore, a change in the number of cells activated by one pathway does not mean that other pathways might also be altered, or that it is the pathway responsible for hormone/ mediator release in GPCR-expressing cells.

In the following chapter, I will examine the ability of stimulated and activated cells to release PYY and serotonin; this will allow us to complete the picture of mRNA expression, cellular expression, cellular activation and peptide and hormone release, that makes up an important nutrient signalling pathway in the colon.

4 Chapter 4: Examining how the release of hormones/ mediators from cells changes upon exposure to GPCR agonists, antagonists or a CaMKII inhibitor.

4.1 Introduction

In Chapter 2 I examined the changes in the mRNA and cellular expression of nutrient receptors and hormones with changing BMI. In Chapter 3 I examined how nutrient receptor stimulation changed the number of activated EECs. In this final chapter, I will examine how these activated cells release hormones/ peptide in response to stimulation.

It is important to look at this final step in the cellular pathway - a decrease in the number of CaSR-activated (CaMKII pathway) PYY-positive cells that I saw in Chapter 3 (Figure 46), might not lead to a decrease in PYY release from these cells. This is important for understanding how nutrient sensing occurs in the normal colon and how it might change in the obese state.

I have chosen to focus on the nutrient receptors CaSR, GPR120 and GPR40 in this chapter. I aim to determine if any changes in GPCR expression seen in previous chapters are matched by a subsequent change in PYY or serotonin release.

Through Chapters 2 and 3, CaSR was the only GPCR which showed robust, consistent staining in tissue. I can therefore better understand the role of CaSR in the nutrient sensing and satiety pathways, from mRNA expression to mediator release. In Chapter 1 I showed that CaSR was expressed at a genetic and protein level in the human colon, however changing BMI did not alter its expression. In Chapter 2, I showed that stimulating murine colonic tissue with the CaSR agonist L-tryptophan significantly decreased the numbers of PYY expressing cells, with concomitant expression of CaMKII, although the total number of CaMKII-positive cells was unchanged. CaSR is expressed on L cells (Reimann et al., 2012), however, PYY release has not been associated with CaSR expression or activation: CaSR activation with L-tryptophan significantly increased CCK and GLP-1 release, but not PYY (Acar et al., 2020). Studies examining CaSR-dependent release of serotonin are lacking. Muramatsu *et al.* have shown that oral and duodenal CaSR agonist administration to rats attenuates glycaemic responses to an oral glucose tolerance test, with duodenal

administration decreasing gastric emptying (Muramatsu et al., 2014). The authors postulated that this was most likely through the actions of CCK, GLP-1, and serotonin (Muramatsu et al., 2014).

GPR40 was the only GPCR that showed a significant change in mRNA expression in overweight/obese individuals in Chapter 2, being significantly increased in the sigmoid colon of individuals with a BMI >25. In Chapter 3, stimulation of GPR40 significantly increased the numbers of cells co-expressing pERK and serotonin, but not PYY. Studies mostly associate colonic activation of the receptor with GLP-1 and GIP secretion (Edfalk et al., 2008, Pujol et al., 2018b) and there are currently no studies demonstrating that GPR40 activation leads to serotonin or PYY release from colonic tissue. I cells in the colon express GPR40 and activation of the receptor with dietary fat promoting CCK release (Liou et al., 2011b).

In Chapter 2, I demonstrated that overweight/obese BMI does not change mRNA expression of GPR120 in either the sigmoid or proximal colon, while Chapter 3 data demonstrated that the GPR120 agonist TUG-891 did not significantly alter the numbers of CaMKII/ pERK or PYY/ serotonin-expressing cells.

I have been able to examine the expression of both PYY and serotonin at the mRNA level (of the enzyme TPH1 for serotonin) in humans of normal vs. overweight/obese BMI, as well as their protein expression levels in murine tissue after GPCR stimulation. The aim of this chapter is to determine how the release of PYY and serotonin is affected by activating or inhibiting the nutrient-sensing GPCRs CaSR, GPR120 and GPR40 in murine tissue. In addition, with the use of a CaMKII inhibitor, I aim to determine if these effects are dependent on the CaMKII pathway. This will give us an understanding of the activity of nutrient-sensing GPCRs in peripheral pathways that control appetite.

4.2 Methods

4.2.1 Mice

Experiments were performed using adult female C57BL/6 mice maintained on standard laboratory chow and water in a controlled environment (12 h light/dark cycle, 22±0.5°C, 40–60% humidity) within a pathogen-free facility. Mice were killed by CO_2 asphyxiation.

4.2.2 Tissue stimulation experiments

The murine colonic tract was removed and prepared as in Chapter 2. Briefly, the tract was cleaned by flushing with PBS and opened by cutting ventrally down its length and stretched in carbogenated Krebs solution for 30 minutes, on ice.

Tissue samples from 8 mice, with an area of ~0.5 by 1 cm^2, were excised from the upper colonic region and weights recorded. Tissue was exposed to 250 µl of carbogenated Krebs solution containing nutrient receptor agonists or antagonists and a CaMKII antagonist (Table 16) in a 96 well plate at 37°C, 5% CO_2 for 20 minutes. Supernatants were collected and 1% of stabiliser solution (BA E-5937, Labor Diagnostika Nord) added to protect serotonin against oxidative degradation. Aliquots of supernatant were stored at -20°C.

The “No drug control” condition had no agonist or antagonist of CaMKII inhibitor and acted as the experimental negative control. Each GPCR was stimulated with a solution containing either the relevant agonist/ antagonist with/ without a CaMKII antagonist, to determine if GPCR signalling activation or abrogation affects the release of hormone from cells, and whether this release was dependent on CaMKII signalling pathways.

Each GPCR also had an “All drug control” condition. This condition included the relevant GPCR agonist and antagonist, as well as the CaMKII antagonist. This condition was included to demonstrate the specificity of agonist binding: the presence of a GPCR and CaMKII antagonist should prevent hormone/ peptide release in the presence of an agonist. If it does not, this could indicate that the agonist is binding to other receptors and activating cells independent of the CaMKII intracellular pathway or GPCR. Agonists might bind to other

receptors at a weaker affinity and induce activation of alternate intracellular pathways such as the cAMP pathway. Detailed description of all conditions in Table 15.

Table 15: Conditions for tissue stimulation: receptor targets and their agonists and antagonists. Each GPCR target (columns) had 6 different experimental conditions (rows).

	GPR40	**GPR120**	**CaSR**
Agonist	AS2034178	TUG891	L-tryptophan, L-phenylalanine
Agonist + CaMKII antagonist	AS2034178 + Autocamtide-2-related inhibitory peptide,	TUG891 + Autocam.	L-t, L-p + Autocam.
Antagonist	DC 260126	AH 7614	NPS2143 hydrochloride
Antagonist + CaMKII antagonist	DC 260126 + Autocam.	AH 7614 + Autocam.	NPS2143 + Autocam.
No drug control	-	-	-
GPCR Agonist + Antagonist + CaMKII antagonist	AS2034178 + DC 260126 + Autocam.	TUG891 + AH 7614 + Autocam.	L-t, L-p + NPS2143+ Autocam.

4.2.3 Nutrient and drug solutions

All solutions contained 124.05 mM NaCl, 4.78 mM KCl, 1.33 mM NaH_2PO_4, 2.44 mM $MgSO_4$, 5.50 mM D-glucose, 2.5mM $CaCl_2$ and 25 mM $NaHCO_3$ (Sigma-Aldrich) and were carbogenated with 95% O2 and 5% CO_2. Tissue was exposed to Krebs buffer containing one of six different agonists/ antagonist with or without the presence of the CaMKII inhibitor, autocamtide-2-related inhibitory peptide, a selective and potent inhibitor of the enzyme. The concentrations of these compounds are listed in Table 16.

Table 16: Nutrients and drugs used in release assay experiments.

Drug	Role	Concentration	Manufacturer	Product Code
AS2034178	GPR40 agonist	10µm	Tocris	5035
DC 260126	GPR40 antagonist	1µm	Tocris	5357
TUG891	GPR120 agonist	10µm	Tocris	4601
AH 7614	GPR120 antagonist	10µm	Tocris	5256
L-Tryptophan	CaSR agonist	3mM	Sigma	T0254
Phenylalanine	CaSR agonist	3mM	Sigma	P1150000
NPS2143 hydrochloride	CaSR antagonist	10µm	Tocris	3626
Autocamtide-2-related inhibitory peptide	CaMKII antagonist	1µm	Tocris	5959

4.2.4 Hormone/peptide release assays

4.2.4.1 Serotonin

The concentration of serotonin in the supernatant from the conditions listed in Table 15 was determined by competitive ELISA (BA E-54900R, Labor Diagnostika Nord), as guided by the kit manufacturer. This kit is a competitive ELISA: instead of using a conjugated detection antibody, a conjugated antigen is used to compete for binding with the serotonin present in the sample. Therefore, the more serotonin in the supernatants, the less conjugated antigen binds to capture antibody and the signal is inversely proportional to the amount of serotonin present.

Supernatants were kept on ice to slow the degradation of serotonin at room temperature. Supernatant samples were diluted and acylated in a separate plate to increase the affinity of the anti-antigen antibodies used in subsequent steps and increase the overall sensitivity of the ELISA. Standards and samples were acylated for 30 minutes at room temperature with the acylation buffer' provided. Supernatants and standards were then pipetted into the pre-coated ELISA plate (96 well, Bio-One microlon single break-strip plate), 25 µl of serotonin antiserum added, the plate sealed and covered with foil and left to incubate at 4°C for 18 hours. Samples were diluted in diluent buffer (1:5). The contents of the plate were discarded, the plate washed and enzyme conjugate added to wells for 30 minutes at room temperature. The contents of the plate were discarded, the plate washed and substrate added to wells and the plate covered with foil for a further25 minutes at room temperature. After 25 minutes, the plate had developed and stop solution was added.

The plate was then read on a Bio-Tek Synergy HT plate reader and the optical densities (at 450nm) of wells recorded on Gen 5 3.08 software (Bio-Tek).

Recorded optical densities for each sample were converted into concentrations by applying the equation from a standard curve (standards' optical density vs. known concentration) for each plate. These concentrations were then corrected for the initial dilution of the samples and the weight recorded for each sample used to ascertain the concentration of serotonin per gram of tissue. In addition, because this ELISA is a competitive ELISA, data is inverted (1/value) to obtain the concentration of serotonin.

4.2.4.2 PYY

The concentration of PYY in supernatant from the conditions listed in Table 15 was determined by standard ELISA. A Mouse Peptide YY ELISA Kit (colorimetric) was used (Novus bio, NBP2-76714), following instructions provided by the manufacturer.

