

The role of short chain fatty acids in appetite regulation

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Arianna Psichas

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Section of Investigative Medicine

Division of Diabetes, Endocrinology and Metabolism

Imperial College London

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Abstract

The current obesity epidemic poses a major challenge to public health. Apart from bariatric surgery, a safe and effective long-term treatment for obesity has yet to be identified. Therefore, an improved understanding of the physiology of energy homeostasis is now more critical than ever.

Epidemiologically, there is an inverse association between consumption of fermentable fibre and weight gain. This is supported by experimental studies, which demonstrate that increasing the consumption of fermentable fibre can reduce appetite and body weight. It is thought that these effects may be attributable, at least in part, to the bacterial fermentation of fibre in the colon yielding short chain fatty acids (SCFAs). SCFAs have been identified as ligands for the G-protein coupled receptors GPR41 and GPR43.

There are fundamental gaps in our current understanding of the physiological roles of SCFAs and their receptors in energy homeostasis, and of their specific effects in different endocrine tissues. This thesis investigates the effects of colonic SCFAs on anorectic gut hormone release from enteroendocrine L cells, and the role of GPR43 in mediating these effects.

I established mouse and human primary L cell models in our laboratory and used them to demonstrate that SCFAs, and particularly propionate, stimulate the release of anorectic gut hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1). I confirmed that colonic administration of propionate increases plasma gut hormone concentrations *in vivo*, in rats and mice. I then explored the mechanisms underlying propionate-induced gut hormone release *in vitro*. I demonstrated that Gpr43^{-/-} L cells exhibit significantly attenuated PYY and GLP-1 secretion in response to propionate; a result which was also confirmed for the first time *in vivo* using Gpr43^{-/-} mice.

These findings suggest that colonic SCFA signalling via GPR43 may play an important physiological role. Further investigation is now required to characterise the intracellular signalling mechanisms underlying GPR43-activated gut hormone secretion and to determine the wider role of GPR43 in energy homeostasis.

Declaration of Contributors

The majority of the work described in this thesis was performed by the author. All collaborations and assistance are detailed below:

All radioimmunoassays were established and are maintained by Professor Mohammad A. Ghatei (Section of Investigative Medicine, Imperial College London) with the assistance of Andrew Hogben.

Chapters 2, 3 and 4

The consenting of patients and collection of human colonic biopsies were carried out by Professor Julian Walters, Dr Ian Johnston and Dr Jonathan Nolan, Imperial College London.

Chapters 3 and 4

In vivo work was carried out with the assistance of Dr Michelle Sleeth.

Miss Lucy Brooks maintained the Gpr43^{-/-} colony and carried out initial genotyping.

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List of Abbreviations

AC	Adenylyl cyclase
Ach	Acetylcholine
Adr	Adrenaline
AgRP	Agouti-related peptide/protein
ANOVA	Analysis of variance
AP	Area postrema
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
AUC	Area under curve
βAR	β-adrenergic receptor
BB2R	GRP receptor
BBB	Blood brain barrier
BETA2	Also known as neuronal differentiation 1 (NeuroD1)
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CART	Cocaine-and amphetamine-related transcript
CaSR	Calcium-sensing receptor
CCK	Cholecystokinin
CGRP	Calcitonin-gene related peptide
CHO-K1	Chinese-hamster ovary K1 cell line
CNS	Central nervous system
CFMB	(S)-2-(4-chlorophenyl)-N-(5-fluorothiazol-2-yl)-3-methylbutanamide
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl sulfoxide
DMX	Dorsal motor nucleus of the vagus
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
DPPIV	Di-peptidyl peptidase IV
EDTA	Ethylene diamine tetraacetic acid
EEC	Enteroendocrine cell
eGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
Epac2	Exchange protein activated by cAMP
ERK1/2	Extracellular-signal-regulated kinase 1 and 2
FACS	Fluorescence-activated cell sorting
FAO	Food and Agriculture Organisation (of the United Nations)
FCS	Foetal calf serum
FDA	Food and Drug Administration (US)
FFAR	Free fatty acid receptor
For	Forskolin
Gal1R	Galanin receptor 1
GDW	Glass distilled water
GI	Gastrointestinal
GIP	Glucose-dependent insulintropic polypeptide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GPCR	G-protein coupled receptor
GRP	Gastrin-releasing peptide
HEK-293	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HET	Heterozygous genotype

HFD	High fat diet
HPLC	High pressure liquid chromatography
HSE	Health Survey for England
5-HT	5-hydroxytryptamine, serotonin
IBMX	3-isobutyl-1-methylxanthine
ICV	Intracerebroventricular
INSERM	French National Institute for Medical Research
INT	Iodonitrotetrazolium
i.p.	Intraperitoneal
IP₃	Inositol 1,4,5-triphosphate
i.v.	Intravenous
JV	Jugular vein
KO	Knockout genotype
LCFA	Long-chain fatty acid
LDH	Lactate dehydrogenase
LHA	Lateral hypothalamic area
M2R	Muscarinic acetylcholine receptor M2
MATH1	Also known as atonal homolog (Atoh1)
MCFA	Medium-chain fatty acid
MCH	Melanin-concentrating hormone
MCT	Monocarboxylate transporter
MEK1/2	Mitogen-activated protein kinase kinase ('MAPK/ERK kinase')
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NA	Noradrenaline
NAD⁺/NADH	Nicotinamide adenine dinucleotide oxidised/reduced form
NDC	Non-digestible carbohydrate
NFκB	Nuclear Factor-κB
NGN3	Neurogenin 3

NHS	National Health Service
NMB	Neuromedin B
NPY	Neuropeptide Y
NSB	Non-specific binding
NTS	Nucleus tractus solitarius
OD	Optical density
OEA	Oleoylethanolamide
2-OG	2-oleoylglycerol
OXM/Oxyn	Oxyntomodulin
PACAP	pituitary adenylate cyclase-activating protein
Pax4/6	Paired box gene 4/6
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHEN/TPM	Phentermine/topiramate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PPARγ	Peroxisome proliferator-activated receptor gamma
PTX	Pertussis toxin
PV	Portal vein
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine (YY)
QC	Quality control
RCT	Randomised controlled trial
RIA	Radioimmunoassay
RPM	Revolutions per minute

RYGB	Roux-en-Y gastric bypass
SCFAs	Short chain fatty acids
SCG	Sympathetic cervical ganglion
SEM	Standard error of the mean
SGLT	Sodium-glucose co-transporter
SI	Small intestinal
siRNA	Small interfering ribonucleic acid (RNA)
SMCT1	Sodium-coupled monocarboxylate transporter 1
SNARE	SNAP (Soluble SNF Attachment Protein) Receptor
SNF	N-ethylmaleimide-sensitive factor
SNS	Sympathetic nervous system
SST	Somatostatin
SST5R	Somatostatin receptor 5
TAE	Tris-acetate-EDTA
TRPM5	Transient receptor potential cation channel subfamily M member 5
VIP	Vasoactive intestinal peptide
VMN	Ventromedial nucleus
VPACR	PACAP receptor
WHO	World Health Organisation
WT	wild type
Y2R	Y2 receptor

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Chapter I:

General Introduction

1.1 Obesity

1.1.1 Definition, prevalence and economic burden

The World Health Organisation (WHO) defines overweight and obesity as “abnormal or excessive fat accumulation that may impair health” (WHO 2012). The most commonly used index for the classification of overweight and obesity is the body mass index (BMI), calculated as weight divided by height squared (kg/m^2) (Table 1.1), which is arguably the most efficient method of measuring the prevalence of obesity at a population level.

Table 1.1 **Body Mass Index (BMI) definitions** (NHS 2012b)

Definition	BMI range (kg/m^2)
Underweight	Under 18.5
Normal	18.5 to less than 25
Overweight	25 to less than 30
Obese	30 to less than 40
Morbidly obese	40 and over

Obesity, which has more than doubled worldwide since 1980 (WHO 2012), is a major public health challenge. Based on an analysis of epidemiological studies from 199 countries, 1.46 billion adults were estimated to be overweight in 2008, of which just over half a billion would be classified as obese (Finucane *et al.* 2011). Despite signs of stabilisation in some populations (Rokholm *et al.* 2010, Sperrin *et al.* 2013), the consequences of obesity remain extensive.

The UK is amongst the countries which have experienced a dramatic rise in the prevalence of overweight and obesity in the past few decades (Wang *et al.* 2011b). Consequently, ~25% of the adult population in England is now classified as obese, with a further 41% of men and 33% of women classified as overweight (HSE 2011). Furthermore, a report published in the

Lancet in 2011, projected that there would be 11 million additional obese adults in the UK by 2030 (Wang *et al.* 2011b). This projection was based on historic trends in BMI obtained from data published in the Health Survey for England (HSE, 1993-2008).

According to the WHO, obesity is the fifth leading risk for deaths globally. The WHO estimates that at least 2.8 million adults die every year as a consequence of being overweight or obese (WHO 2012). Excess body weight within populations foreshadows increased prevalence of several chronic diseases, most notably cardiovascular diseases (Wormser *et al.* 2011), type 2 diabetes (Vazquez *et al.* 2007) and certain cancers (e.g. endometrial, breast and colon) (Renehan *et al.* 2008). Excess body weight also contributes to non-fatal but expensive and disabling disorders such as osteoarthritis (Guh *et al.* 2009). As a result, Western societies, many of which have ageing populations, suffer from premature mortality, obesity-associated morbidities and reduced productivity and quality of life.

The sheer scale of obesity and its multiple associated co-morbidities, translate into a substantial economic burden for national healthcare systems. A report produced by the UK's Office for Science Foresight Programme in 2007 projected that the continuing rise in obesity will add £5.5 billion in medical costs to the National Health Service (NHS) by 2050 (Kopelman *et al.* 2007).

1.1.2 Aetiology

By definition, the cause of excess weight gain is deceptively simple; it is an energy imbalance between calories consumed and calories expended (WHO 2012). However, the aetiology of obesity is highly complex and has multiple determinants. The dramatic rise in obesity has been linked to changes in food production and the increased availability of energy-dense foods, and at the same time to a decrease in physical activity and energy

expenditure due to the increasingly sedentary nature of urban life (Butland *et al.* 2007). This 'obesogenic environment' (Swinburn & Egger 2002), has exposed the propensity of humans to accumulate energy and conserve it (Yeo & Heisler 2012). Our drive to consume food is a very powerful instinct, intimately linked with the promotion of survival. As such, multiple redundant mechanisms have evolved to respond to, and deal with, frequent periods of food scarcity (Yeo & Heisler 2012). Therefore, at the core of the problem is a now ill-adapted physiological system, which has been unable to cope with rapid environmental change.

Due to the complex nature of the aetiology of obesity, the development of anti-obesity treatments has proved challenging.

1.1.3 Treatment

Lifestyle modification

The first line of treatment for the management of overweight and obesity is lifestyle intervention, incorporating dietary restriction and increased physical activity to improve energy balance. Lifestyle interventions can be successful in inducing weight loss (Tuomilehto *et al.* 2001). However, more often than not, individuals struggle with maintaining this weight loss (Crawford *et al.* 2000, Weiss *et al.* 2007). Therefore, at a population level, anti-obesity agents appear necessary as an adjunctive therapy to lifestyle modification to ensure successful weight loss and maintenance.

Pharmacotherapy

The only anti-obesity drug currently licensed for prescription use in the UK and within the European Union is Orlistat. Orlistat is a pancreatic lipase inhibitor that acts within the

gastrointestinal tract to inhibit the absorption of fat by approximately 30% (Drew *et al.* 2007). Orlistat is associated with a modest but significant weight reduction (~3%) above that achieved with dietary modification alone in obese individuals (Drew *et al.* 2007). A Cochrane meta-analysis encompassing 11 randomised controlled trials (RCTs) found that a larger number of subjects in the Orlistat group achieved clinically significant weight loss: 21% and 12% achieved $\geq 5\%$ and $\geq 10\%$ weight loss, respectively (Padwal *et al.* 2003). However, the Cochrane review also reported high attrition rates (14-52%) during the Orlistat studies. Orlistat treatment is associated with adverse gastrointestinal effects, experienced by 16-40% patients (Padwal *et al.* 2003). One of the unpleasant and unavoidable consequences of fat malabsorption in the gut is steatorrhea. In addition, the magnitude of weight loss achieved is, in many cases, insufficient to prevent the development of complications associated with obesity (Field *et al.* 2010a, Powell *et al.* 2011).

The development of pharmacological treatments to combat obesity has been riddled with setbacks related to efficacy and safety issues. As a consequence, no new drugs were licensed for the treatment of obesity in over a decade. However, the summer of 2012 saw the approval of two novel anti-obesity drugs by the United States Food and Drugs Administration (FDA). The first drug to be granted approval was Lorcaserin, commercially marketed as Belviq, which is an entirely centrally-acting serotonin 2c (5-HT_{2c}) receptor agonist (Yao 2012). Lorcaserin selectively activates central 5-HT_{2c} receptors and increases satiety via effects on the melanocortin system (Lam *et al.* 2008, Xu *et al.* 2008). A recent meta-analysis of three RCTs (lasting ≥ 1 year, total sample size: 7,789) demonstrated that the use of lorcaserin results in statistically significant but moderate absolute weight loss compared with placebo (~3%), similar in magnitude to the weight loss following orlistat treatment (Chan *et al.* 2013). However, lorcaserin too is associated with adverse effects, the most common ones being transient headache, nausea and dizziness, which were found to be statistically significant in the meta-analysis (Chan *et al.* 2013).