A standard range (1000 (Std 1), 500, 250, 125, 62.5, 31.25, 15.63 (Std 11) pg/mL) was created by diluting the top standard and pipetted onto the plate with a blank, in duplicate. Diluted experimental samples (1:3) were added to remaining wells and the plate incubated at 37°C for 90 minutes. Liquid was removed, biotinylated detection antibody solution was added to each well and the plate further incubated for 1 hour at 37°C. The plate was decanted and washed. HRP conjugate solution was added to each well and the plate incubated for 30 minutes at 37°C. The plate was washed again and substrate reagent added for 25 minutes at 37°C. Once the plate was developed, stop solution was added. The plate was then read on a Bio-Tek Synergy HT plate reader and the optical densities (450nm) of wells recorded on Gen 5 3.08 software (Bio-Tek).

Recorded optical densities for each sample were converted into concentrations by applying the equation from a standard curve (standards' optical density vs. known concentration) for each plate. The concentrations of all wells were then corrected for the blank wells and the supernatant samples for the initial dilution of the samples. The weight recorded for each sample was used to ascertain the concentration of PYY per gram of tissue.

4.2.5 Statistical and data analysis

For both PYY and serotonin data, outliers for each condition across all repeats (different mice) were identified by IQR as previously discussed and removed. The mean (±SEM) concentration for all mice repeats was plotted for each nutrient receptor. A Kruskal-Wallis multiple comparison test was used to determine statistical significance between conditions, within each nutrient receptor group as for Chapter 3 (6 independent experimental conditions).

Normality was confirmed with a Shapiro-Wilk test - data not shown.

4.3 Results

4.3.1 With CaSR stimulation, the release of serotonin is significantly increased in murine tissue in the 'all drug control', while PYY release is not changed.

I see a significant (p = 0.0279) increase in the concentration of serotonin/ gram of tissue from 8 mice, in the 'all drug control' condition, compared to the 'no drug control' condition (Figure 47, A). The 'all drug' condition contains CaSR agonists (L-tryptophan and L-phenylalanine), antagonist (NPS2143 hydrochloride) and CaMKII inhibitor (autocamtide-2-related inhibitory peptide (autocam). No change was seen in the concentration of serotonin released in the other conditions, compared to the 'no drug' control. See Table 15 for the breakdown of agents for each experimental group.

PYY release from tissue stimulated with CaSR agonist or antagonist was not altered compared to the 'no drug' control. PYY release was higher in the 'no drug control' (Figure 47, B) condition compared to the other conditions, but this did not reach significance (p = >0.9999). Incubation in the presence of a CaMKII inhibitor did not significantly change serotonin or PYY release compared to conditions without.

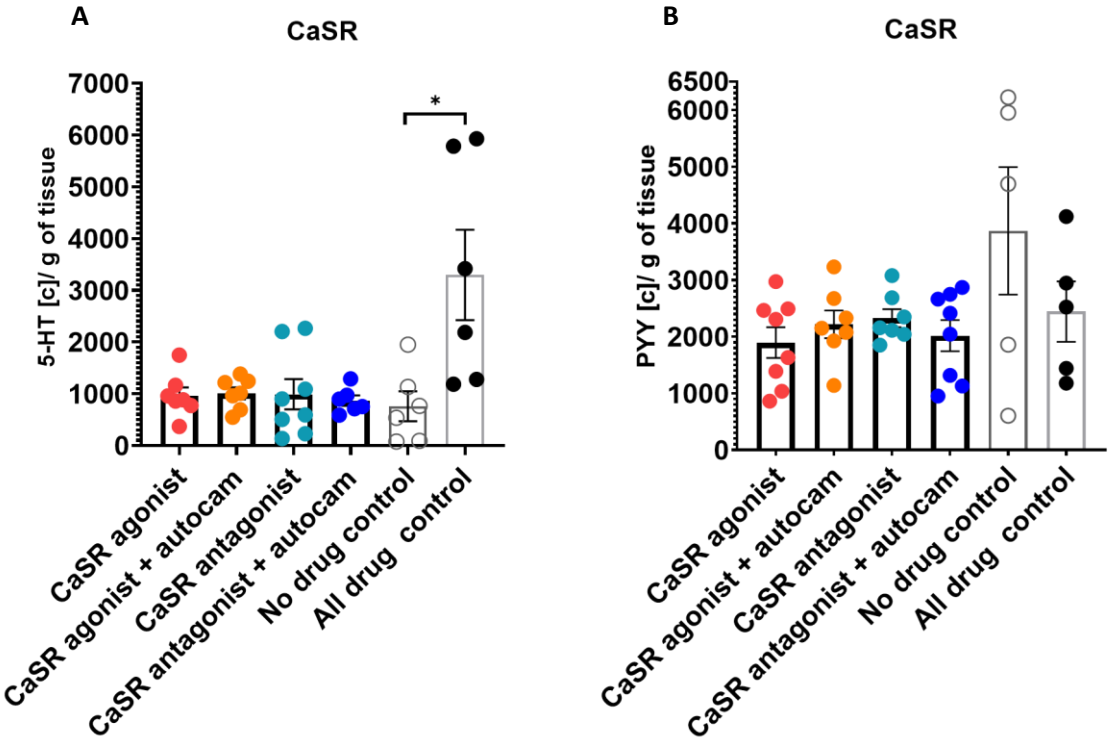


Figure 47: Release of serotonin (serotonin, A) and PYY (B) (concentration/ gram of stimulated tissue) in murine tissue stimulated with: CaSR agonists/ antagonists, with/ without CaMKII inhibitor (autocam) (N = 6-8); no drug (only Krebs); All drug controls (N = 5). Significance *p* = ≤ 0.05.

4.3.2 Release of serotonin and PYY is not changed under any experimental conditions of GPR120 stimulation

I saw no change in the release of serotonin or PYY (Figure 48A and B, respectively) in mouse colonic tissue stimulated with the GPR120 agonist (TUG891) or antagonist (AH 7614), under any experimental condition when compared to the buffer ('no drug') control. Incubation with a CaMKII inhibitor did not significantly change serotonin or PYY release compared to conditions without the inhibitor. See Table 15 for the breakdown of agents for each experimental group.

PYY release was slightly higher in the buffer ('no drug') control compared to the other experimental conditions (Figure 48, B), but this did not reach significance (*p* = >0.9999).

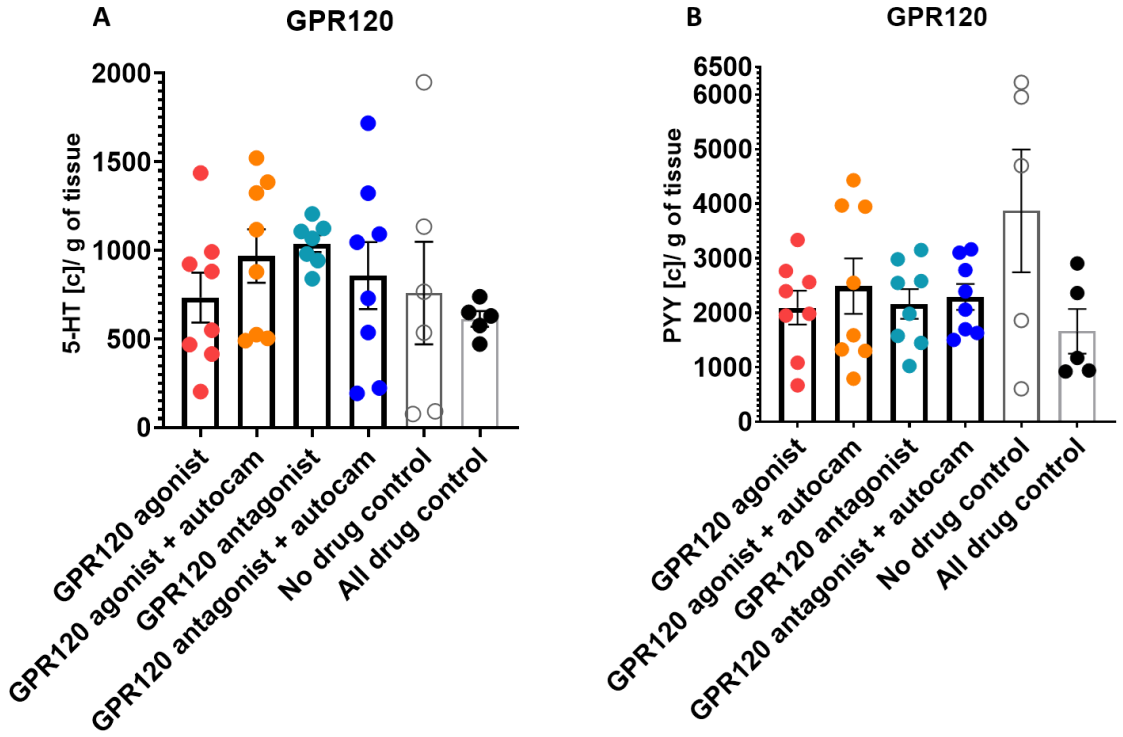


Figure 48: Release of serotonin (serotonin, A) and PYY (B) (concentration/ gram of stimulated tissue) in murine tissue stimulated with: GPR120 agonists/ antagonists, with/ without CaMKII inhibitor (N = 6-8); no drug; All drug controls (N = 5). Significance *p* = ≤ 0.05).

4.3.3 Release of serotonin and PYY is not changed under any experimental conditions of GPR40 stimulation.

I saw no change in the release of serotonin or PYY (Figure 48, A and B, respectively) in mouse colon tissue exposed to the GPR40 agonist (AS2034178) or antagonist (DC 260126), under any experimental condition, compared to a 'no drug control'. Incubation with a CaMKII inhibitor did not significantly change serotonin or PYY release compared to conditions without. See Table 15 for the breakdown of agents for each experimental group.

PYY release was higher in the 'all drug control' (Figure 49, B) condition compared to the other conditions, but this was not statistically significant (*p* = >0.9999).