The second anti-obesity drug approved by the FDA in July 2012 was Qsymia, previously known as Qnexa. Qsymia is a combination of two drugs: the appetite suppressant, phentermine and the anticonvulsant, topiramate. The SEQUEL study of phentermine/topiramate (PHEN/TPM, Qsymia) demonstrated sustained weight loss over 108 weeks of treatment in combination with lifestyle modification. A significant weight loss of 10% was achieved by >50% of PHEN/TPM-treated subjects, whereas less than 12% of individuals receiving placebo achieved this goal (Garvey *et al.* 2012). Phentermine is a well-established drug which reduces appetite by stimulating hypothalamic release of noradrenaline (Powell *et al.* 2011). As with many centrally acting drugs, Qsymia is not without unwanted side effects; patients reported depression and/or anxiety-related adverse events. The safety profile of drugs is currently the biggest obstacle in anti-obesity drug development (McGavigan & Murphy 2012).

Bariatric surgery

Advances in the field of bariatric surgery over the past decade have been a major breakthrough in the treatment of the very obese. Bariatric surgery is now well-recognised as the most effective therapeutic option currently available for morbidly obese individuals. It results in significant and sustained weight loss in the region of ~30% (Maggard *et al.* 2005, Sjostrom *et al.* 2007) with a demonstrated mortality benefit [adjusted all cause long term mortality ~40% (Adams *et al.* 2007)]. The Roux-en-Y gastric bypass (RYGB) procedure, which involves the creation of a small stomach pouch and bypass of the proximal small intestine (Fig. 1.1), is considered the gold-standard operative treatment for morbid obesity (Buchwald & Oien 2009, 2013).

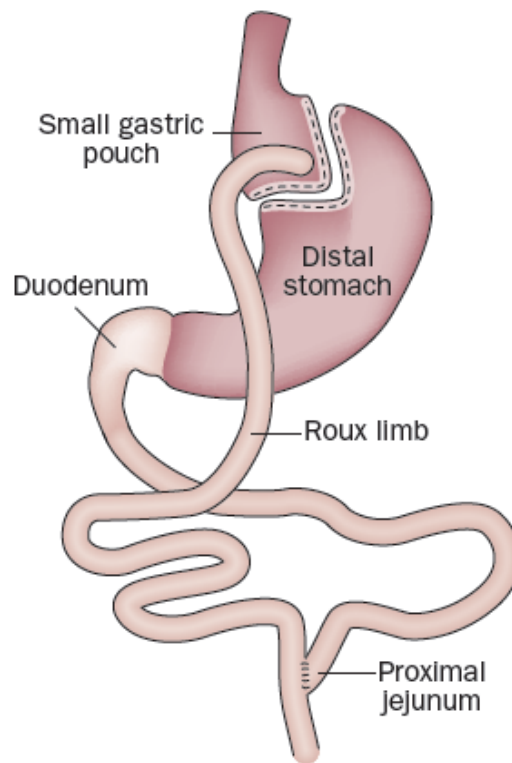


Figure 1.1. **The Roux-en-Y gastric bypass surgical procedure.** This surgery creates a small gastric pouch (~30ml) directly linked to the distal jejunum by the Roux limb. The distal stomach, duodenum and proximal part of the jejunum are subsequently anastomosed 1.5m below the gastrojejunal anastomosis. Adapted from: (Aron-Wisnewsky *et al.* 2012)

However, bariatric surgery is highly invasive and carries a mortality rate in the region of 0.5-2% (Flancbaum & Belsley 2007). Furthermore, in the UK, obesity surgery is only available to morbidly obese individuals on the NHS, who currently only account for ~2-4% of total obese and overweight in England (NHS 2012a). For those who do not meet the requirements for the procedure, and yet who are at significant risk from the complications of being obese, the options remaining are limited and largely ineffective. Consequently, understanding the mechanisms by which gastric bypass induces and maintains weight loss, in order to potentially target these mechanisms pharmacologically, would be invaluable.

Initially it was thought that RYGB-induced weight loss was mediated by mechanical restriction and malabsorption. However, additional mechanisms are now emerging, including bile flow alterations (Pournaras *et al.* 2012), increased satiety (Borg *et al.* 2006), altered taste (Burge *et al.* 1995, Tichansky *et al.* 2006) and a reduced preference for energy dense foods (Olbers *et al.* 2006). Furthermore, a recent study by Liou *et al.* provided the first empirical evidence to suggest that alterations in gut microbiota following RYGB in mice are involved in inducing RYGB-weight loss (Liou *et al.* 2013). RYGB, but not sham surgery or caloric restriction, led to a rapid and sustained modulation of the relative abundance of certain microbial populations, particularly in the distal gut. Transplantation of gut microbiota from the RYGB group to non-operated germ-free mice resulted in weight loss and reduced adiposity in the recipient mice, compared to the recipients of gut microbiota from the sham surgery group (Liou *et al.* 2013). This effect may have been mediated, at least in part, by an alteration in the microbial production of short chain fatty acids (discussed in greater detail in Section 1.4).

The rearrangement of the gastrointestinal tract has also been proposed to increase satiety via increased delivery of nutrients to distal regions of the small intestine, thereby enhancing the secretion of appetite-suppressing gut hormones (Beckman *et al.* 2011, Korner *et al.* 2005, le Roux *et al.* 2006, le Roux *et al.* 2007). These peripherally-derived signals stimulate central anorexigenic pathways to reduce food intake via the so-called gut-brain axis.

An improved understanding of the physiology of energy homeostasis and appetite regulation would potentially enable us to mimic the physiological consequences of gastric bypass, such as the favourable SCFA and anorectic gut hormone profile, without the need for invasive surgery, which would have profound implications for the development of anti-obesity treatments.

1.2 The Gut-Brain Axis in the Regulation of Appetite

Appetite is regulated by a sophisticated homeostatic system, at the centre of which lies a complex neuroendocrine network known as the gut-brain axis (Dockray 1988, Track 1980). The gut-brain axis is a bidirectional communication system that relays information between the gut and the brain to tailor gastrointestinal function (secretion and motility) and food intake to the nutrient content of the intestinal milieu. This serves to maximise the efficient digestion and assimilation of ingested nutrients and to induce postprandial satiety, which ultimately contributes to the long term maintenance of energy balance. The major regions orchestrating appetite modulation within the brain are the hypothalamus and the brainstem, which integrate peripherally-derived neural and hormonal signals (Fig. 1.2).

1.2.1 The hypothalamus

The hypothalamus is a major site of peripheral signal integration within the central nervous system (CNS); receiving information from metabolic, endocrine and neural pathways, which concertedly modulate feeding behaviour. The hypothalamus is situated at the base of the brain, surrounding the third ventricle, and comprises several distinct but highly interconnected nuclei and regions, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and the lateral hypothalamic area (LHA), all of which are implicated in the regulation of energy homeostasis. Of these hypothalamic regions, the ARC perhaps has the most prominent role in energy homeostasis.

The arcuate nucleus (ARC)

The ARC is pivotal to the convergence and integration of peripheral energy homeostatic signals (Stanley *et al.* 2005). This is partly due to its anatomical location, adjacent to the median eminence, an area of incomplete blood-brain barrier, enabling the ARC to interact with circulating metabolic and endocrine signals. These signals include nutrients, and hormones derived from the gut, pancreas and adipose tissue (Lam *et al.* 2005, Murphy & Bloom 2006, Porte *et al.* 2002, Wang *et al.* 1997). The ARC also receives input from the caudal brainstem and is thus indirectly privy to neural signals originating from the gastrointestinal tract. These collated signals act on two distinct neuronal populations within the ARC to modulate appetite.

A population of neurons in the medial ARC co-express the orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY) (Broberger *et al.* 1998), whereas a population in the lateral ARC co-express the anorexigenic neuropeptides cocaine- and amphetamine-regulated transcript (CART) peptide and pro-opiomelanocortin (POMC) (Elias *et al.* 1998). These neurons are known as “first-order” neurons, the axons of which project to “second-order” neurons within the ARC, other hypothalamic nuclei and extra-hypothalamic regions.

POMC and CART

POMC is the polypeptide precursor of several molecules including the melanocyte-stimulating hormones (α -, β -, and γ -MSH), of which α -MSH has the most prominent role in energy homeostasis, as an anorexigenic agent (Tsujii & Bray 1989). MSH hormones act on the melanocortin receptors and particularly the melanocortin 4 receptor (MC4R), which is widely expressed in the CNS, including in the PVN and ARC (Mountjoy *et al.* 1994).

Melanocortin receptor agonism is fundamental in the regulation of energy homeostasis (Cone 1999, 2005). In humans, specific mutations affecting components of the melanocortin system are associated with morbid obesity (Farooqi *et al.* 2003, Krude *et al.* 1998, Yeo *et al.* 1998, Yeo & Heisler 2012) .

CART was originally identified as a consequence of its upregulation by cocaine and amphetamine (Kuhar & Dall Vechia 1999). However, 90% of CART neurons are co-localised with POMC neurons in the ARC, which suggested a prominent role in energy homeostasis. Consistent with its co-localisation with POMC, CART has been demonstrated to possess strong anorexigenic qualities. Central administration of CART in rodents leads to a reduction in food intake, an effect reversed by central infusion of anti-CART antibodies (Lambert *et al.* 1998). Fasting, on the other hand, reduces CART levels in the ARC of lean animals (Kristensen *et al.* 1998).

NPY and AgRP

NPY is one of the most potent central enhancers of appetite identified to date. It is widely expressed in the CNS (Allen *et al.* 1983), and its expression is abundant in the ARC where ~90% of NPY neurons co-express AgRP. Central administration of NPY induces marked hyperphagia and reduces energy expenditure (Billington *et al.* 1991, Clark *et al.* 1984, Stanley & Leibowitz 1985). Furthermore, fasting increases NPY mRNA expression in the ARC (White & Kershaw 1990). The hyperphagic effects of NPY are mediated by specific NPY receptor subtypes. To date, five NPY receptors have been identified. The present consensus is that Y1 and Y5 act synergistically to mediate the orexigenic effects of NPY (Harrold *et al.* 2012, Mashiko *et al.* 2009).

AgRP is another potent orexigenic peptide. Its expression is restricted to the ARC of the hypothalamus, where it is co-synthesised in NPY neurons (Ollmann *et al.* 1997). As with NPY, AgRP expression in the ARC is elevated following fasting (Hahn *et al.* 1998). Moreover, central administration of AgRP also induces prolonged hyperphagia and body weight gain (Ebihara *et al.* 1999, Small *et al.* 2001). AgRP is thought to stimulate food intake mainly via competitive antagonism/inverse agonism of central melanocortin receptors (Ollmann *et al.* 1997), though alternative mechanisms of action have also been suggested (Schwartz *et al.* 2000, Wu *et al.* 2008).

1.2.2 The caudal brainstem and the vagus nerve

The brainstem

The caudal brainstem is another region of fundamental importance to the regulation of energy homeostasis and the relaying and integration of information in the gut-brain axis. The brainstem, located in the hindbrain, receives neural signals from vagal nerve afferents and cervical spine afferents from the gastrointestinal tract, the abdominal viscera and the oral cavity (Young 2012). Ingestion of a meal generates mechanosensory, chemosensory and endocrine signals from the gastrointestinal tract, which slow gastric emptying and gut motility to ensure efficient digestion and absorption, and bring about a sense of fullness and satiation. These signals mediate their effects predominantly by activating vagal afferent fibres which synapse in the brainstem (van der Kooy *et al.* 1984). The brainstem contains three structures which are important in the control of energy balance; the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMX) and the area postrema (AP)(Young 2012). The NTS, situated in the dorsal medulla, is the primary sensory relay nucleus for the gastrointestinal tract (and other viscera). It integrates information related to

the gustatory properties of food (Travers *et al.* 1987) as well as neural input stimulated by gastrointestinal distension (Emond *et al.* 2001, Traub *et al.* 1996), intraluminal nutrients (Zittel *et al.* 1994) and gut hormones.

The NTS also receives projections from the adjacent AP, an anatomically discrete chemosensory structure surrounded by the NTS. Like the median eminence of the hypothalamus, the AP is a circumventricular organ with fenestrated capillaries and is therefore capable of sensing blood- and cerebrospinal fluid-borne signals (Ellacott & Cone 2004). The AP possesses a plethora of receptors for such signals, including receptors for gut hormones.

The third component of the gut-brain axis within the brainstem is the DMX, which lies directly ventral to the NTS. The DMX is the orchestrator of the major descending limb of the vagus and functions as a centre for the integration of motor and secretory drive to the viscera (Young 2012). The AP, NTS and DMX are extensively interconnected. The NTS and DMX therefore form a relatively simple reflex arc for the regulation of gastrointestinal function.

The vagus nerve

The vagus nerve is a major pathway for signals originating from the small intestine and proximal colon, whereas sacral parasympathetic nerves innervate the distal colon. Subdiaphragmatic vagotomy or abdominal afferent denervation, using the neurotoxin capsaicin, significantly suppresses or completely abolishes nutrient-induced satiety (Walls *et al.* 1995, Yox & Ritter 1988). Vagal sensory innervation of the gastrointestinal tract arises from afferent neurons with cell bodies in the nodose and jugular ganglia, the endings of which are concentrated either within the muscular layers or the intestinal mucosa. Mucosal vagal afferent terminals are located within the parenchyma of mucosal villi in close proximity to the basal lamina, but not the epithelial surface (Berthoud *et al.* 1995) and can be either

mechanosensitive or chemosensitive. They typically receive signals from specialised epithelial endocrine cells in a paracrine manner. Many gut hormones are thought to act, at least in part, by activating receptors on vagal nerve afferents (Abbott *et al.* 2005a, Date *et al.* 2002, Moran *et al.* 1990).

1.2.3 Gut hormones

The gastrointestinal tract responds to the presence of intra-luminal nutrients in a coordinated manner, with multiple physiological responses occurring to maximise the efficient digestion and assimilation of ingested food. Enteroendocrine cells (EECs) are the primary sensors of luminal content; they are specialised endocrine epithelial cells that secrete regulatory gut peptides, which in turn modulate the gut-brain axis. Anticipation of a meal, gastrointestinal distension and luminal nutrient composition all affect the secretion of these gut hormones, which in turn tailor gastrointestinal function (secretion and motility) and tend to suppress further food intake. An impressive number of gut hormones have been implicated in these processes and multiple examples of overlapping functions and redundancy can be observed (Cummings & Overduin 2007, Murphy & Bloom 2006). However, certain gut hormones have emerged as instrumental components of homeostatic appetite regulation and these are discussed below.