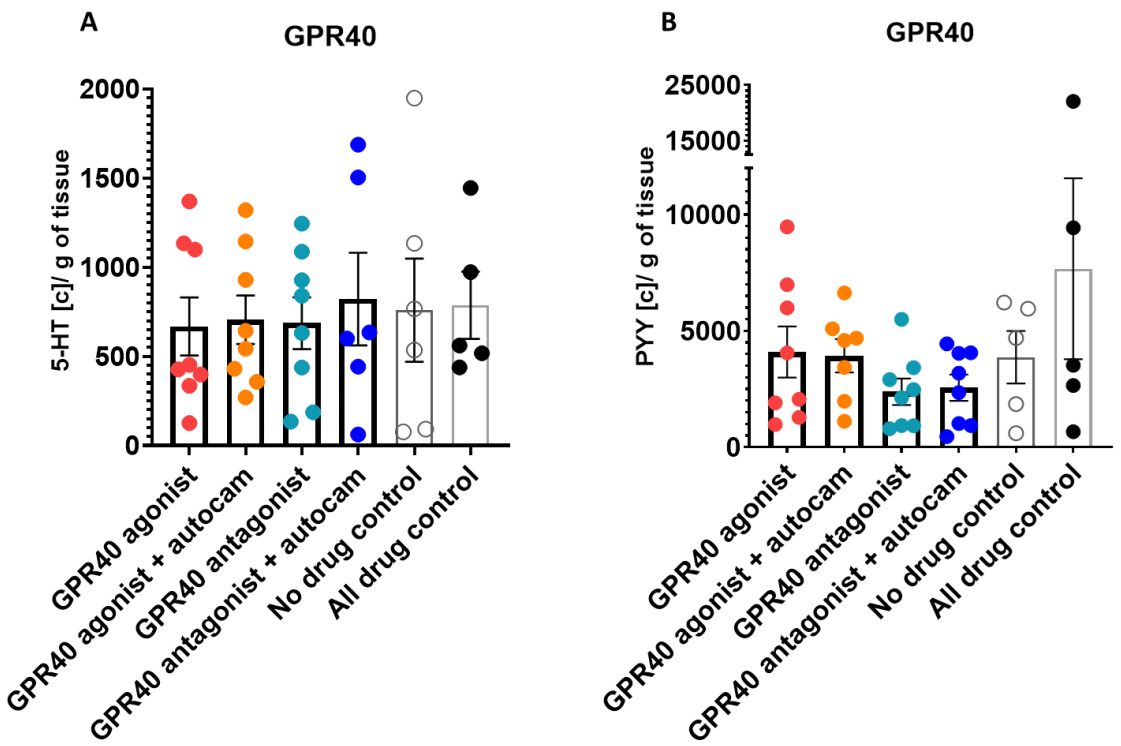


Figure 49: Release of serotonin (serotonin, A) and PYY (B) (concentration/gram of stimulated tissue) in murine tissue stimulated with: GPR40 agonists/ antagonists, with/ without CaMKII antagonist (N = 6-8); no drug; All drug controls (N = 5). Significance *p* = ≤ 0.05).

Table 17: Summary of changes in analyte release vs. no drug control in Chapter 4.

	CaSR	GPR120	GPR40
PYY	No change	No change	No change
Serotonin	Significant increase: All drug	No change	No change

4.4 Discussion

Overall, stimulation of the GPR120 and GPR40 receptors did not affect PYY or serotonin release from murine colonic tissue. However, a significant increase in serotonin release was observed following exposure to a combination of CaSR agonist, antagonist and a CaMKII inhibitor (Table 17). Our findings suggest that CaSR-, GPR120- and GPR40-dependent hormone release is independent of the CaMKII signalling pathway, as incubation with a CaMKII inhibitor did not alter the release of PYY or serotonin in the presence of GPCR agonists/antagonists.

CaSR has been shown to be expressed on murine colonic serotonin-positive ECs in a FACS-based study (Lund et al., 2018) as well as in our own research: the majority of cells in the human colon co-expressed CaSR and serotonin. I have also shown that CaSR is expressed on PYY-positive human colonic cells; however, this population of cells is rarer. I therefore postulated that agonist stimulation of CaSR is likely to lead to serotonin release from EC cells, but not PYY release from L cells. There is no current evidence for CaSR stimulation causing the release of serotonin, and PYY release is not associated with CaSR activation on L cells (Acar et al., 2020); however, research on their release from CaSR-stimulated cells, is lacking. Our data suggests that though serotonin and PYY are expressed with CaSR in EECs, their release is CaSR-independent.

Interestingly, I saw a significant increase in the release of serotonin in the 'all drug' control (CaSR agonists + antagonist + CaMKII inhibitor). This condition served as a measure of specificity of the agonist-mediated effect on this pathway. This result suggests that in the mouse, L-tryptophan, L-phenylalanine or both agonists, are activating CaSR- and CaMKII-independent pathways, leading to serotonin release. Human data by Symonds *et al.* demonstrated that L-tryptophan, L-phenylalanine stimulation of human proximal colonic tissue did not increase serotonin or PYY release but did increase GLP-1 release significantly (Symonds et al., 2015). This difference could be species dependent; however, future research is needed to fully elucidate if this trend observed by Symonds *et al* is a significant one, in a larger data set. If the antagonist was unable to effectively abrogate CaSR-ligand binding and this increased serotonin release was a response to CaSR activation, I would see a corresponding increase in the experimental condition with only tryptophan and

phenylalanine. On the other hand, if increased release was due to an abrogation of CaSR as well as CaMKII signalling, I would expect to see an increased release in the CaSR antagonist and CaMKII inhibitor condition. There was no change in serotonin release in either condition. The 'all drug control' condition contains the same concentration of agonists and antagonists as other conditions - they should have the same effect as in the other experimental conditions.

As discussed previously, CaSR and TPH1 may compete for L-tryptophan, however by co-stimulating with L-phenylalanine (another CaSR agonist), I aimed to ensure sufficient CaSR stimulation. Despite the fact that tryptophan and phenylalanine have been shown to increase the numbers of activated cells, and specifically, decrease the numbers of PYY positive cells (Acar et al., 2020), I saw no change in the release of serotonin or PYY apart from that seen in the 'all drug' control condition. Phenylalanine has been shown to increase GLP-1 and calcium release from murine STC-1 cells in a CaSR-dependent manner (Wang et al., 2019a). In the porcine duodenum, L-phenylalanine has been shown to stimulate CCK and GIP release as well as increasing the mRNA expression of these hormones and the CaSR receptor, in a CaSR-dependent manner (Feng et al., 2019). It is therefore possible that these CaSR agonists are not responsible for altering serotonin or PYY release, but instead CCK and GLP-1 – indeed this increased GLP-1 is seen in the Symonds *et al.* paper discussed earlier.

GPR120 has been shown to be expressed (protein level) on a small (5-6%) population L cells as well as on a large (70%) population of EC cells in the human ascending colon (Peiris et al., 2021). Stimulation of the receptor with TUG891 alone is associated with increased GLP-1 release, but not PYY or serotonin (Peiris et al., 2021). In Chapter 2 I did not see any change in the expression of activated (CaMKII or pERK positive) cells expressing either PYY or serotonin in response to TUG891 stimulation in murine colonic tissue. I see no change in the release of serotonin or PYY under any of our experimental conditions, thus further complementing studies showing that GPR120 stimulation does not change the release of PYY or serotonin from these cells. I see a trend of decreased PYY release in the experimental conditions compared to the 'no drug control'. This trend is quite strong and could also reach significance with more murine samples, though it would be surprising that both a GPR120 agonist and antagonist would act to lower PYY release.

Research on PYY and serotonin release after GPR120 agonist stimulation is currently lacking. Stimulation of GPR120 has been shown to increase GLP-1 (Holst, 2007) and CCK release (Rasoamanana et al., 2012) in a receptor-dependent manner, with an increase in CaMKII (but not pERK) expressing cells (Peiris et al., 2021). Peiris *et al.* reported a non-significantly increased release of GLP-1 and PYY after TUG891 stimulation, which reached significance when TUG891 and lauric acid were co-administered, for GLP-1, PYY and serotonin (Peiris et al., 2021). GLP-1 release via GPR120 has been shown to be independent of ERK activation (Katsuma et al., 2005a), however reduced free fatty acid-induced CCK release was observed when STC-1 cells were transfected with a GPR120-specific shRNA (Tanaka et al., 2008). It is therefore likely that GPR120 facilitates CCK release but not GLP-1 or, by extension, PYY release (PYY and GLP-1 are co-released). It is therefore likely that a GPR120 agonist alone is not sufficient to drive or block the release of serotonin or PYY from colonic cells. Based on research from other studies, it seems that stimulation of GPR120 instead drives satiety via increased GLP-1 and CCK release.

In Chapter 3, I demonstrated that a GPR40 agonist increased the numbers of pERK activated EC cells but did not change the numbers of CaMKII activated L- or EC cells (Figure 44). In this Chapter, I show that despite an increased number of activated EC cells, neither serotonin nor PYY release were changed by GPR40 stimulation in any of our experimental conditions, compared to a 'no drug' control. The role of GPR40 in nutrient sensing is unclear, though the receptor is implicated in GLP-1 and GIP release (Pujol et al., 2018b, Edfalk et al., 2008). Gribble *et al.* demonstrated that human colonic L cells stimulated with the GPR40 and GPR120 agonist (GW9508) increased GLP-1 release (Habib et al., 2013). Though PYY release was not examined as part of the study, the authors had previously shown that GPCR-independent activation of cells induced GLP-1 and PYY co-secretion, it is likely that PYY release was therefore also increased (Habib et al., 2013).

Serotonin release was almost identical across all our conditions. Therefore, GPR40 activation may preferentially induce GLP-1 and CCK release (Pujol et al., 2018b, Liou et al., 2011b). Whilst GPR40 is expressed on PYY containing L cells, and data shows PYY and GLP-1 are co-released from these cells (Elliott et al., 1993), our data shows no change in the release of PYY under any of our experimental conditions. GPR40 co-localises with NPY and

POMC-expressing neurons in the brain (Freitas and Campos, 2021), and can be activated by n-3 fatty acids: it is possible that GPR40-dependent PYY release is more likely to occur via a central mechanism.

As discussed previously, depending on the specific MCFA/LCFA, GPR40 activation might alter PYY or serotonin release. AS2034178 is a GPR40-specific agonist that has been shown to increase the release of insulin in response to oral glucose (Pujol et al., 2018b), and the release of GLP-2 (GPR40-dependent) in a murine model of colitis (Kato et al., 2019) and from murine small intestine L cells (Tsukahara et al., 2015). It is possible that other GPR40 agonists (eicosapentaenoic acid, α-linolenic acid, decanoic acid and lauric acid) might cause an increased serotonin or PYY release. Lauric acid is a GPR40 and GPR84 agonist and has been shown to increase PYY and GLP-1 release; however, whether this is due to a GPR40 specific effect is not clear (Peiris et al., 2021).

To conclude, overall, stimulation with agonists of the CaSR, GPR120 and GPR40 receptors does not lead to a significant change in the release of serotonin or PYY from murine colonic tissue. I did see a significant increase in serotonin release in our 'all drug control', CaSR-stimulated condition. Future work examining the release in response to exposure to a pERK inhibitor could determine the potential involvement of this pathway in mediator release.

5 Overall Discussion

5.1 Summary of data

Data presented in this thesis has characterised the expression of nutrient-sensing GPCRs, hormones and peptide mediators associated with satiety pathways, in the lean and overweight/obese human colon. I showed that of the GPCRs analysed in the human colon, GPR40 was the only one to change, at the mRNA level, between the lean and overweight/obese BMI patient groups. I also showed that the number of serotonin- or PYY-expressing cells was unchanged between these two BMI groups, as was the number of cells co-expressing these mediators with CaSR. I showed that there was a large population of CaSR-expressing serotonin-positive cells, while CaSR-expressing PYY-positive cells were rare. It has been previously shown that colonic TPH1 mRNA expression is lowered in obese mice; however, expression in humans has not previously been demonstrated. In addition, it has been shown that PYY circulating levels and post-prandial release is lowered in obese humans (le Roux et al., 2006b); I show no change at the mRNA or protein cellular level, therefore it is likely that PYY release is altered, rather than a change in expression.

With the expression of GPCRs and hormones characterised in the colon, I formulated functional experiments to determine how nutrient receptor stimulation might change cellular activation; by which pathway and in which population of cells (PYY- or serotonin-expressing), and then correlate this with obesity in human samples. Unfortunately, due to the SARS-CoV-2 pandemic I could not access human tissue. Instead, I was able to characterise how nutrient receptor stimulation changed the number of cells activated specifically via either the CaMKII or ERK pathways in subsets of PYY- or serotonin-expressing cells in the mouse colon. Overall, there was not a significant increase in the total number of cells showing activation of the CaMKII or pERK pathways after nutrient receptor agonist stimulation. However, GPR40 stimulation significantly increased the number of pERK-positive EC cells. Conversely, CaSR stimulation significantly decreased the number of CaMKII-positive, PYY-positive cells. This increase in activated serotonin-expressing cells could be linked to the increased mRNA expression I saw in our human obese patients. This study demonstrates for the first time that GPR40 activation can change signalling in EC cells and that this specifically occurs via the pERK pathway.

I then set out to investigate how the release of PYY or serotonin might be changed in cells stimulated via agonists of the GPR40, GPR120 and CaSR receptors. I also ran experimental conditions blocking the CaMKII pathway as well as the GPCR, in order to determine if hormone/mediator release was GPCR- or CaMKII-dependent. Taken together, our data show that stimulation of these GPCRs did not lead to a significant change in PYY or serotonin release. I had previously shown that GPR40 expression is altered in obesity and that stimulation of GPR40 increased the numbers of serotonin-positive cells; however, this data seems to suggest that this does not lead to a change in either serotonin or PYY release in the colon. I will further discuss these findings, their limitations and their ramifications in elucidating nutrient sensing and activation pathways in the colon.

6 Limitations and future work

6.1 Limitations

The areas of interest covered by this study are novel and have not been previously examined in depth. Therefore, some of the experimental techniques and/or reagents are novel or adapted from existing techniques. In our immunohistochemistry studies this was most apparent with the lack of validated antibodies against GPCRs as well as hormones, mediators. In Chapter 2 I show our optimisation experiments with several different antibodies (GPR120, GPR43, GPR119) for the GPCRS that had been examined by qPCR. Antibodies against human GPCRs are rare, and published papers based on them, rarer still. These antibodies were often not tested by the manufacturer in IHC, having been validated for western blot or for differently processed IHC samples (paraffin/ wax embedded), therefore it was not surprising so many failed to elicit a specific staining signal in our frozen tissue. On the other hand, antibodies which had been tested and published with definitive staining were no longer sold by the time of this study (e.g., GPR120 antibodies AB97272, AB188954 – Abcam). I was therefore only able to examine the GPCR CaSR, the hormone PYY and the peptide serotonin at both the mRNA and protein (IHC) level. I was unable to examine other GPCRs and hormones which have previously been shown to be changed either at the mRNA or protein cellular level or circulating in the plasma. I therefore have an incomplete picture of expression.

Western blotting is a technique that allows for the separation of proteins in a tissue based on size and then the staining with antibodies to identify the target protein(s). The antibodies that did not work in IHC, might have worked for western blot, and this would have given me an indication of the presence and abundance of our target proteins and whether changes in mRNA expression led to changes in translation. However, a limitation of western blot would have been that I would not have been able to discern from where these proteins had come. With IHC not only can we localise proteins within the crypts, mucosa etc. but also colocalise with other markers to localise proteins within specific cells (like L cells, EC cells etc). This study is focused on enteroendocrine cells, and with western blot, we would have been unable to link protein expression to these cells.

One of those hormones was GLP-1, which is an important hormone expressed by colonic EECs and for which there is evidence for co-release with PYY and which is also reduced post-prandially in obesity (Muscelli et al., 2008, Ranganath et al., 1996). I was only able to measure GLP-1 expression at the mRNA level and only indirectly via glucagon expression, as GLP-1 is produced following translation and post-translational modifications of the glucagon/ pro-glucagon gene (Holst, 2007, De Silva and Bloom, 2012) into GLP-1 and GLP-2. GLP-2 has been shown to have a role in regulating lipid absorption and energy uptake as well as glycaemic control (Amato et al., 2016), therefore it is not possible to directly attribute changes in glucagon expression to either the expression or activity of GLP-1. Like GLP-1, serotonin is not produced from the translation of a gene. Instead, it is produced in cells by the activity of the enzyme TPH1 which synthesises the monoamine from the amino acid tryptophan (Watanabe et al., 2010). This is the rate-limiting step (France et al., 2016, Watanabe et al., 2010), but it is not possible to correlate directly serotonin production/levels to the expression of TPH1.

In Chapter 2, I separated our patient cohort into 2 groups: those with a BMI BMI<25, and those with a BMI≥ 25. There are a couple of potential issues with this method. Firstly, BMI is an inexact way to measure if someone is overweight or obese. BMI is determined by dividing a person's weight by the square of their height (2014). BMI is used by the NHS in the UK as a patient metric, with a BMI of over 40 being categorised as severely/ morbidly obese, 18.5 – 24.9 considered a healthy weight and under 18.5 classified as underweight (UK). However, this system does not measure body fat and is therefore not a clinical

classification of obesity: someone who is relatively short but might be fit and muscled will be seen as having a high BMI, despite them having very little body fat. In general waist circumference is seen as a more exact way to measure body fat and obesity; however, this is not something routinely carried out by the NHS, therefore I did not have access to this data. The other issue with dividing patients into two BMI groups in this way is that a patient with a BMI of 24.99 and 25.01 will be in two different groups, despite how close they are in BMI. To avoid this, I initially plotted our data as a linear regression, without dividing our subjects into groups (see Appendix, all Figures in section 7.3). However, the issue with this kind of analysis is that it was not easy to identify significance nor trends in the data set and this did not help answer the question of how expression might change with obesity. For this reason, the data analysis method where patients were divided into two BMI groups was ultimately used as this was the best possible option.

Another limitation of this study could be sample size; I was limited in patient recruitment due to the cessation of operations at the hospital and the closure of the hospital to research staff because of the Covid-19 pandemic. I was able to get full thickness tissue for IHC from 26 patients (13 BMI >25, 13 BMI <25); however, ideally, I would have wanted to recruit closer to 50 patients, over 12 months. The 26 patients I did manage to recruit is a higher number than those reported in other GPCR, human, gene expression studies such as work by Symonds *et al.* (N = 15), and Little *et. al* (N = 23). However, other studies (particularly sequencing studies) have looked at much larger cohorts, such as Ichimura *et al.* (N = 312). Therefore, whilst our study population is smaller than I had anticipated, by comparison to most studies referenced throughout this thesis (with the exception of population genotyping studies) our population is larger.

The data set used in Chapter 2 came from an IBS study in the Netherlands. I was sent patient data and samples that the team in Maastricht were able to share. Among these IBS patients were also healthy controls. The aim was therefore to analyse whether there is any difference in mRNA expression caused by the IBS diagnosis in patients. As shown in the Appendix, Figure 52 and Figure 53, I compared mRNA expression between IBS sub-types and healthy controls. However, I was only sent 1-2 of the 12 healthy control samples recruited and so were unable to carry out any meaningful analysis to determine whether the IBS state affected GPCR or hormone/ mediator expression. IBS is associated with diarrhoea and/ or

constipation and low-grade chronic inflammation (Saha, 2014). The disease is also associated with altered EC expression and increased or decreased circulating levels of serotonin in IBS diarrhoea and constipation sub-types, respectively (Saha, 2014). Whilst I was unable to compare the expression between IBS and healthy patients, I had enough data to compare between IBS sub-types and I saw no change in the mRNA expression of TPH1 between any of the IBS sub-types. Much remains unknown about IBS and it is very possible that the typical visceral hypersensitivity and gastrointestinal motor disturbances could affect mRNA expression of GPCRs and hormones, though there is of yet no evidence for it. It is possible that additional healthy patient samples could be obtained; this would allow us to determine the role of IBS on mRNA expression which would not only add to existing research on IBS but also increase the validity of our data.

In Chapters 3 and 4, I used mouse tissue instead of human tissue. The mouse colon differs structurally and functionally from the human colon. The mouse colon is not divided like the human into proximal, sigmoid or distal sections, has a smaller (20x) surface area and smaller (3x) small intestine: colon ratio (Nguyen et al., 2015). Mice as omnivores are able to eat a larger amount of plant matter than humans and this is reflected in an enlarged caecum, which is an important site of fermentation, whereas in humans the caecum function is unclear (Treuting and Dintzis, 2012). Fermentation instead occurs throughout the human large intestine (Treuting and Dintzis, 2012). This will have the largest impact on the composition and diversity of the microbiome within the human and mouse colon, with the mouse colon probably having SCFA-producing bacteria concentrated within the caecum – indeed 85% of the bacterial species expressed in mice are absent in humans (Ley et al., 2005). In addition, diet-switching (high fat, low fibre / low fat, high fibre) is able to change the microbiota enterotype relatively rapidly in mice (around a 7 days) (Wang et al., 2015) whereas even after 10 days, this was not apparent in humans (Wu et al., 2011). The human and murine colon differ in several other significant ways that would impact research into nutrient sensing and obesity. This is of particular importance to this study because data presented in Chapter 2 is from human tissue from the proximal and sigmoid colon, whereas Chapters 3 and 4 are derived from murine samples from post-caecal (ascending) colon tissue. Human and murine colonic functions and activity are therefore not directly

comparable. In addition, our murine tissue was sourced from exclusively female mice. Female and male mice have differences in their microbiomes (for instance females have much lower Firmicutes, Bacteroidetes, Actinobacteria species than males) and this is driven by sex hormones like testosterone (Yurkovetskiy et al., 2013). Other species like *Clostridium leptum et rel.* are enriched in females (Elderman et al., 2018). The microbiome has an important role in satiety, particularly SCFA receptors, therefore it's possible that SCFA receptor expression could be sex dimorphic. Our human samples were derived from both female and male participants, therefore additional murine, male samples should be sampled to determine whether GPCR and satiety hormone expression is gender-specific.