1.2.3.1 Ghrelin

Ghrelin is the only orexigenic gut hormone isolated so far; a 28-amino acid acylated peptide secreted by “A-X like” cells of the oxyntic glands in the stomach. Ghrelin may be partly responsible for the initiation of hunger; circulating ghrelin levels increase almost two-fold prior to the ingestion of food and fall rapidly in the postprandial phase (Tschop *et al.* 2001). Peripheral or central administration of ghrelin to rodents acutely stimulates food intake (Wren

et al. 2000), and chronically, promotes weight gain (Tschop *et al.* 2000, Wren *et al.* 2001b). In humans, intravenous infusion of ghrelin at physiological doses stimulates hunger and increases food intake acutely (Wren *et al.* 2001a). Ghrelin exists in one of two forms: an acylated or a des-acylated peptide (Kojima *et al.* 1999). The enzyme responsible for the acylation of ghrelin is gastric O-acyl transferase (GOAT). The acylated form of ghrelin is thought to account for the gut hormone's potent orexigenic effects, while the role of des-acylated ghrelin remains contentious.

Ghrelin is the main endogenous ligand for the growth hormone secretagogue receptors (GHS-Rs) and particularly the GHS-R1a receptor, which is expressed in the hypothalamus and the anterior pituitary. Within the hypothalamus, the GHS-R1a receptor is expressed on NPY neurons (Date *et al.* 2000, Lucidi *et al.* 2005), and in accordance with this, the orexigenic action of ghrelin is thought to be mediated by the stimulation of NPY/AgRP pathways and the inhibition of POMC neurons in the ARC (Lucidi *et al.* 2005, Nakazato *et al.* 2001). However, the vagus nerve is also likely to be a critical facilitator of the appetite-enhancing effects of ghrelin (Date *et al.* 2002, van der Lely *et al.* 2004).

1.2.3.2 Cholecystokinin (CCK)

CCK is the prototypical gut hormone (Gibbs *et al.* 1973, Jorpes & Mutt 1959). It is produced and secreted from a subset of enteroendocrine cells known as I cells, located within the duodenal and jejunal mucosa, in response to intraluminal protein and lipids (Liddle *et al.* 1985, Polak *et al.* 1975). Multiple bioactive forms of CCK exist, depending on the specific post-translational processing of the polypeptide precursor, preprocholecystokinin. CCK delays gastric emptying, slows intestinal transit, stimulates pancreatic enzyme secretion and gall bladder contraction and induces a sense of fullness (Dockray 2012). Peripheral administration of CCK-8 has been demonstrated to reduce food intake in both lean and obese individuals (Kissileff *et al.* 1981, Lieveise *et al.* 1995, Pi-Sunyer *et al.* 1982). The

anorexigenic effects of CCK are predominantly mediated through activation of CCK-1 receptors on vagal afferent fibres and the subsequent innervation of the NTS in the brainstem (Gutzwiller *et al.* 2000, Moran *et al.* 1990). In accordance with this, interruption of the vagus nerve in rats abolishes the satiating effect of CCK (Lorenz & Goldman 1982, Smith *et al.* 1981). Furthermore, CCK-1R antagonism has been demonstrated to increase food intake in both animals and humans (Beglinger *et al.* 2001, Hewson *et al.* 1988, Moran *et al.* 1993).

1.2.3.3 Glucagon-like peptide-1 (GLP-1) and Oxyntomodulin (OXM)

The gut peptides glucagon-like peptide 1 (GLP-1) and oxyntomodulin (OXM) are products of the post-translational processing of proglucagon, a 160-amino acid polypeptide precursor synthesised by enteroendocrine L cells in the gastrointestinal tract, pancreatic α -cells, and neurons within the NTS (Holst 2007, Kieffer & Habener 1999).

GLP-1

GLP-1 is secreted by enteroendocrine L cells, found throughout the gastrointestinal tract but in highest density in the colon, in response to nutrients, especially carbohydrates and fatty acids (Kreymann *et al.* 1987, Layer *et al.* 1995, Orskov *et al.* 1994). GLP-1 circulates in two different forms: GLP-1₇₋₃₇ and the major circulating form, GLP-1₇₋₃₆ amide. Both forms are equally potent at the GLP-1 receptor (GLP-1R) (Orskov *et al.* 1994). GLP-1 has multiple physiological roles: it delays gastric emptying and inhibits gastric acid secretion (Tolessa *et al.* 1998), acts as an endogenous incretin (Kreymann *et al.* 1987), suppresses glucagon secretion (Naslund *et al.* 1999) and has trophic effects in pancreatic cells (Edvell & Lindstrom 1999). GLP-1 also has an established role in the induction of satiety. Peripheral administration of GLP-1 reduces acute food intake in rodents and in humans (Abbott *et al.*

2005a, Flint *et al.* 1998), whereas the opposite effect is observed with peripheral administration of the GLP-1R antagonist Exendin 9-39 (Williams *et al.* 2009).

The mechanisms through which GLP-1 inhibits food intake, however, require further investigation. As GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPP-IV) on its route into the circulation, newly secreted GLP-1 is likely to at last partly mediate its anorexigenic effects through activation of peripheral GLP-1 receptors expressed on vagal nerve afferents in the gastrointestinal tract or even in the hepatoportal region (Hansen *et al.* 1999, Holst & Deacon 2005, Vahl *et al.* 2007, Williams *et al.* 2009). In accordance with this, brainstem NTS neurons are activated in response to peripheral GLP-1 administration (Baumgartner *et al.* 2010). Furthermore, either subdiaphragmatic vagotomy (Abbott *et al.* 2005a), or selective vagal deafferentation (Hayes *et al.* 2011, Ruttimann *et al.* 2009), significantly inhibit peripheral GLP-1-induced hypophagia. In humans, the acute inhibitory effect of peripheral GLP-1 on food intake is lost in patients who have had a truncal vagotomy (Plamboeck *et al.* 2013).

However, there is also evidence to support the concept that GLP-1 may have direct CNS effects (Pannacciulli *et al.* 2007, van Dijk & Thiele 1999) as it is able to reach the brainstem via the AP, where GLP-1Rs are also expressed (Kastin *et al.* 2002). A direct central effect of GLP-1 is supported by the observation that only the anorectic effect of intraperitoneal, and not intravenous, GLP-1 requires vagal afferent signalling (Ruttimann *et al.* 2009). In fact, the GLP-1R is widely expressed in the brain (Goke *et al.* 1995), including the brainstem (Larsen *et al.* 1997), and work by Turton *et al.* and Tang-Christensen *et al.* has established central GLP-1 as a physiological regulator of appetite in its own right (Tang-Christensen *et al.* 1996, Turton *et al.* 1996). They demonstrated potent reduction in food intake following central administration of GLP-1, an effect inhibited by Exendin 9-39. Furthermore, knockdown of GLP-1 producing neurons, and chronic administration of Exendin 9-39, are associated with hyperphagia and increased adiposity in rats on a high fat diet (Barrera *et al.* 2011). The effects of ICV administration of GLP-1 likely reflect the action of GLP-1 produced and

released by neurons of the NTS which express the proglucagon gene and display a processing pattern similar to that of enteroendocrine L cells (Holst 2013, Larsen *et al.* 1997, Shimizu *et al.* 1987). However, the relationship between the peripheral and central GLP-1 systems, and their contribution to nutrient-stimulated appetite suppression, are currently unclear.

OXM

OXM is another anorexigenic gut hormone synthesised by enteroendocrine L cells along with GLP-1, and released in proportion to the energy content of ingested food (Le Quiellec *et al.* 1992). As with GLP-1, OXM has also been shown to delay gastric emptying and reduce food intake (Dakin *et al.* 2001, Schjoldager *et al.* 1989). Both central and peripheral administration of OXM potentially reduce food intake and induce weight loss in rats (Dakin *et al.* 2001, Dakin *et al.* 2004, Dakin *et al.* 2002). Similarly in humans, peripheral OXM promotes satiety and results in long-term weight loss (Cohen *et al.* 2003, Wynne *et al.* 2006, Wynne *et al.* 2005). OXM has been demonstrated to bind to the GLP-1R, though with much lower affinity than GLP-1 (Fehmann *et al.* 1994), and the glucagon receptor (Baggio *et al.* 2004). However, there are discrepancies between the pathways mediating the effects of GLP-1 and OXM (Badman & Flier 2005, Parkinson *et al.* 2009) and at present, the precise mechanisms underlying the hypophagic action of OXM remain obscure.

1.2.3.4 Peptide YY (PYY)

PYY is a 36-amino acid peptide that belongs to the PP fold family, along with pancreatic polypeptide (PP) and NPY. All three peptides bind to the Y family of receptors (Y1-Y5), albeit with varying affinities (Blomqvist & Herzog 1997). PYY is an anorexigenic peptide synthesised and secreted from enteroendocrine L cells in response to luminal nutrients (Adrian *et al.* 1985). High concentrations of PYY are found in the terminal ileum, colon and

rectum (Adrian *et al.* 1985). Two forms of PYY exist: PYY₁₋₃₆ and the truncated PYY₃₋₃₆, which is produced from the cleavage of full length PYY by DPP-IV (Eberlein *et al.* 1989, Mentlein *et al.* 1993) and is the major circulating form (Grandt *et al.* 1994).

PYY has several physiological functions, including the delay of gastric emptying, the slowing of gut transit and the induction of satiety. Peripheral administration of PYY₃₋₃₆ at physiological doses significantly reduces food intake in both lean and obese individuals (Batterham *et al.* 2003, Batterham *et al.* 2002, Chelikani *et al.* 2005). Surprisingly, ICV administration of PYY₃₋₃₆ has the opposite effect and increases food intake (Morley *et al.* 1985). This orexigenic effect has been attributed to pharmacological activation of Y1 or Y5 receptors in the PVN (Hagan 2002).

PYY₃₋₃₆ selectively binds to and activates the Y2 receptor (Y2R) (Browning & Travagli 2009, Grandt *et al.* 1992, Keire *et al.* 2000). However, the exact mechanisms underlying the anorectic effects of PYY₃₋₃₆ remain unclear. PYY₃₋₃₆ may reduce appetite by inhibiting the release of orexigenic NPY via autoinhibitory Y2Rs and by disinhibiting POMC neurons in the ARC (Acuna-Goycolea & van den Pol 2005, Batterham *et al.* 2002). Central administration of a Y2R agonist has been demonstrated to suppress food intake in rodents (Leibowitz & Alexander 1991). Furthermore, Y2R-deficient mice are hyperphagic and PYY₃₋₃₆ is unable to reduce food intake in these animals, suggesting that the anorectic effects of PYY are mediated by this receptor (Naveilhan *et al.* 1999).

Peripheral PYY₃₋₃₆ administration increases c-fos expression in the ARC and reduces hypothalamic NPY mRNA expression (Batterham *et al.* 2002). Furthermore, intra-arcuate administration of a specific Y2R antagonist attenuates the anorectic effect of peripherally administered PYY₃₋₃₆ in rats (Abbott *et al.* 2005b). However, peripherally administered PYY₃₋₃₆ is also thought to act via Y2Rs expressed on vagal afferent fibres. In support of this, vagotomy abolishes the reduction in food intake induced by peripheral PYY₃₋₃₆ (Abbott *et al.* 2005a, Koda *et al.* 2005).

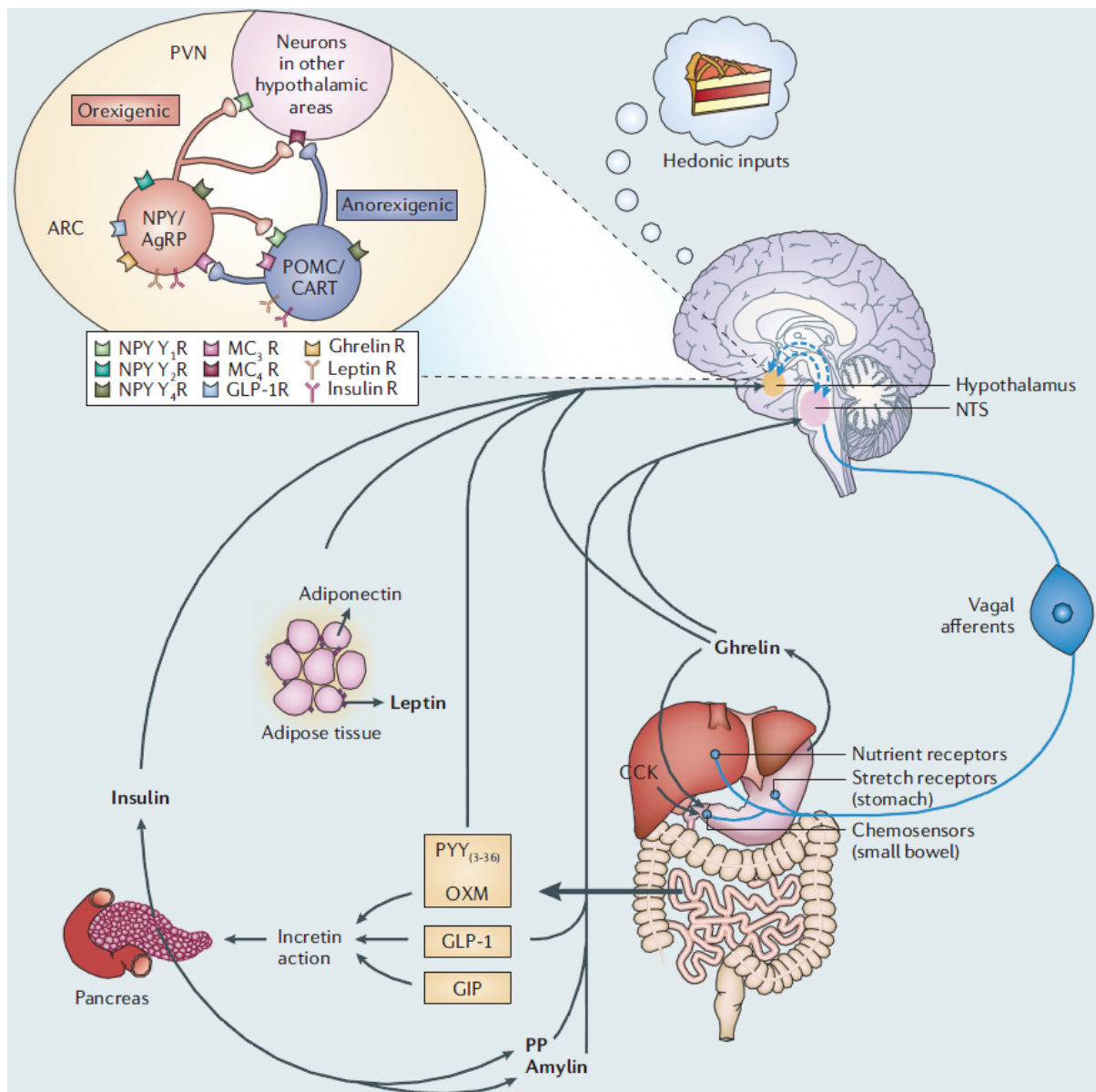


Figure 1.2. The regulation of appetite and energy homeostasis. The brain integrates peripheral and neural signals to regulate energy homeostasis. Peripheral factors indicative of long-term energy status are produced by adipose tissue (leptin, adiponectin) and the pancreas (insulin), whereas the acute hunger signal ghrelin, and satiety signals such as the gut hormones peptide YY₃₋₃₆ (PYY₃₋₃₆), pancreatic polypeptide (PP) and oxyntomodulin (OXM) indicate short term energy status. The incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) improve the response of the endocrine pancreas to absorbed nutrients. Further feedback is provided by nutrient receptors in the upper small intestine, and neural signals indicating distension of the stomach's stretch receptors, which are primarily conveyed by the vagal afferent and sympathetic nerves to the nucleus of the tractus solitarius (NTS) in the brain stem. The arcuate nucleus (ARC) of the hypothalamus integrates these energy homeostatic signals. Two distinct subsets of neurons control food intake in the ARC; the first co-expresses the orexigenic agouti-related peptide (AgRP) and neuropeptide Y (NPY) neurotransmitters, the second co-expresses the anorexigenic cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) peptide neurotransmitters. Both neuronal populations innervate the paraventricular nucleus (PVN) followed by other areas of the brain. CCK, cholecystikinin; MCR, melanocortin receptor; NPY YR, neuropeptide Y receptor Y. Adapted from: (Cooke & Bloom 2006)

1.2.3.5 Current understanding of gut hormone expression in EECs

The enteroendocrine cell population represents just 1% of intestinal epithelial cells and their lack of specific cell-surface markers has made these cells challenging to study. However, our understanding of EECs has evolved significantly in recent years. This has been driven by the engineering of transgenic mouse models possessing fluorescently-tagged gut hormone genes enabling the isolation and characterisation of these previously elusive cell populations (Liou *et al.* 2011, Parker *et al.* 2009, Reimann *et al.* 2008, Sykaras *et al.* 2012, Wang *et al.* 2011a). The adoption of this enabling technology by the field of gastrointestinal endocrinology has already provided invaluable insights.

Recent work revealed extensive overlap in the gut hormone content of small intestinal (SI) L cells and K cells, the EECs which secrete the gut hormone and incretin glucose-dependent insulinotropic polypeptide (GIP) (Baggio & Drucker 2007, Brown *et al.* 1975, Pederson *et al.* 1975). Surprisingly, colonic and upper SI proglucagon-expressing L cells appeared to share less in common, despite being considered the same cell type, than the traditionally distinct cell subtypes of upper SI L cells and K cells (Habib *et al.* 2012). Using intestinal tissue from transgenic mice in which the expression of the yellow fluorescent protein (YFP) Venus is driven by the proglucagon promoter, upper SI L cell populations were demonstrated to express mRNA for gut hormones classically found in different enteroendocrine cell types, including GIP, CCK, secretin and neurotensin (Habib *et al.* 2012). By immunostaining and fluorescence-activated cell sorting (FACS) analysis, the majority of proglucagon-expressing colonic L cells were shown to contain GLP-1 and PYY, as anticipated. In contrast, in the upper SI, most proglucagon-expressing L cells also contained CCK, ~10% were GIP-positive and only ~20% were PYY-positive (Habib *et al.* 2012). A similar pattern of expression was also demonstrated using small intestinal tissue from transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of the CCK promoter (Egerod *et al.* 2012). Further work is required to address the issue of whether SI L cells store and release

these peptides at physiologically meaningful levels. However, these data enable the speculation that upper SI L, K and I cells may in fact comprise a single cell type, with individual cells exhibiting a 'hormonal spectrum', potentially influenced by their location along the gastrointestinal tract and exposure to different luminal factors.

1.2.4 Beyond the gut brain-axis: non-homeostatic influences on food intake

The homeostatic mechanisms controlling food intake evolved during frequent periods of food scarcity and as a result, are highly efficient at defending the lower limits of body weight and adiposity. However, as previously alluded to, we currently live in an 'obesogenic' environment, saturated with highly-palatable and energy-dense foods (Berthoud 2012). The ability to override anorectic homeostatic signals and 'overconsume' calorie-dense palatable foods, demonstrates that food consumption goes beyond meeting metabolic demand and points towards the presence of a non-homeostatic component. This is referred to as 'hedonic' food intake and is driven by the pleasure-reward system (Berthoud 2011, Petersen *et al.* 2013). Therefore, the current consensus is that eating behaviour is co-ordinated by a combination of both homeostatic and non-homeostatic inputs. The crosstalk between homeostatic feedback and hedonic circuits provides a mechanism by which highly palatable food may overwhelm energy regulation but also opens up the possibility of strengthening satiety signals to assist with resisting the urge to overindulge (Petersen *et al.* 2013).

1.3 Non-Digestible Carbohydrates (NDCs) and Obesity

1.3.1 NDC nomenclature and classification

Traditionally, carbohydrate classification and terminology have been based on chemical divisions. In 1998, a joint expert consultation by the Food and Agriculture Organisation of the United Nations (FAO) and the WHO on carbohydrates in human nutrition proposed the use of the terms “sugars, oligosaccharides and polysaccharides” with appropriate subgroups to include all dietary carbohydrates (Clausen & Mortensen 1995) (Table 1.2).

The terms dietary fibre, non-digestible carbohydrate (NDC) and fermentable carbohydrate, all allude to carbohydrates which escape digestion in the mammalian small intestine and reach the caecum (Cummings 1997). These include members of the oligosaccharide and polysaccharide groups, which contain glycosidic linkages that cannot be hydrolysed by amylases (Table 1.2). Examples include the β 1-4 linkages in cellulose and the α -galactosidic linkages of raffinose (Flint *et al.* 2012a). In addition to the nature of the glycosidic bond, the rate and extent of carbohydrate digestion are also determined by the structure of the starch granule, the amylose:amylopectin ratio, the degree of gelatinisation and the integrity of the plant cell wall (Chambers *et al.* 2011, Flint *et al.* 2012a). All of these factors will ultimately affect the susceptibility of the carbohydrate to enzymatic digestion.

Table 1.2 **Classification of dietary carbohydrates**

Group	DP (1)	Sub-group		Digestion in the small intestine
Sugars	1	Monosaccharides	Glucose, fructose, galactose, sorbitol, mannitol	Usually well absorbed except sugar alcohols
	2	Disaccharides	Sucrose, lactose, maltose	Usually well absorbed except lactose
Oligosaccharides	3–9	α -glucans	Mostly starch hydrolysis products	Well digested
		Non α -glucans (NDO)	Fructo-oligosaccharides, galacto-oligosaccharides, raffinose, stachyose, polydextrose (partly)	Probably all reach the caecum
Polysaccharides	≥ 10	Starch (α -glucans)	Amylose, amylopectin	Some forms of resistant starches reach the caecum
		Non-starch polysaccharides (non α -glucans)	Cell wall Cellulose Hemicellulose Pectins, etc.	All reach the caecum
			Storage Guar Inulin	All reach the caecum
			Gums, exudates, mucilages Ispaghula Sterculia Karaya	All reach the caecum

(1) DP, degree of polymerisation

Adapted from: (Cummings 1997)

1.3.2 The beneficial effects of NDCs on metabolic health

Epidemiological evidence suggests that there is an inverse correlation between consumption of NDCs and weight gain (Liu *et al.* 2003, Ludwig *et al.* 1999), and with fat mass (Kromhout *et al.* 2001, Nelson & Tucker 1996, Tucker & Thomas 2009). This is supported by experimental studies in both animals and humans, which demonstrate that increasing the consumption of NDCs can reduce appetite, body weight (Cani *et al.* 2004a, Cani *et al.* 2006, Cani *et al.* 2005, Perrigue *et al.* 2009, Rigaud *et al.* 1990, Rytting *et al.* 1989) and adiposity (Keenan *et al.* 2006, Pawlak *et al.* 2004, So *et al.* 2007). There is also evidence to suggest that a high intake of NDCs may improve insulin sensitivity independently of effects on body weight and adiposity (Robertson *et al.* 2005, Robertson *et al.* 2003).

As reviewed by Howarth *et al.*, the majority of NDC intervention studies indicate that an increase in NDC intake is associated with enhanced post-meal satiety and a decrease in subsequent hunger (Howarth *et al.* 2001). However, while evidence from NDC supplementation studies in animals is largely consistent, not all NDC intervention studies in humans have demonstrated that NDC consumption increases perceived satiety or weight loss (Howarth *et al.* 2003). This observation may be accounted for by the use of insufficient NDC levels in human studies compared to animal studies (Chambers *et al.* 2011, Parnell & Reimer 2009).

NDCs have been proposed to reduce caloric intake by a number of different mechanisms. These include the displacement of energy-dense foods from the diet, interference with the absorption of macronutrients from the gut lumen, an altered luminal environment, and increased gastric distension (Heaton 1973). However, the hypophagic effects of NDCs are also thought to be mediated by the release of anorexigenic gut hormones (Sleeth *et al.* 2010). NDC supplementation has been associated with increased circulating PYY and GLP-1 levels (Cani *et al.* 2006, Cani *et al.* 2009, Delzenne *et al.* 2005, Keenan *et al.* 2006, Zhou

et al. 2008) as well as with enhanced PYY and proglucagon intestinal gene expression (Zhou *et al.* 2006, Zhou *et al.* 2008).

The molecular mechanisms underlying the beneficial metabolic effects of NDCs are poorly understood. However, these effects may be attributable, at least in part, to the bacterial fermentation of NDCs in the colon yielding short chain fatty acids.

1.4 Short Chain Fatty Acids (SCFAs)

1.4.1 Sources and production

The mammalian gastrointestinal tract is host to over one hundred trillion (10^{14}) bacteria and approximately 1,000 bacterial species have been identified to date (Turnbaugh *et al.* 2007). The majority of the gut microbiota (>90%) belong to the Bacteroidetes (gram-negative) and Firmicutes (gram-positive) phyla. The species and quantities of gut bacteria vary along the gastrointestinal tract (Fig. 1.3). By far the largest and most diverse population of gut bacteria is found in the large intestine. Despite a considerable degree of inter-individual variation in gut microbiota, humans share an underlying common 'core microbiome' (Arumugam *et al.* 2011, Qin *et al.* 2010, Turnbaugh & Gordon 2009).

Short chain fatty acids (SCFAs), along with gases (H_2 , CO_2 , CH_4), are the major end-products of the colonic fermentation of NDCs by gut microbiota. In this context, fermentation refers to the anaerobic, energy-yielding process by which organic compounds are broken down into simpler compounds. The majority of this fermentation occurs in the proximal large intestine, mainly due to substrate availability (Macfarlane & Macfarlane 2003). The straight-chain SCFAs acetate (C2), propionate (C3) and butyrate (4) account for over 85% of total SCFAs produced (Fig. 1.4). Concentrations of SCFAs in the lumen are in the range of 70-130mmol/L (Cummings *et al.* 1987, Mortensen & Clausen 1996) and occur in the approximate molar ratio of 60% acetate, 25% propionate and 15% butyrate (McNeil *et al.* 1978). However, the production of SCFAs is subject to great inter-individual variation, predominantly dependent on the type of fermentable NDC (chemical composition, physical form, quantity), the profile of the host's microbiota and gut transit time (Macfarlane & Macfarlane 2003). In addition, there are numerous other host-related factors affecting bacterial fermentation and SCFA production at any given time, including luminal contents, mucus production, stress, disease and antibiotics (Cho *et al.* 2012, Macfarlane & Macfarlane 2003).