In Chapter 3 I measure, as an outcome of cellular activation, changes in the numbers of cells expressing CaMKII or pERK. However, there are other pathways of cellular activation such as cAMP and intracellular calcium levels via PLC and DAG, therefore the absence of cell activation via the studied pathways may indicate that other intracellular mechanisms are active in response to nutrient-sensing GPCR stimulation. The CaMKII and ERK pathways were selected as target pathways because they are well elucidated in EECs and previous studies have shown that GPCRs on these cells can signal via one or both pathways.

In Chapter 3, ~4500 images were collected, with each image having hundreds of stained cells (e.g., for GPR119, per each of the 15-20 FOVs there were 0-200 CaMKII-positive, 0-20 5-HT-positive, 0-10 PYY-positive and 0-35 peRK-positive cells – notwithstanding co-staining in cells), therefore automated counting using ImageJ was used. This method has several limitations. For example, the settings to automate cell counting were chosen to work in the majority of cases, however the min-max pixel size settings in particular often had to be changed due to the presence of differently sized cells, often within the same condition. This was done in order to not miss clearly positively stained cells, but also it makes the method less consistent. In addition, in trying not to miss positively stained cells, it is possible that non-positive cells were included, inadvertently. This was a general weakness of the method, overall. In addition, because I was analysing tissue that had been stimulated *in vitro,* cells were only counted 3 crypts deep of the luminal surface of the colon. This was done to ensure I was counting activated cells; indeed, the majority of staining occurred in this region. However, active cells in the more basal layers could be under-represented in our data, these include serotonin-positive cells which occurred more

frequently than PYY in these regions. I saw rare staining of PYY in our tissue, therefore any process which might lead to us either missing or overcounting true staining would disproportionately affect this population of cells. The methods that I used were designed to provide a balance between accuracy and accessibility, therefore some trade-offs had to be accepted as unavoidable.

In Chapter 3 I used TUG891 as a GPR120 agonist. TUG891 has been shown to also have affinity for GPR40. In human HEK293T cells, TUG891 has a greater affinity for GPR120 compared to GPR40, however in mice this selectivity differential is reduced (Hudson et al., 2013). Treatment of murine EECs with a GPR40 antagonist reduced the TUG891-dependent GLP-1 release by 26% compared to GLP-1 release in the absence of an antagonist, therefore this may indicate that TUG891 showed only limited selectivity for GPR120 (Hudson et al., 2013). To eliminate the possibility that TUG891 could have been stimulating GPR40 as well as GPR120, a GPR40 antagonist should have been used. This would have ensured that any changes in expression caused by TUG891 stimulation were only GPR120 dependent. This could have been done for all other GPCR conditions, with an agonist for one GPCR given in tandem with antagonists for all other GPCRs - should non-selective binding be a significant risk with the agonists chosen.

In Chapter 4 I stimulated cells with a GPCR agonist, antagonist and CaMKII inhibitor, as well as combinations of these conditions. The CaMKII inhibitor condition was included in order to determine if hormone release was CaMKII-dependent. A pERK inhibitor could also have also been included, to determine if hormone release is pERK dependent. A combination of pERK and CaMKII inhibitors would have allowed us to better answer the question from Chapter 2: whether GPCR stimulation activates cells via a pERK- or CaMKII-independent pathway. Our choice of GPCR agonists was predicated on those that have been previously shown to elicit specific GPCR activation. However, they are not the only compounds capable of activating GPCRS. As previously discussed, some GPCRs exhibit different activation patterns in response to activation with different agonists. GPR40 activation for instance, has been shown to cause the release of GLP-1 and CCK (but not PYY) (Pujol et al., 2018b) upon stimulation with AS2034178, while lauric acid has been shown to increase PYY and GLP-1 release (Peiris et al., 2021). Additional experiments with different

GPCR agonists could be carried out, to ascertain the finer detail of the specific activation pathway for a specific agonist.

6.2 Future Work

I have contributed to elucidation of the role of various GPCRs and hormones/ mediators in the nutrient sensing pathways in the colon. Further work is needed to characterise the expression of other GPCRs and hormones, mediators that I did not have the time nor capability to examine in detail as part of this study. For instance, assessing the cellular expression and activation of leptin receptor-expressing cells in the colon would determine if leptin has a role within the colon (either on vagal afferents or elsewhere in the tissue), to affect hormone release from EECs. Any changes in the obese state in either leptin receptor expression or the excitability of cells to leptin, might help answer what drives leptin resistance in the obese state. As discussed previously, GLP-1 and CCK are also important hormones in mediating satiety, so ideally expression and release of these hormones would be characterised not just in the colon, but elsewhere in the GIT. Other hormones and mediators are expressed at low levels (CCK) or not at all (leptin) in the colon, therefore it is important to look at the GIT as a whole, to assess nutrient sensing pathways in the obese state.

There is also an opportunity to increase the number of patient samples for Chapter 1 data, as well as repeat of the experiments carried out in Chapter 2 and 3, but with human tissue. This would allow us to understand if the results I saw were specific just to mice or were similar in humans. If these results were repeated in humans, then the mouse might prove to be a robust model of colonic hormone release. I would also be able to see if hormone release is changed not only by GPCR stimulation, but also by altered BMI.

7 Conclusions

In summary, our data suggest that it is likely that obesity does not affect the expression of GPCRs or hormones at the mRNA or protein cellular level in the human colon. The nutrient receptor GPR40, however, would benefit from additional study, as its expression is significantly changed in humans and this may drive, in part, the reduced satiety characteristic of the obese state. Further studies with human tissue could further elucidate the role of the MCFA/LCFA receptor in driving a reduced release of satiety hormones or reduced vagal activation. The importance of GPR40 is further demonstrated in the murine colon, where stimulation of the receptor with a high concentration of an agonist significantly increased the numbers of EC cells activated via the ERK pathway. It is likely that the increased GPR40 expression I saw in obese humans could also drive the increased activation of EC cells that I observed in the mouse. The high concentration of GPR40 agonist used could mimic the high caloric intake (specifically of LCFA saturated fatty acids (Nettleton et al., 2017)), common in obesity. Further work in human tissue could determine if this increased activation is abnormal and associated with obesity. On the other hand, stimulation with other GPCR agonists did not change the activation expression profiles of cells, despite other studies showing, for instance increased ERK activation in GPR120-expresing cell lines (Hirasawa et al., 2005). It is surprising that stimulation of GPR119, GPR120 and GPCRc6a receptors in the murine colon did not change the numbers of overall activated cells, nor activated L or EC cells. Further work with GPCR-antagonists and additional agonists would help clarify whether this result was a consequence of the specific agonists used in this study.

I aimed to investigate whether the activated cells led to changes in the release of PYY and serotonin, two important satiety mediators in the colon and centrally. I expected that activation of nutrient receptors on L and EC cells would lead to a change in release of these mediators; however, I saw no change. This result is an interesting one, as it demonstrates for the first time that post-prandial PYY and serotonin release may be independent of GPR120, GPR40 and CaSR activation. It is very likely (based on work in other studies) that the release of other hormones, such as CCK and GLP-1, are dependent on the activation of these GPCRs.

This body of work suggests that GPR40 could be an important nutrient receptor both in obesity in humans, and in normal weight adult mice. Further research could elucidate the role of the GPR40 receptor more fully in both the obese state as well as the normal satiety signalling mechanisms in the colon. This study also highlights that at the genetic and protein level, many of the systems that regulate satiety, from receptor expression to mediator release, are likely to be tightly conserved even in the obese/overstimulated state. However, additional studies would be needed to expand on this relatively small cohort study.

8 Appendix

8.1 Food Frequency Questionnaire

A food frequency questionnaire (FFQ) was given to participants in the Maastricht study. A habitual dietary intake was assessed in IBS patients and healthy controls between May 2012 and April 2015 by a self-administered FFQ, using intake over the previous month as a reference period. This FFQ was developed and validated by the division of Human Nutrition, Wageningen University using standardized procedures (Tigchelaar et al., 2017b) and comprised of 80 questions that explored the food habits of individuals, divided into 1-5 sub-questions. I had access to filled food questionnaires from 21 patients, each totalling 282 questions.

The food frequency questionnaire provided did not provide enough granular detail, nor is the FFQ validated to be able to compare food intake against receptor/ hormone expression shown in Chapter 2. Data is included here for transparency and a demonstration of work undertaken.

This food questionnaire presented several challenges. Firstly, it required translating from the original Dutch. Because I was sent scanned copies of the original paper transcript, automated translation required the converting of each PDF scanned questionnaire into a Word document and then uploading for translation in Google Translate. It was then necessary to liaise with Dutch speaking members of the team in Maastricht involved in the study who could help turn the Google translation into understandable questions. These translated questions were then copied into an Excel sheet.

3c What types of breakfast cereals have you eaten?

	rarely / never	once in a while	often	usually / always
muesli, cruesli	O	O	O	O
cornflakes	O	O	O	O
All Bran	O	O	O	O
other breakfast products	O	O	O	O

Figure 50: A typical question from the Maastricht FFQ (note issues with imperfect translation and graphical errors during the translation process).

Whilst Figure 50 is set out as one question in the questionnaire, for data entry purposes this proved unsuitable; instead, it was divided into four different questions (Table 18).

Table 18: The question in Figure 50 is converted into several separate questions in an Excel format.

3c	**3c**	**3c**	**3c**
How often eaten breakfast muesli, cruesli	**How often eaten breakfast cornflakes**	**How often eaten breakfast all Bran**	**How often eaten other breakfast products**
rarely/ never	rarely/ never	rarely/ never	rarely/ never
once in a while	once in a while	once in a while	once in a while
often	often	often	often
usually/ always	usually/ always	usually/ always	usually/ always

This format was common throughout the 21-page questionnaire and so almost every question was edited to function within an Excel data sheet.