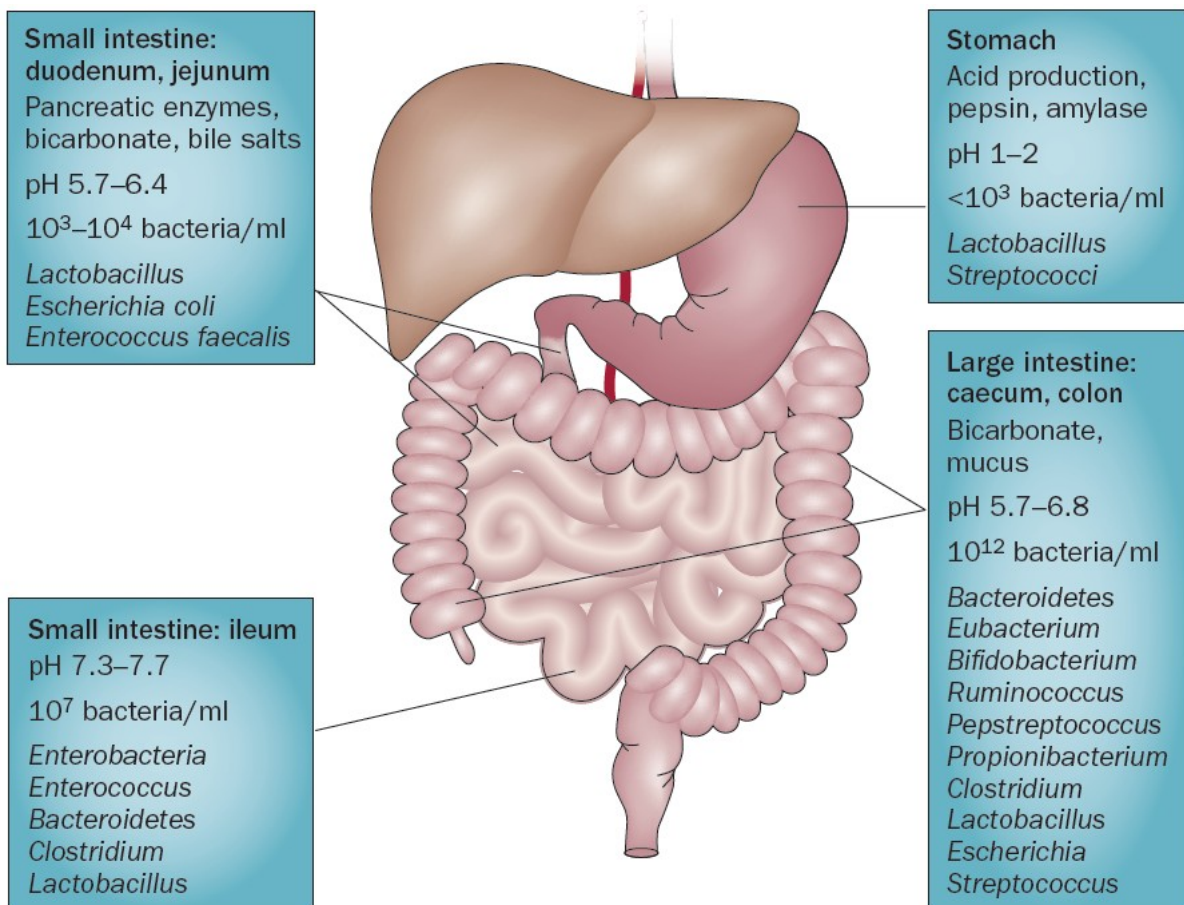


Figure 1.3. **Characteristics of the normal gastrointestinal tract.** The various organs of the gastrointestinal tract differ according to digestive secretions and pH. Different species and quantities of bacteria are found at different points along the digestive tract according to these major variations in the environmental niche. Adapted from: (Aron-Wisniewsky *et al.* 2012)

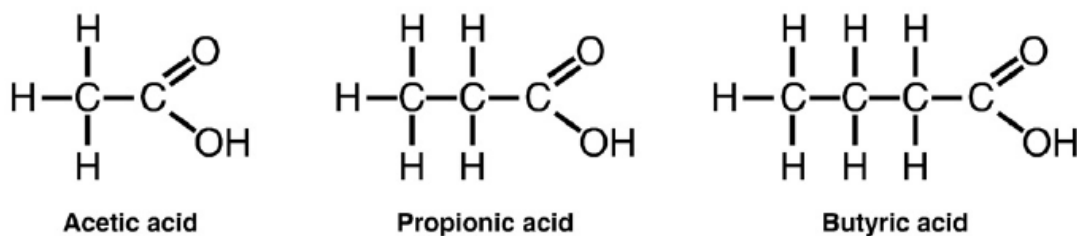


Figure 1.4. **The chemical structure of the three major short chain fatty acids.**

Source: (Al-Lahham *et al.* 2010)

Gut bacteria possess a wide range of hydrolytic enzymes which are able to depolymerise the large macromolecular structures of NDCs, allowing them to ferment their component sugars (Flint *et al.* 2012a). Despite the diversity of carbon sources used as fermentation substrates by bacteria in the large intestine, they are catabolised in a relatively small number of biochemical pathways, as summarised in the simplified diagram shown in Figure 1.5 (Macfarlane & Macfarlane 2003).

There are two major bacterial metabolic routes by which monosaccharides are converted into phosphoenolpyruvate (PEP): the Embden-Meyerhof-Parnas pathway (glycolysis, hexose sugars) and the pentose-phosphate pathway (pentose sugars) (Cummings 1981, den Besten *et al.* 2013, Miller & Wolin 1996). PEP is converted into pyruvate and acetyl-CoA, key metabolites which are subsequently converted into a wide range of end-products, including SCFAs (Flint *et al.* 2012b, Macfarlane & Macfarlane 2003).

Acetate is typically formed by the oxidative decarboxylation of pyruvate and hydrolysis of acetyl-CoA, or via the 'Wood-Ljungdahl pathway' using formate, whereas butyrate formation involves the condensation of two molecules of acetyl-CoA (Cummings 1981, Jorgensen *et al.* 1997). Propionate is produced by two main routes; through fixation of carbon dioxide to form succinate, which is subsequently decarboxylated (the 'succinate decarboxylation pathway') (Blackburn & Hungate 1963, Paynter & Elsdon 1970), or via the reduction of lactate and acrylate (the 'acrylate pathway')(Ladd & Walker 1965, Wallnofer & Baldwin 1967).

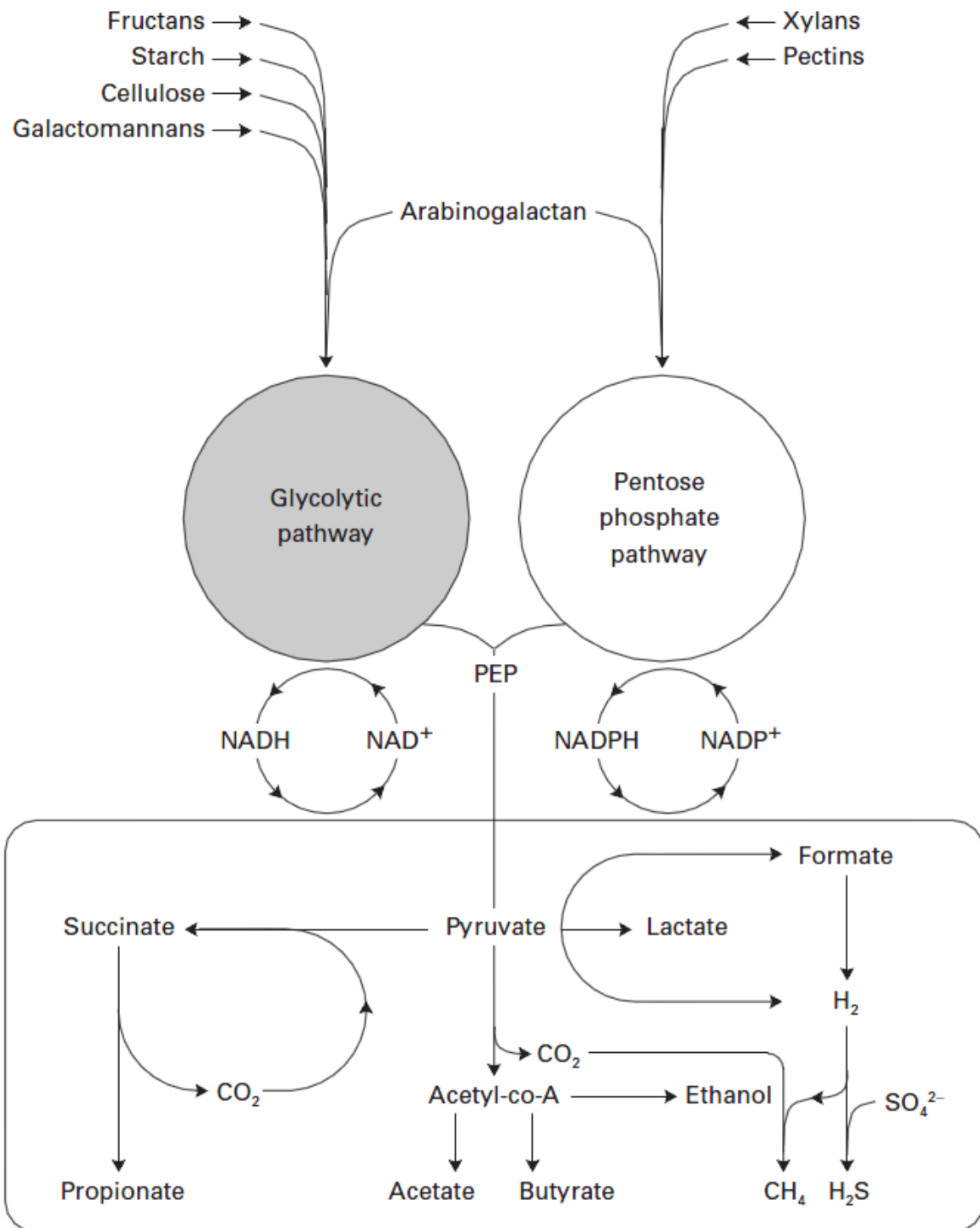


Figure 1.5. **Simplified diagram of non-digestible carbohydrate breakdown and the main routes of its fermentation in the large intestine.** CH₄, methane; CO₂, carbon dioxide; H₂, hydrogen; H₂S, hydrogen sulfide; NAD⁺, nicotinamide adenine dinucleotide (NADH, reduced form of NAD⁺); PEP, phosphoenolpyruvate; SO₄, sulfate (Macfarlane & Macfarlane 2003)

1.4.2 Absorption and transport

The absorption of all three major SCFAs (C2-C4) in the colon is highly efficient (>95%) (McNeil *et al.* 1978) and occurs by both passive diffusion and via specific transporters (Miyachi *et al.* 2004, Rajendran & Binder 2000, Ritzhaupt *et al.* 1998, Sellin 1999). There are three known major forms of SCFA transport across the mammalian large intestine: bicarbonate (HCO_3^-)-coupled, proton-coupled and sodium-coupled transport (Fig. 1.6). The identity of the HCO_3^- exchanger is currently unknown (Harig *et al.* 1996, Harig *et al.* 1991, Mascolo *et al.* 1991, Vidyasagar *et al.* 2005). At present, the contribution of these pathways to total SCFA absorption by the distal gut is poorly understood.

H⁺-coupled transport

To date, four proton-coupled SCFA transporters have been identified, monocarboxylic acid transporters 1-4 (MCT1-4), which belong to the solute carrier family 16 (SLC16) (Kekuda *et al.* 2013). Their expression has been demonstrated in the rodent and human intestinal epithelium (Dimmer *et al.* 2000, Gill *et al.* 2005, Ritzhaupt *et al.* 1998, Tamai *et al.* 1999). However, the identity of the major apical H⁺-dependent SCFA transporter is unclear. MCT1 and MCT4 are the prime candidates but their cellular location in colonic cells (basolateral vs. apical) remains controversial (Gill *et al.* 2005, Iwanaga *et al.* 2006, Kekuda *et al.* 2013). As MCT function is coupled to a transmembrane proton gradient, these transporters are not very active since the magnitude of the gradient across the colonocyte apical membrane is negligible. Therefore, the driving force for the uphill entry of SCFAs via MCTs from the gut lumen is minimal. Moreover, the H⁺/substrate stoichiometry for MCTs is 1:1, rendering the transport process electroneutral (Kekuda *et al.* 2013). Interestingly, it has recently been proposed that transporter function in the large intestine may be modulated by luminal SCFA levels (Borthakur *et al.* 2012), a mechanism best described for the intestinal sugar

transporter GLUT2 which is recruited to the apical surface in response to high concentrations of sugars (Kellett *et al.* 2008).

Na⁺-coupled transport

An important route for SCFA transport from the gut lumen into colonocytes is via sodium-coupled monocarboxylate transporter 1 (SMCT1, Slc5a8). SMCT1 transporters are selectively located on the luminal membrane of enterocytes in the terminal ileum and large intestine (Takebe *et al.* 2005). Proposed affinities for its primary substrates are in the order: butyrate>propionate>lactate>>acetate. The absorption of SCFAs is electrogenic and obligatorily coupled to co-transport of sodium ions with a Na⁺/substrate stoichiometry of 2:1 (Coady *et al.* 2004).

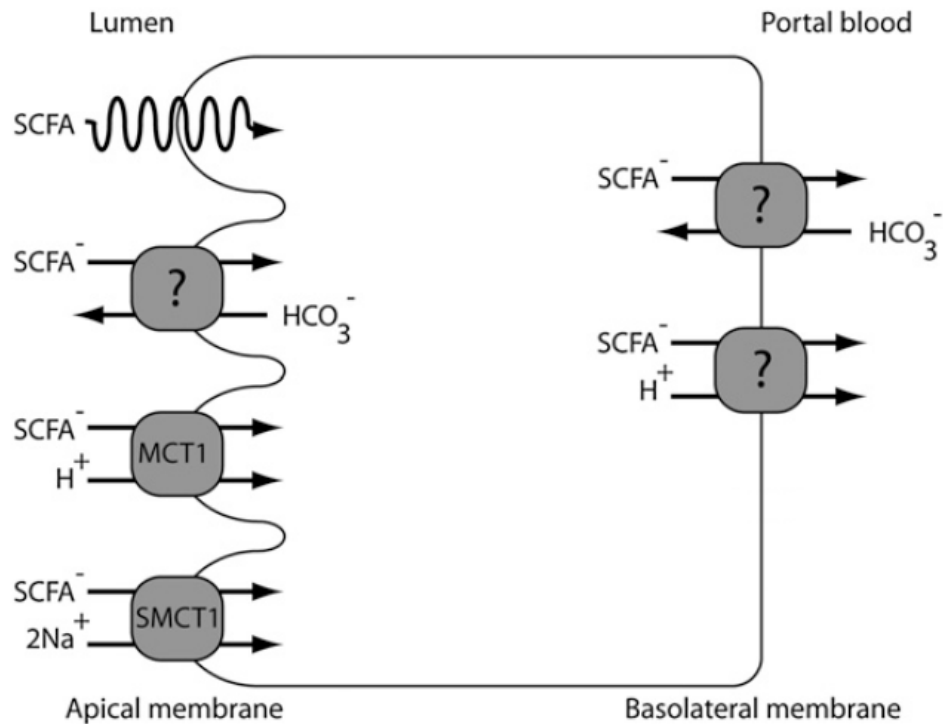


Figure 1.6. **Short chain fatty acid transport in colonocytes.** Short chain fatty acids (SCFAs) are generated from dietary fibre by bacterial fermentation in the colonic lumen. SCFAs can be transported into colonocytes by passive diffusion or via one of three transport mechanisms: (1) a bicarbonate (HCO₃⁻) exchanger of unknown identity, (2) monocarboxylate transporters (MCT) or (3) sodium-coupled monocarboxylate transporter 1 (SMCT1). The identity of the transporters involved in SCFA transport across the basolateral surface remains unclear.

Adapted from: (den Besten *et al.* 2013)

1.4.3 SCFA metabolism

Once inside the cell, butyrate is almost exclusively utilised by colonocytes as their preferred energy source (Roediger 1980). Using isolated human colonic epithelial cells, Roediger demonstrated that butyrate is the preferred energy source for the colonic mucosa, even in the presence of other SCFAs or glucose (Roediger 1980). However, subsequent work using isolated human and rat colonocytes demonstrated that under physiological conditions, with a higher relative colonic concentration of acetate compared with butyrate, acetate is at least as important as butyrate for the energy supply of colonocytes (Clausen & Mortensen 1995, Jorgensen *et al.* 1997). In contrast, acetate and propionate drain into the portal vein and are largely taken up by the liver (Bloemen *et al.* 2009, Cummings *et al.* 1987, Dankert *et al.* 1981). Acetate is utilised as an energy source or as a substrate for the synthesis of cholesterol, long-chain fatty acids, glutamine and glutamate (Desmoulin *et al.* 1985, Reilly & Rombeau 1993). Propionate is converted into propionyl-CoA followed by succinyl-Co, which enters the citric acid cycle and is converted into oxaloacetate, the precursor of gluconeogenesis (Bloemen *et al.* 2010, McNeil *et al.* 1978, Reilly & Rombeau 1993, Wolever *et al.* 1989) (Fig. 1.7). Circulating SCFAs can then also be metabolised by other tissues including adipose tissue and muscle (Knowles *et al.* 1974, Robertson *et al.* 2005). Consequently, peripheral plasma levels are relatively low under normal physiological conditions: 100-200 μ mol/L for acetate, 4-5 μ mol/L for propionate and 1-3 μ mol/L for butyrate (Pouteau *et al.* 2001, Wolever *et al.* 1997). However, four weeks of NDC supplementation (30g/d) has been shown to almost double fasting plasma propionate concentrations in humans (Robertson *et al.* 2005).

1.4.4 The role of SCFAs in colonic homeostasis

SCFAs are the major anions present in the distal gut lumen and play a fundamental role in maintaining 'normal' colonic function (Cummings 1997, Topping & Clifton 2001). This is likely to be achieved through a host of concerted actions within the colonic milieu and may be linked to events such as changes in luminal pH, GPCR activation, carrier-mediated transport and intracellular metabolism.

SCFAs stimulate fluid and electrolyte uptake by the colonic mucosa (Ruppin *et al.* 1980, Topping & Clifton 2001), as well as colonic blood flow in isolated human colonic segments *in vitro* and in surgical patients (Mortensen *et al.* 1991, Mortensen *et al.* 1990). The enhanced blood flow is predicted to improve tissue oxygenation and facilitate the transport of absorbed nutrients from the gut. Furthermore, SCFAs modify colonic motility, though whether their effects are stimulatory or inhibitory is unclear as they vary under different experimental conditions (Cherbut *et al.* 1998, Yajima 1985). Both effects would serve important functions; slowing the rate of food passage in the proximal intestine would maximise nutrient absorption, whereas increasing gut transit would improve laxation and reduce exposure to intraluminal carcinogens (Fung *et al.* 2013). The mechanisms underlying the effects of SCFAs on intestinal motility are poorly understood. There is evidence to suggest that SCFAs directly influence neurons of the enteric nervous system (Bertrand *et al.* 1997, Neunlist *et al.* 1999, Soret *et al.* 2010). Alternatively, it is possible that SCFAs modify intestinal motility indirectly, via the stimulation of PYY (Cherbut *et al.* 1998) or 5-hydroxytryptamine (5-HT) release (Fukumoto *et al.* 2003). SCFAs are also thought to have beneficial roles in the regulation of intestinal inflammation and cancer cell proliferation (Donohoe *et al.* 2012, Fung *et al.* 2013, Hinnebusch *et al.* 2002, Maslowski *et al.* 2009, Smith *et al.* 2013, Vinolo *et al.* 2011). Therefore, through multiple actions, SCFAs form an integral component of large intestinal physiology.

1.4.5 SCFA receptors: GPR41 (FFAR3) and GPR43 (FFAR2)

SCFAs have been identified as ligands for two G-protein coupled receptors (GPCRs), GPR41 and GPR43, originally cloned in 1997 (Sawzdargo *et al.* 1997). These related receptors (52% similarity, 43% identity) were de-orphanised in 2003 by three independent groups (Brown *et al.* 2003, Le Poul *et al.* 2003, Nilsson *et al.* 2003). The activation and signalling of the SCFA receptors are discussed in Chapter IV, Section 4.1.1.

1.4.5.1 Tissue distribution and physiological role

GPR41 and GPR43 expression has been demonstrated in a variety of tissues and cell types including immune cells, adipose tissue, pancreatic islets and the gastrointestinal tract (Brown *et al.* 2003, Covington *et al.* 2006, Ge *et al.* 2008, Hong *et al.* 2005, Kimura *et al.* 2013). The first physiological role attributed to SCFA receptors concerned innate immunity and neutrophil chemotaxis (Le Poul *et al.* 2003, Nilsson *et al.* 2003). However, multiple functions for GPR43 and GPR41 have since emerged. The putative physiological roles of SCFA receptors in tissues implicated in energy homeostasis are discussed below.

Adipose tissue

Hong *et al.* were the first to investigate the role of GPR43 in adipose tissue. Their experiments using 3T3-L1 cells suggested that SCFAs stimulate expression of GPR43 and of peroxisome proliferator-activated receptor gamma (PPAR γ), a regulator of adipocyte differentiation (Hong *et al.* 2005). This effect was reduced by GPR43 siRNA transfection. However, the physiological relevance of these results has been brought into question by a recent paper that was unable to replicate these findings using human primary adipocyte cultures (Dewulf *et al.* 2013). Subsequently, SCFAs were demonstrated to suppress isoproterenol-induced lipolysis in wild type but not Gpr43-deficient adipocytes (Ge *et al.*

2008). In addition, *in vivo* activation of the Gpr43 receptor by acetate in mice resulted in a reduction in plasma free fatty acid levels, an effect associated with improved insulin sensitivity (Boden 2011, Ge *et al.* 2008). Interestingly, a recent study demonstrated that overexpression of Gpr43 in adipose tissue protected mice from HFD-induced obesity (Kimura *et al.* 2013). These findings support a role for GPR43 in adipose tissue physiology and in the regulation of energy homeostasis.

The putative physiological role for GPR41 in adipose tissue is controversial. Xiong *et al.* reported that activation of GPR41 in adipocytes stimulated secretion of the adipokine leptin from adipocytes *in vitro* (Xiong *et al.* 2004). Leptin plays an important role in energy homeostasis, as exemplified by the phenotype of mice lacking the *ob* gene (coding for leptin) which are severely hyperphagic and obese (Zhang *et al.* 1994). Subsequent experiments indicated that leptin secretion from murine primary adipose tissue cultures increased following treatment with SCFAs, and that oral administration of propionate in mice was associated with increased circulating leptin levels eight hours post administration (Xiong *et al.* 2004). However, several groups have since been unable to detect the presence of GPR41 in adipose tissue (Hong *et al.* 2005, Zaibi *et al.* 2010).

Gastrointestinal tract

GPR43 and GPR41 mRNA expression has recently been detected in the rat (Karaki *et al.* 2006) and human colonic mucosa (Karaki *et al.* 2008) and specifically within enteroendocrine L cells (Karaki *et al.* 2006, Tazoe *et al.* 2009, Tolhurst *et al.* 2012a). The general consensus was that GPR43 is widely expressed in L cells, whereas GPR41 is only expressed in a small percentage (4%) of these cells (Tazoe *et al.* 2009, Tolhurst *et al.* 2012a). However, this concept was recently challenged by Nohr *et al.* who showed evidence to suggest that GPR41 expression in enteroendocrine cells is more extensive (Nohr *et al.* 2013). L cells are distributed throughout the gastrointestinal tract, with high concentrations

found in the colon (Eissele *et al.* 1992), where SCFAs are most abundant. Consequently, they are ideally located to sense changes in the luminal concentrations of nutrients, such as SCFAs, in the distal intestine. Indeed, several studies demonstrate that SCFAs can trigger the release of PYY and GLP-1, suggesting a potential role in appetite regulation and the potentiation of insulin secretion (Anini *et al.* 1999, Cherbut *et al.* 1998, Tolhurst *et al.* 2012a). In practice however, the exact localisation of SCFA receptors within L cells (apical vs. basolateral) is unknown and complicates speculation regarding their physiological function.

Interestingly, Sykaras *et al.* recently reported that duodenal enteroendocrine I cells, traditionally associated with CCK release, are also highly enriched in mRNA encoding both GPR41 and GPR43 (Sykaras *et al.* 2012). However, once again, the localisation of the receptors is uncertain. Furthermore, the levels of SCFAs in the duodenal lumen are low, as fermentation is thought to occur predominantly in the large intestine. In light of this, it is possible that GPR41 and GPR43 in duodenal I cells may sense circulating plasma SCFAs to modulate I cell function, rather than SCFAs in the duodenal lumen.

Recent data suggest that GPR41 is also expressed in enteric neurons, in both submucosal and myenteric ganglia (Nohr *et al.* 2013). Counter staining with antibodies against the neuropeptide vasoactive intestinal peptide (VIP) revealed co-localisation of VIP and GPR41, indicating that SCFAs may be sensed on secretomotor neurons involved in the regulation of water and electrolyte secretion from enterocytes (Nohr *et al.* 2013).

Sympathetic nerve ganglia

GPR41 is abundantly expressed in sympathetic nerve ganglia, in mice and humans, and propionate in particular is able to directly activate the sympathetic nervous system to enhance noradrenaline release, suggesting a potential role for SCFAs in energy expenditure (Kimura *et al.* 2011). However, these findings have yet to be confirmed by independent groups.

Energy homeostasis in global GPR43 knockout mice

The wider role of GPR43 in energy homeostasis is poorly understood. While there is a general consensus that *Gpr43*^{-/-} mice on a normal chow diet are healthy and do not display an altered energy homeostasis phenotype (Bjursell *et al.* 2011, Kimura *et al.* 2013), there are conflicting reports from two studies regarding how *Gpr43*^{-/-} mice fare on a high fat diet (HFD) background compared to their wild type (WT) littermates (Bjursell *et al.* 2011, Kimura *et al.* 2013) (Table 1.3). In the first study, HFD-fed *Gpr43*^{-/-} mice displayed no difference in phenotype compared to HFD-fed WT mice in the first 15-20 weeks. From then onwards, the *Gpr43*^{-/-} mice were hyperphagic but exhibited reduced body fat mass and improved glucose tolerance (Bjursell *et al.* 2011), as the increase in food intake was counteracted by an increase in energy expenditure. The second study also reported hyperphagia in the *GPR43*^{-/-} mice. However in this study, the *Gpr43*^{-/-} mice exhibited significantly increased adiposity and reduced energy expenditure compared to their HFD-fed WT littermates (Kimura *et al.* 2013). These contradictory findings may reflect the different housing environments and genetic backgrounds of the knockout mice (Kimura *et al.* 2013). Further work is therefore required to clarify the role of GPR43 in energy homeostasis in an obesogenic context.

Table 1.3. **Energy homeostasis in Gpr43 receptor knockout mice on a high fat diet (HFD) background.**

A. Study Design and Characteristics

Reference	Generation of KO (targeting vector)	Genetic background	Diet (% energy)	Duration of diet	Age at start
Bjursell <i>et al.</i> 2011	Homologous recombination (LacZ-PGK-neo)	C57BL/6J0laHsd	HFD: R638 39.9% fat	35 weeks	4 weeks
Kimura <i>et al.</i> 2013	Homologous recombination (LacZ-PGK-neo)	129/Sv	HFD: 58Y1 61.6% fat	12 weeks	4 weeks

B. Phenotypes and Study Results

Reference	Food intake	Body weight/ adiposity	Glucose Tolerance	Energy Expenditure	SCFAs
Bjursell <i>et al.</i> 2011	No difference at 15 wk of age Increased in Gpr43 ^{-/-} (at 38 wk of age)	Reduced in Gpr43 ^{-/-} beyond 25 wk of age	Improved in Gpr43 ^{-/-}	Increased in Gpr43 ^{-/-} (at 25 wk of age)	Not measured
Kimura <i>et al.</i> 2013	Increased in Gpr43 ^{-/-}	Increased in Gpr43 ^{-/-}	Impaired in Gpr43 ^{-/-}	Reduced in dark phase in Gpr43 ^{-/-}	Increased faecal propionate in Gpr43 ^{-/-}

1.5 Summary

There are fundamental gaps in our current understanding of the physiological roles of SCFAs and their receptors in energy homeostasis, and of their specific effects in different endocrine tissues. This thesis determines the effect of colonic SCFAs on PYY and GLP-1 release, and investigates the underlying mechanisms, focusing on the role of the GPR43 receptor using Gpr43 knockout mice. This field offers the exciting possibility of identifying a more physiological method of treating obesity by stimulating the endogenous secretion of anorexigenic gut hormones.