In order to assess how food intake was related to nutrients and their related nutrient sensing GPCRs, questions were assigned to food groups. For example, “How often did you eat cheese in the past month?” was associated with calcium (Babinsky et al., 2016), tryptophan (Conigrave et al., 2000b), oleoylethanolamide (Hansen et al., 2012), lysophosphatidylcholine (Barber et al., 2012), osteocalcin (Michaelsson et al., 1995), L-arginine and L-lysine (Górska-Warsewicz et al., 2018) and the nutrient receptors involved in sensing these nutrients would then be CaSR (Babinsky et al., 2016), GPRC6a (Babinsky et al., 2016, Michaelsson et al., 1995, Górska-Warsewicz et al., 2018) and GPR119 (Barber et al.,

2012, Hansen et al., 2012). CaSR has the highest EC50 score for calcium (2.57 ± 0.03(Babinsky et al., 2016)) and therefore the question that was most associated with foods high in calcium was selected.

With the questions and possible answers translated and transferred to Excel, each answer was then converted into a number. Some answers were already numerical, some were not; taking the example above, “rarely/ never”, “once in a while”, “often”, “usually/ always” was turned into 1, 2, 3, 4 respectively (Table 19). In order to turn these answers into a numerical score, they were converted into a score over 4 weeks: “1 day per 4 weeks” was turned into 0.25 (1÷4 weeks); “2-3 days per 4 weeks” into 0.625 (2-3 days average at 2.5÷4); “7 days per week” into 28 (7*4), (Table 19).

This was then converted into a frequency (Table 19) and amount score (Table 20).

Table 19: Calculating a frequency score from answered questions.

Answer provided	Numerical conversion	Number of days/ 4 weeks
None	1	0
1 day per 4 weeks	2	0.25
2-3 days per 4 weeks	3	0.625
1 day per week	4	4
2 days per week	5	8
3 days per week	6	12
4 days per week	7	16
5 days per week	8	20
6 days per week	9	24
7 days per week	10	28

Table 20: Different questions have different options that can be selected for "amount". 43b, 44a and 45b refer to 3 different questions which had 3 different measures of quantity.

43b: "tablespoons"	44a: "portions"	45b: "pieces"
1	0.5	1-3
2	1	4-6
3	1.5	7-9
4	2	10-12
5	2.5	13-15
6	3	16-18
7		19-21
8		22-24
9		25-27
10		28-30
11		more than 30
12		

Table 21 shows what this might look like for two genes, CaSR and GPR40; calcium (EC50 2.57 ± 0.03(Babinsky et al., 2016)) and eicosapentaenoic acid (EC50 5.17 ± 0.08(Briscoe et al., 2003)) were chosen respectively. Of note is that palmitic acid has a higher EC50 (5.30 ± 0.12(Briscoe et al., 2003)) than eicosapentaenoic acid for GPR40, however palmitic acid is found mostly in palm oil (PubChem), which is a less common food stuff than fish and fish oil [26]. Also of note is the "?" in the first in the amount column of question 44b in Table 21. This is a case of a question being left unanswered by the participants; for instance, there are some cases where the frequency might be marked as 'once/ week' but the amount is blank; in this case the data is not included.

Table 21: Frequency and amount score for foodstuffs and nutrients that are strong agonists for CaSR and GPR40.

Gene target	CASR		GPR40	
Nutrient with highest EC50 rating	Calcium		Eicosapentaenoic acid	
Question chosen	16a	16b	44a	44b
Question chosen (full text)	How often did you drink milk and buttermilk in the past month?	How many glasses / cups did you drink on average on such a day?	How often did you eat fish in the past month?	How many portions of fish did you eat on average on such a day?
Frequency and amount scores	**Frequency**	**Amount**	**Frequency**	**Amount**
	0.65	1	4	?
	0		8	1
	8	1	0.25	2
	0		8	2
	28	2	8	4
	0		0	
	0.65	1	0	
	28	2	0.625	2
	0.25	1	0	
	0.25	1	12	2
	16	1	4	2
	12	1	0	
	8	1	0.25	2
	12	2	4	1
	0		0	
	0		8	2
	0.25	1	8	2
	0		4	1
	16	8	0	
	0		4	2
	0.65	1	8	2

This score was calculated by quantifying the frequency answer and multiplying by the approximate intake of said foodstuff, as previously described. Some questions have different available options for 'amount'; therefore, this value is relative and different GPCRs cannot be compared with each other. This was repeated for each of the 10 nutrient receptors examined by qPCR: CASR, GPR40, GPR41, GPR43, GPR120, TGR5, GPR119, GPCR6a, GPR109a and PGE4 (Table 22). In general, a higher score means that more food stuffs relevant to the nutrient receptor were consumed.

Table 22: Calculated food intake scores for nutrients, food stuffs against various target nutrient receptors. Higher score equates to higher intake.

	Nutrient receptor gene of interest								
Patient ID	**CASR**	**GPR40**	**GPR43 and GPR41**	**GPR120**	**TGR5**	**GPR119**	**GPRC6a**	**GPR109a**	**Prostaglandin E receptor 4**
565	0.65		2.00		4.00		24.00	12.00	12.00
591	8.00	0.50	16.00	0.50	0.25	0.50	16.00	16.00	0.00
592	0.00	16.00	8.00	16.00	0.00	16.00	16.00	12.00	0.00
601	56.00	32.00	16.00	32.00	56.00	32.00	8.00	20.00	0.00
605	0.00	0.00	4.00	0.00	0.25	0.00	0.00	30.00	72.00
607	0.65	0.00	1.25	0.00	8.00	0.00	0.50	8.00	0.00
610	56.00	1.25	8.00	1.25	8.00	1.25	0.50	20.00	8.00
611	0.25	0.00	1.25	0.00	16.00	0.00	1.25	3.00	0.00
612	0.25	24.00	0.63	24.00	16.00	24.00	32.00	0.00	40.00
617	16.00	8.00	1.25	8.00	1.25	8.00	8.00	20.00	0.25
619	12.00	0.00	0.63	0.00	0.25	0.00	0.00	0.00	12.00
620	8.00	0.50	4.00	0.50	28.00	0.50	0.25	20.00	1.88
626	24.00	4.00	0.00	4.00	0.25	4.00	1.25	18.00	
633	0.00	0.00	8.00	0.00	0.00	0.00	0.00	20.00	0.00
645	0.00	16.00	4.00	16.00	4.00	16.00	24.00	14.00	0.00
650	0.25	16.00		16.00	28.00	16.00	0.00	40.00	84.00
656	0.00	4.00	0.50	4.00	12.00	4.00	1.25	20.00	36.00
663	128.00	0.00	48.00	0.00	12.00	0.00	0.50	4.00	0.75
674	0.00	8.00	16.00	8.00	8.00	8.00	1.25	18.00	4.00
680	0.65	16.00	0.63	16.00	4.00	16.00	0.63	20.00	48.00

8.1.1 A Food Frequency Questionnaire showed no significant differences in food intake for nutrient receptors between normal and overweight/ obese BMI groups.

A food intake score was calculated for each gene examined by PCR, based on the results of a food frequency score, as previously discussed. Patients were separated into healthy BMI (<25) and overweight/obese BMI (≥25) groups. When food intake score was compared to the two BMI groups (Figure 51), there was no significant differences. GPR43 and GPR41 are grouped together because both nutrient receptors have high EC50 scores for propionate, butyrate and acetate, which are associated with indigestible dietary fibre (plants, cereal grains, fruits, vegetables) (Ang and Ding, 2016a, Dhingra et al., 2012).

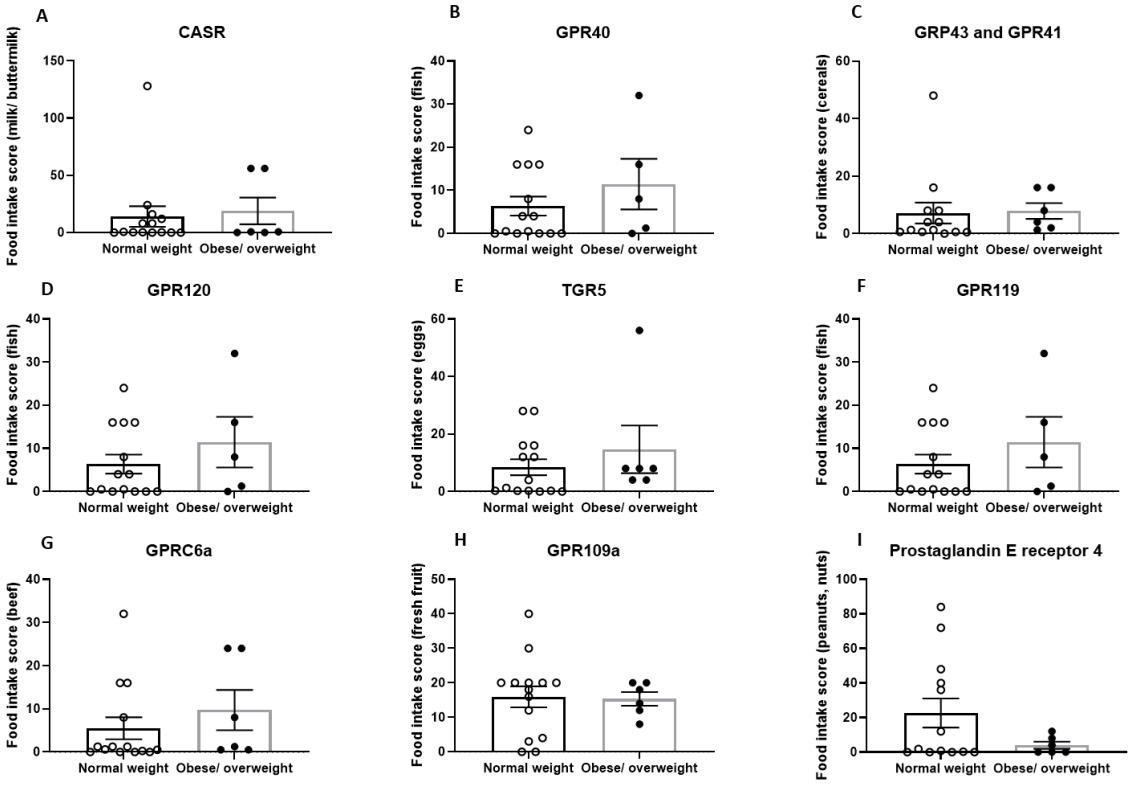


Figure 51: Food intake score for various nutrient receptors in normal weight and obese/ overweight subjects. The foodstuff examined is marked on the Y axis for each gene.