1.6 Hypotheses

I hypothesised that:

- SCFAs stimulate the release of the anorectic gut hormones PYY and GLP-1 from enteroendocrine L cells.
- SCFA/propionate-induced gut hormone release is mediated via a GPR43-dependent mechanism.

1.7 Aims

The aims of this research project were:

1. To develop and validate murine and human primary L cell models.
2. To establish the effect of colonic SCFAs, and in particular propionate, on PYY and GLP-1 release
 - a. *In vitro*, from human and murine primary colonic L cells and
 - b. *In vivo*, in mice and rats.
3. To determine the signalling mechanisms underlying propionate-mediated gut hormone release
 - a. *In vitro*, from murine primary colonic L cells and
 - b. *In vivo*, in wild type and Gpr43 knockout mice.

Chapter II:

Development of a primary L cell model

2.1 Introduction

2.1.1 L cell distribution and morphology

Enteroendocrine cells (EECs) are scattered throughout the intestinal mucosa and comprise as little as 1% of cells lining the gut epithelium (Cheng & Leblond 1974). Therefore, EECs differentiate in tissues where the overwhelming majority of surrounding cells are non-endocrine, including absorptive enterocytes, goblet cells and Paneth cells (Schonhoff *et al.* 2004). EECs differentiate from common pluripotent stem cells within intestinal crypts (Cheng & Leblond 1974) (Fig. 2.1 and 2.2). Tritiated thymidine (^3H -thymidine) labelling after continuous infusion of the isotope, revealed that cells deep within the crypt were the first to label, followed by an increased number of differentiated cells moving out of the crypt. These data suggest that enterocytes, goblet cells and EECs differentiate as they migrate up the crypt-villus axis with a turnover of 3-4 days (Cheng & Leblond 1974). As mature EECs migrate to the tips of the villi, they are thought to undergo apoptosis before they are shed into the gut lumen (Simon & Gordon 1995).

PYY- and GLP-1-secreting L cells increase in number distally along the intestinal axis, with the highest density found in the colon and rectum (Eissele *et al.* 1992, Larsson *et al.* 1975). However, relatively little is known about how the different EEC lineages segregate as they differentiate. Whereas L cells are found in higher numbers in the distal intestine, some EEC types are primarily found in the stomach and proximal intestine (gastrin, ghrelin, GIP, secretin, CCK), and others are located throughout the gastrointestinal tract (somatostatin, serotonin, substance P). The cues that influence the rostro-caudal distribution of each EEC type are poorly understood (Schonhoff *et al.* 2004). Microarray-determined expression of transcription factors in different enteroendocrine cell types and different regional populations may provide novel candidate genes for future investigation (Habib *et al.* 2012).

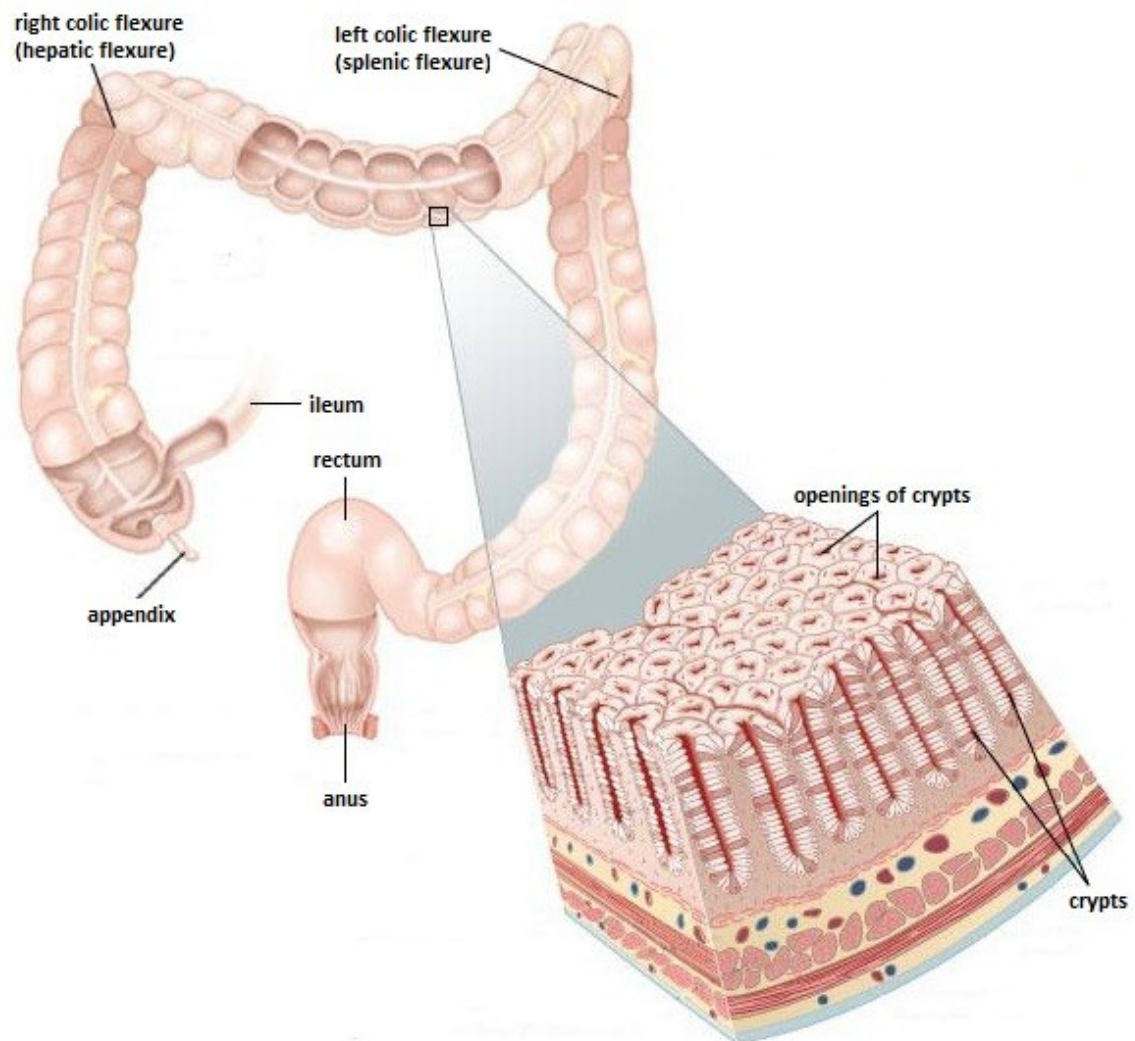


Figure 2.1 **Schematic representation of the large intestine showing the structure and location of crypts in the colonic mucosa.** Adapted from: (Encyclopaedia-Britannica 2003)

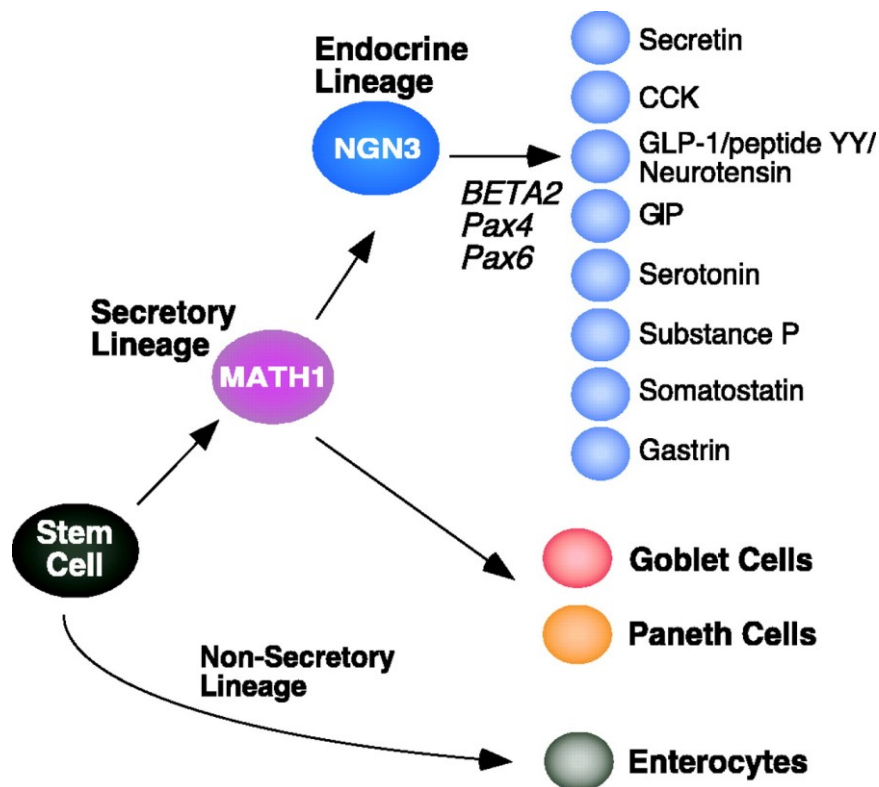


Figure 2.2 **Schematic overview of enteroendocrine cell differentiation in the intestinal tract.** Stem cells located in the crypts differentiate into all four cell types present in the intestinal epithelium. Math1 (also known as Atoh1) expression restricts cells to the secretory lineage and NGN3 restricts cells to the endocrine lineage, whereas the transcription of specific hormones is regulated by several late acting transcription factors such as Pax4, Pax6, and BETA2 (also known as NeuroD1). Adapted from: (Schonhoff *et al.* 2004)

Atoh1, atonal homolog 1; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1, NeuroD1, neuronal differentiation 1; NGN3, neurogenin 3; Pax4/6, paired box gene 4/6

EECs are typically thought of as cone-shaped. In the case of ‘open type’ EECs such as L cells, a narrow apical surface faces the intestinal lumen and gut hormones are exocytosed from dense-core secretory granules at the broader basolateral base (Eissele *et al.* 1992). In addition, EECs often possess basal, ‘dendrite-like’ cytoplasmic processes which serve paracrine and sensory functions (Larsson *et al.* 1975). L cells are therefore ideally situated to function as intestinal nutrient sensors tailoring the secretion of gut hormones to the nutritional composition of the intestinal lumen.

2.1.2 Neural and hormonal stimulation

The activity of L cells is influenced by numerous signals; derived from the gut lumen, from other EECs, as well as from the enteric nervous system (Reimann *et al.* 2012). The enteric nervous system is a network of nerve fibres and ganglia innervating the intestine which interacts with branches of both the parasympathetic and sympathetic nervous systems (Furness 1996, 2000). Signalling within the enteric nervous system relies predominantly on GPCR activation and employs the use of acetylcholine and noradrenaline, as well as peptide neurotransmitters including calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating protein (PACAP), gastrin-releasing peptide (GRP) and galanin (Furness 1996, 2000, Reimann *et al.* 2012) (Fig. 2.3). Autonomic modulation of gut hormone release, via muscarinic M1 receptors, has been demonstrated to influence the secretion of GLP-1 from rats *in vivo* (Anini *et al.* 2002).

Enteric neuro-hormonal circuits are thought to play a role in relaying information between different regions of the gut. For example, proximal-distal crosstalk has been proposed to underlie the rapid increase in plasma GLP-1 observed following nutrient delivery to the proximal small intestine (Rocca & Brubaker 1999). Proximal signals could be conveyed distally via the peptide neurotransmitter PACAP, which is localised throughout the stomach and intestine (Vaudry *et al.* 2000), and has been reported to enhance the secretion of a number of gut hormones including GLP-1 and PYY (Chang *et al.* 1996, Herrmann-Rinke *et al.* 1995, Simpson *et al.* 2007). However, L cells have since also been identified in the small intestine, albeit at lower levels than the distal intestine (Habib *et al.* 2012).