8.2 Comparing the IBS state of samples against expression of nutrient receptors, hormones, mediators.

8.2.1 The IBS state of sigmoid colonic samples was compared against the gene expression of nutrient receptors

In order to determine if the IBS state (constipated, diarrhoea, mixed, no IBS) of patients from Maastricht influenced RNA expression, the mean relative mRNA expression of each IBS group was compared with the mean of a healthy control group who did not have a clinical diagnosis of IBS, by ordinary one-way ANOVA (Dunnett's multiple comparisons test, Figure 52), data was confirmed to pass normality (D'Agostini and Pearson normality test). This was carried out to remove the IBS state as a possible confounder in this data set, as IBS-diagnosed patients might have some form of disruption to normal gut function (which might manifest differently to obesity gut dysregulation), which could change gene expression.

Healthy controls were recruited by our partners in Maastricht, however samples were only taken from the sigmoid colon, and I only received 2 healthy control samples for analysis.

GPRC6a and PTGER4 appear to show a change in expression between the IBS sub-groups and no IBS subgroup. However, because the numbers of patient samples that were part of the No IBS group was so low (1-2 samples) it is problematic to attribute any real statistical inference to this finding. I saw no change in relative gene expression between any of the IBS sub-groups.

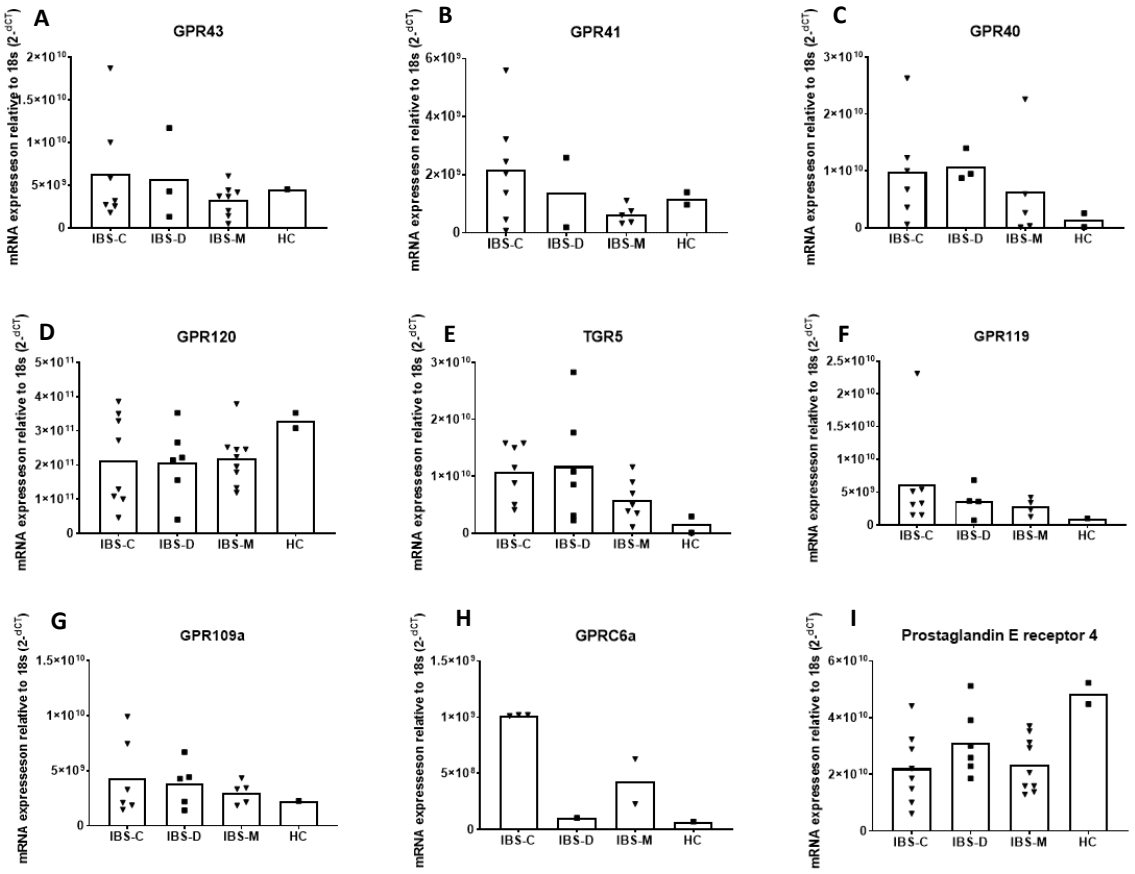


Figure 52: Relative mRNA expression for genes of nutrient receptors including for short-, medium-, long-chain fatty acids in human sigmoidal colon samples. Expression in healthy control patients (HC) compared with each IBS sub-group. IBS-C = IBS with constipation, IBS-D – IBS with diarrhoea, IBS-M = IBS with mixed symptoms. The expression (mean) is described relative to 18s.

8.2.2 IBS state of sigmoid colonic samples did not seem to affect gene expression of hormones or signalling molecules

Gene expression was measured in sigmoid colonic samples from patients separated into IBS-C, IBS-D, IBS-M or healthy control groups (Figure 53) and analysed as previously (Figure 52). Healthy control samples were only taken from the sigmoid colon. The numbers of healthy control samples were low (1-2 samples only); therefore, it is not possible to gather any statistical inference. I saw no change in relative gene expression between any of the IBS sub-groups.

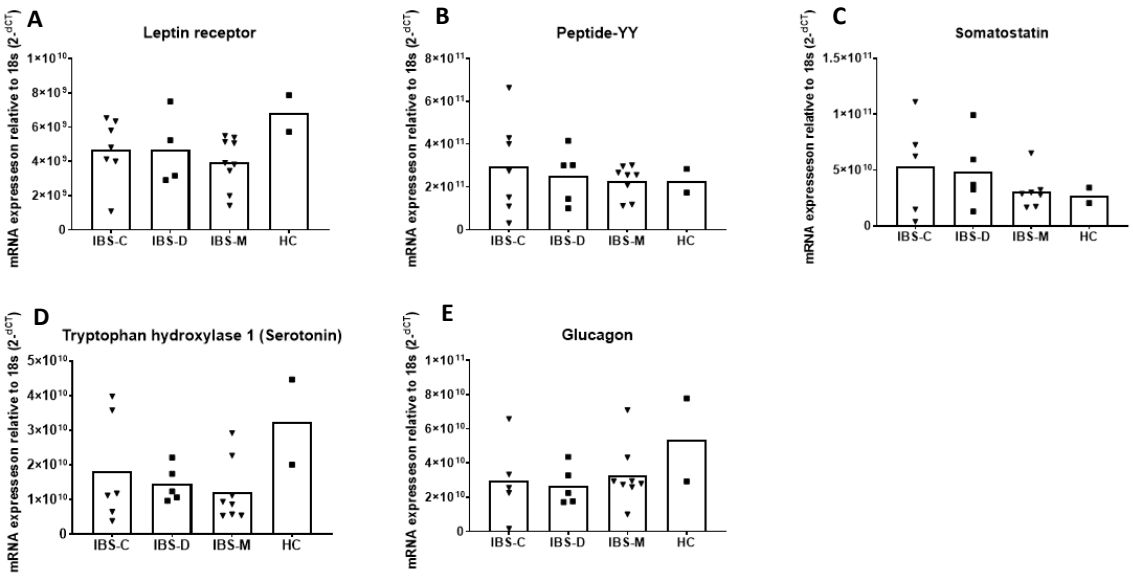


Figure 53: Relative RNA expression for genes of hormones involved in satiety, appetite regulation and gut motility. Expression in control patients compared with each IBS sub-group. No significant difference in gene expression between IBS disease groups vs. controls. IBS-C = IBS with constipation, IBS-D – IBS with diarrhoea, IBS-M = IBS with mixed symptoms. The expression (mean) is described relative to 18s.

8.3 Analysing gene expression data as a linear regression made identifying significant changes in expression difficult

8.3.1.1 Linear regression of relative expression against BMI on samples from the sigmoid colon

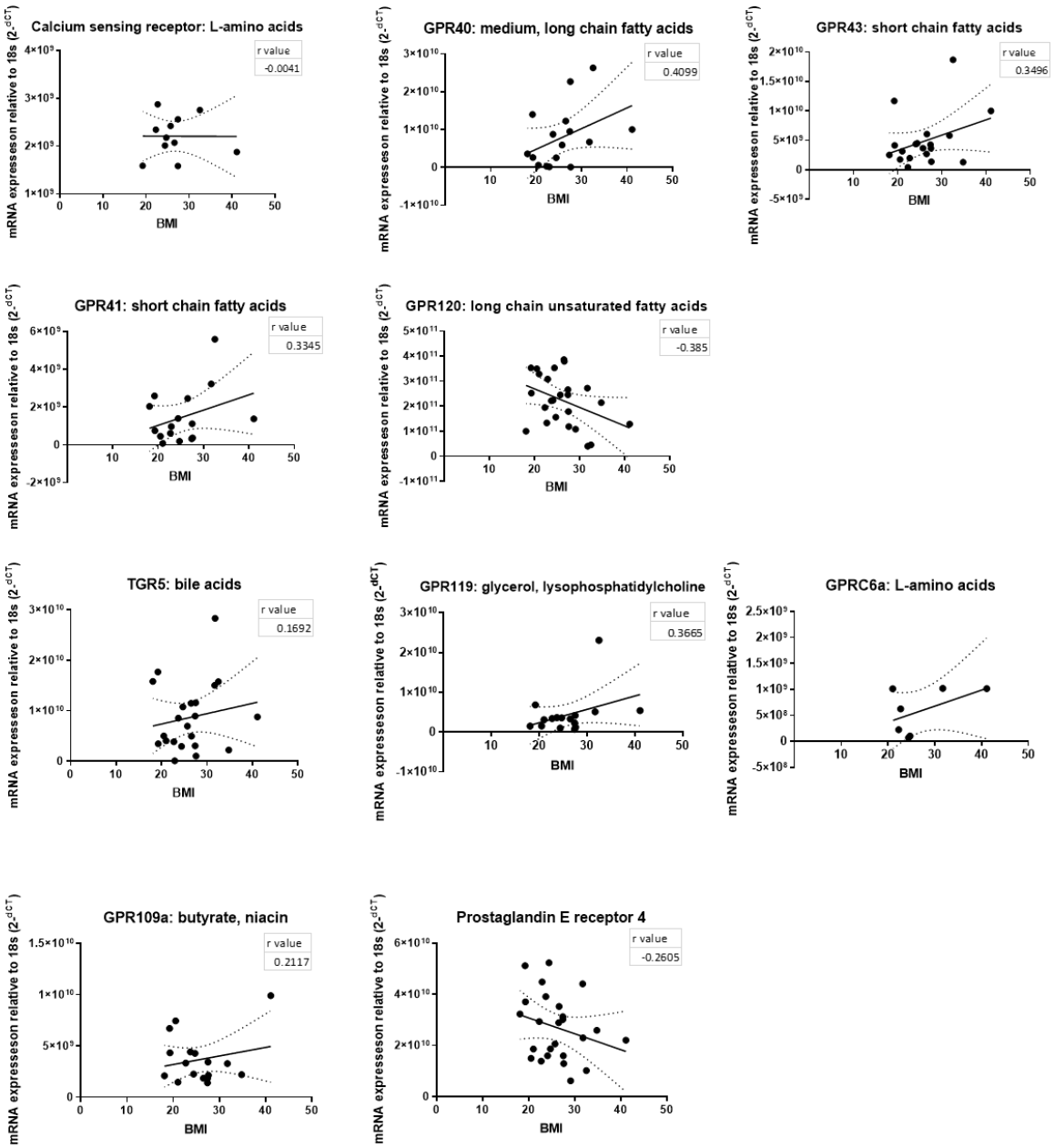


Figure 54: Linear regression analysis of nutrient receptors. Relative expression of genes of interest derived from human sigmoidal colonic tissue. R value shown determined by Simple Linear Regression.

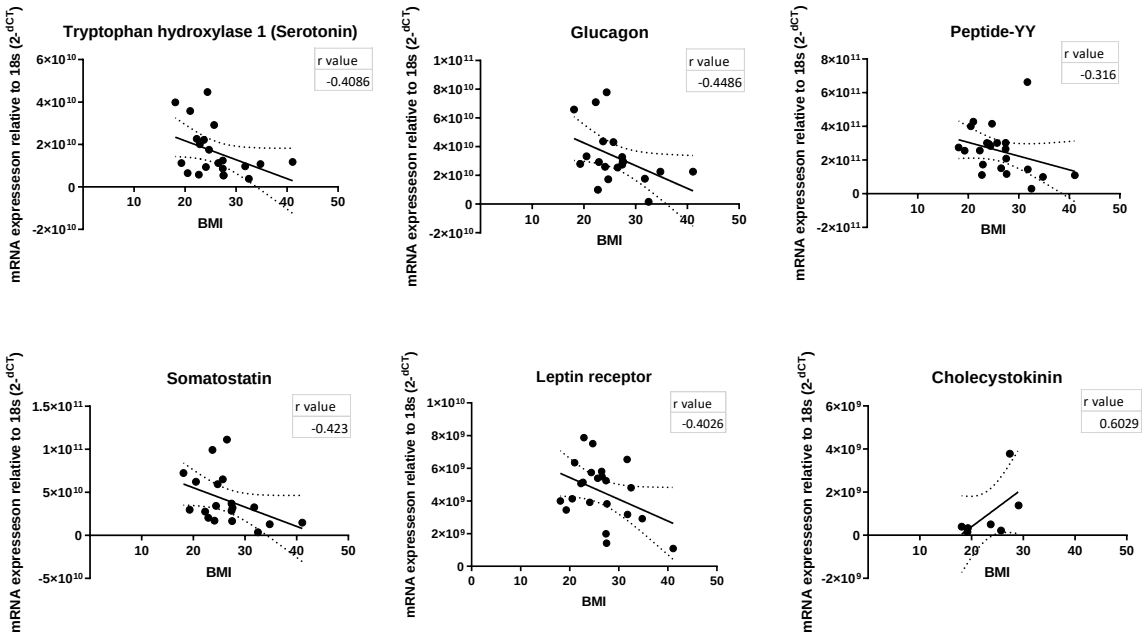


Figure 55: Linear regression analysis of hormones, mediators, hormone receptors. Relative expression of genes of interest derived from human sigmoid colonic tissue. R value shown determined by Simple Linear Regression.

8.3.2 Linear regression of relative expression against BMI on samples from the proximal colon

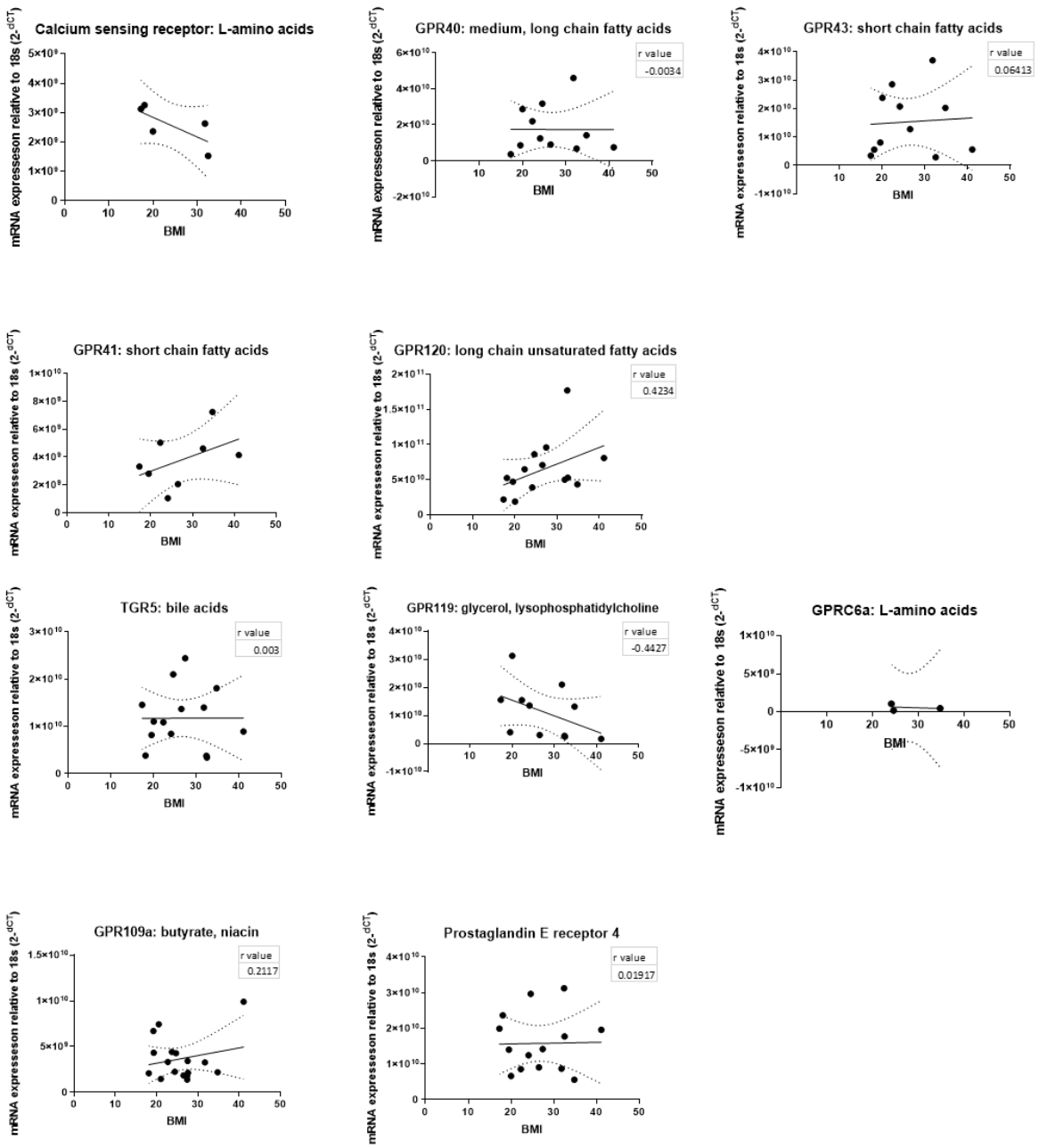


Figure 56: Linear regression analysis of nutrient receptors. Relative expression of genes of interest derived from human proximal colonic tissue. R value shown determined by Simple Linear Regression.

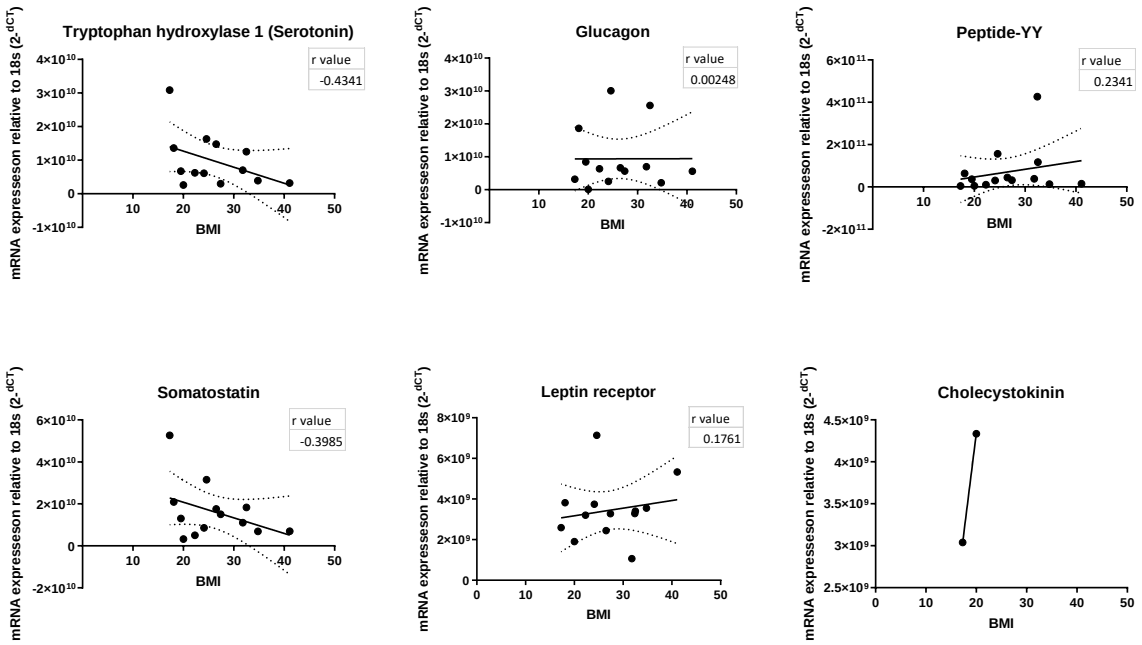


Figure 57: Linear regression analysis of hormones, mediators, hormone receptors. Relative expression of genes of interest derived from human sigmoidal colonic tissue. R value shown determined by Simple Linear Regression.

9 References

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