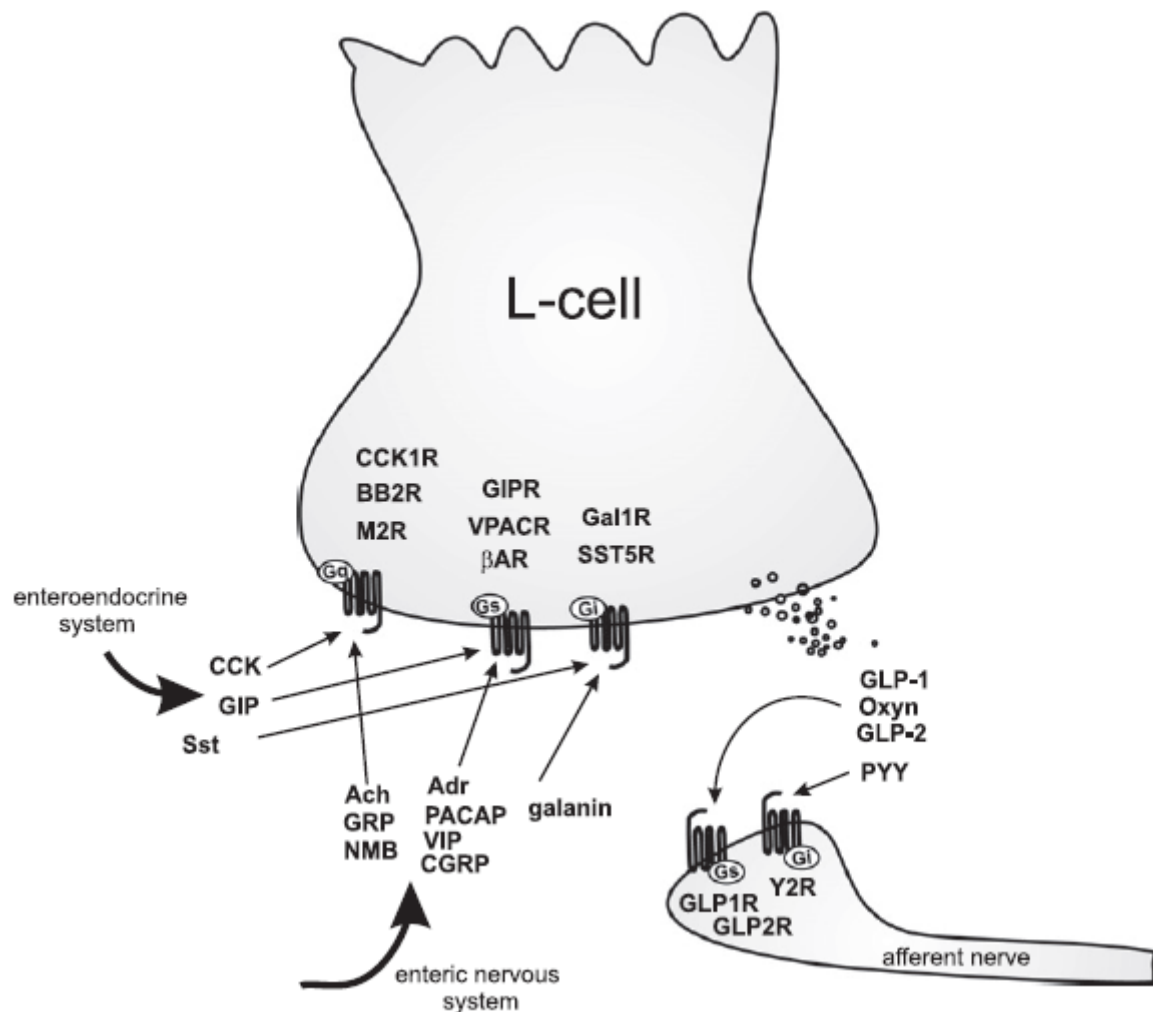


Figure 2.3. L cell stimulation by the enteroendocrine and enteric nervous systems. A number of G-protein coupled receptors (GPCRs) have been identified on enteroendocrine L cells. They are activated by hormones and neurotransmitters released from other enteroendocrine cells and the enteric nervous system, and they recruit the same intracellular signalling pathways as the nutrient-sensing GPCRs. This modulates the release of L cell peptides glucagon-like peptide 1 and 2 (GLP-1, GLP-2) oxyntomodulin (Oxyntomodulin) and peptide YY (PYY), which in turn activate GPCRs shown to be expressed on afferent nerves. Adapted from: (Reimann *et al.* 2012)

Ach, acetylcholine; *Adr*, adrenaline; β AR; β -adrenergic receptor; *BB2R*, GRP receptor; *CCK*, cholecystokinin; *CGRP*, calcitonin gene-related peptide; *Gal1R*, galanin 1 receptor; *GIP*, glucose-dependent insulintropic polypeptide; *GRP*, gastrin-releasing peptide; *M2R*, muscarinic acetylcholine receptor M2; *NMB*, neuromedin B; *PACAP*, pituitary adenylate cyclase-activating protein; *Sst*, somatostatin; *SST5R*, somatostatin 5 receptor; *VIP*, vasoactive intestinal peptide; *VPACR*, VIP and PACAP receptor

2.1.2 Nutrient-sensing in the gut

Nutrient sensors can either sense changes in the composition of the gut lumen, or increased concentrations of nutrients absorbed across the intestinal epithelium. Activation of nutrient sensors can trigger processes including membrane depolarisation, increased intracellular calcium (Ca^{2+}) concentrations and second messenger cascades that ultimately result in the secretion of gut hormones or changes in gene expression (Tolhurst *et al.* 2012b). The three main pathways by which nutrients are 'sensed' involve GPCRs, surface membrane solute transporters, and intracellular metabolism. To date, a large number of GPCRs have been identified as nutrient sensors in the gut (Wellendorph *et al.* 2010) (Fig. 2.4).

Colonic L cells are known to express the bile acid receptor TGR5, the oleoylethanolamide receptor GPR119, the medium/long chain fatty acid receptors GPR40/120, the SCFA receptors GPR41/43 and components of the sweet taste receptor pathway (Table 2.1). However, L cells are also electrically active and alterations in membrane potential leading to Ca^{2+} influx have been associated with nutrient-stimulated gut hormone secretion (Rogers *et al.* 2011, Tolhurst *et al.* 2011).

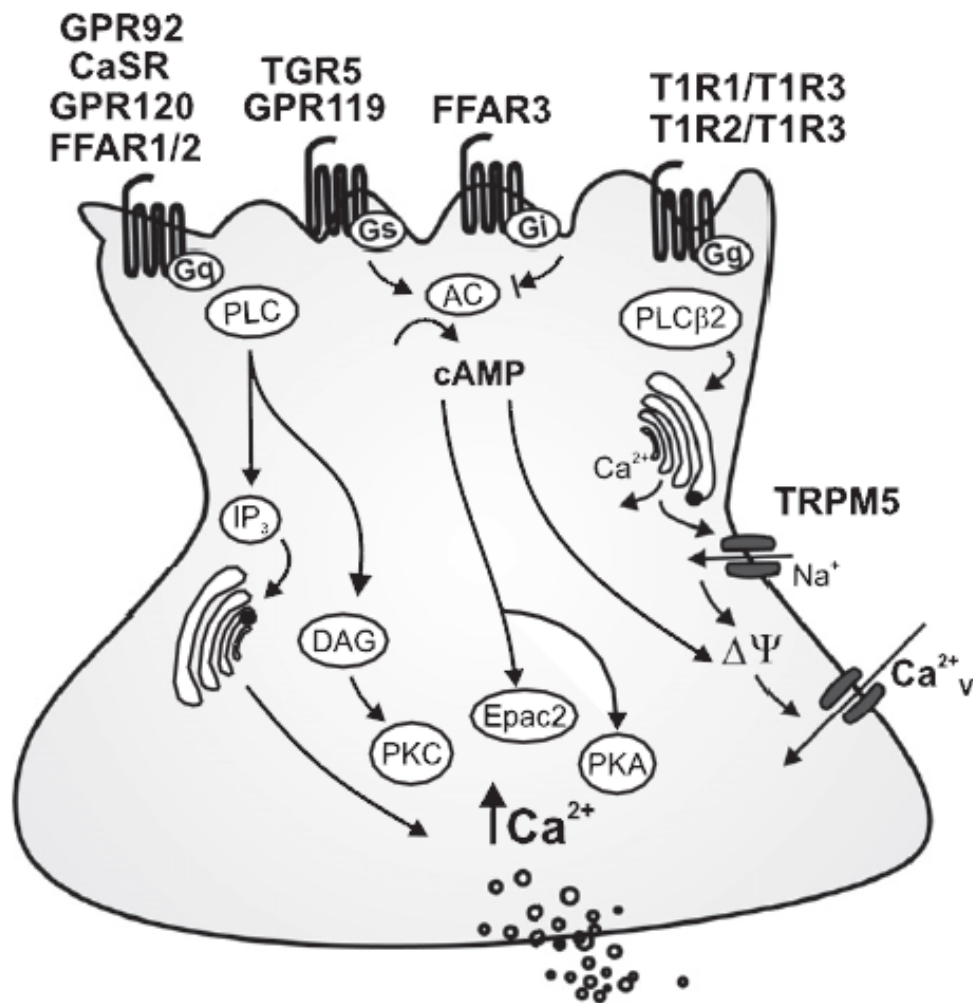


Figure 2.4. **GPCR-mediated nutrient sensing in enteroendocrine cells.** A number of GPCRs have been identified as putative nutrient “sensors”. These receptors stimulate the release of gut hormones via coupling to G_{α_s} , which elevates intracellular cAMP, and G_{α_q} , which results in Ca^{2+} mobilisation and protein kinase C (PKC) activation. Inhibitory influences are exerted by signalling through G_{α_i} pathways that decrease cAMP levels. Adapted from: (Reimann *et al.* 2012)

AC, adenylyl cyclase; *CaSR*, calcium-sensing receptor; *DAG*, diacylglycerol; *Epac2*, exchange protein activated by cAMP; *FFAR*, free fatty acid receptor; *IP₃*, inositol 1,4,5-trisphosphate; *PKA*, protein kinase A; *PLC*, phospholipase C; *TRPM5*, transient receptor potential cation channel subfamily M member 5

Table 2.1 **Chemosensory receptors expressed in enteroendocrine L cells.**

Ligands	Receptor	Species	Reference
Sweet tastants	T1R2/T1R3, α -gustducin	Mouse	(Reimann <i>et al.</i> 2008) (Li <i>et al.</i> 2013)
SCFAs	GPR43	Mouse	(Tolhurst <i>et al.</i> 2012a)
	GPR41	Mouse	(Samuel <i>et al.</i> 2008)
			(Tolhurst <i>et al.</i> 2012a)
MCFAs/LCFAs	GPR40	Mouse	(Edfalk <i>et al.</i> 2008)
			(Reimann <i>et al.</i> 2008)
	GPR120	Mouse Human	(Reimann <i>et al.</i> 2008) (Hirasawa <i>et al.</i> 2005)
OEA	GPR119	Mouse	(Cox <i>et al.</i> 2010)
2-OG		Rat	(Lauffer <i>et al.</i> 2009)
Bile acids	TGR5	Mouse	(Reimann <i>et al.</i> 2008)

MCFAs, medium-chain fatty acids; LCFAs, long-chain fatty acids; OEA, oleoylethanolamide; 2-OG, 2-oleoylglycerol; SCFAs, short-chain fatty acids

2.1.2.1 Immortalised cell lines for the study of nutrient sensing

The study of EEC physiology has been impeded in the past by the scarcity of these cells in the intestinal epithelium and the difficulties associated with accurately identifying them. Consequently, a large proportion of our knowledge of how EECs function and signal originates from immortalised cell lines such as GLUTag (Drucker *et al.* 1994), STC-1 (Abello *et al.* 1994) and NCI-H716 cells (Reimer *et al.* 2001). GLUTag and NCI-H716 cells are used as murine and human GLP-1-secreting L cell models, respectively. However, neither of these cell lines secretes PYY, which is thought to be co-secreted from L cells *in vivo* along with GLP-1 (Habib *et al.* 2013). On the other hand, the STC-1 cell line has been reported to secrete a variety of hormones including CCK, GIP, GLP-1, PYY and secretin, a hormonal

profile also unrepresentative of native colonic L cells (Geraedts *et al.* 2009, Hand *et al.* 2013).

Furthermore, enteroendocrine cell lines often exhibit different nutrient sensitivities, and do not necessarily act as accurate models of the native L cell (Reimann *et al.* 2008). As isolated FACS-sorted primary L cells do not survive in culture, mixed-cell population primary culture appears to be essential (Reimann *et al.* 2008). This primary cell culture method enables the study of gut hormone release from this elusive cell type, while to a certain extent maintaining its physiological micro-environment and proximity to neighbouring cells.

2.1.2.2 Glucose and L-glutamine sensing in primary L cells

Using a novel primary L cell model, isolated from transgenic mice in which proglucagon-expressing cells were labelled by the YFP Venus (Nagai *et al.* 2002), Reimann *et al.* demonstrated that detection of luminal glucose involves membrane depolarisation and action potential firing. This was triggered by the electrogenic activity of sodium glucose co-transporter 1 (SGLT1), which led to a voltage-dependent influx of Ca^{2+} ions (Reimann *et al.* 2008). The same techniques were successfully employed to study the mechanisms underlying amino acid-stimulated GLP-1 secretion. Tolhurst *et al.* demonstrated that L-glutamine also triggers primary L cell membrane depolarisation and voltage-dependent influx of Ca^{2+} ions, via L- and Q-type calcium channels, following their electrogenic uptake (Tolhurst *et al.* 2011).

This method of primary colonic cell isolation was selected for experiments investigating the effects of SCFAs on gut hormone release and the mechanisms underpinning these effects. Therefore, the first aim of this thesis was to establish this method in our laboratory, to optimise procedures associated with it, and to replicate experiments in the literature to validate the primary L cell model.

2.2 Aims and Hypotheses

2.2.1 Aim

To establish a primary L cell model that can be employed to determine the effects of nutrients on the release of the anorectic gut hormones GLP-1 and PYY.

Objectives:

1. To successfully isolate murine colonic crypts.
2. To optimise radioimmunoassay methods for measurement of GLP-1 and PYY in supernatants and cell lysates.
3. To validate this model by replicating published work with nutrients, glucose and glutamine, and agents that increase intracellular cAMP levels.
4. To apply and tailor the murine primary L cell model methodology to the development of a novel human primary L cell model.

2.2.2 Hypotheses

I hypothesised that the method pioneered by Reimann *et al.* would enable the isolation of intact and viable colonic crypts from mouse and human colonic tissue. Furthermore, I hypothesised that the nutrients, glucose and glutamine, and the combination of 3-isobutyl-1-methylxanthine (IBMX) and forskolin leading to an increase in intracellular [cAMP], would stimulate the release of GLP-1 and PYY from murine and human primary L cells.

2.3 Methods

2.3.1 Primary cell culture

2.3.1.1 Murine colonic crypt isolation

The method of primary murine colonic crypt isolation was based on the method published by Professor Fiona Gribble of the University of Cambridge (Reimann *et al.* 2008).

Adult male C57BL6 mice (8 weeks of age or older) were culled and the colon, distal to the caecum, was removed, cleaned and placed into ice-cold L-15 (Leibowitz) medium (PAA, UK). The intestinal tissue was thoroughly cleaned with L-15 medium and digested with 0.4mg/ml collagenase XI (Sigma, UK) in high glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma, UK) at 37°C. Resulting cell suspensions were centrifuged (5 min, 500 × g) and the pellets were re-suspended in DMEM (supplemented with 10% foetal calf serum and 1% antibiotics, 100 U/ml penicillin and 0.1mg/ml streptomycin). The digestion process was repeated four times and the combined cell suspensions were filtered through a nylon mesh (pore size 250 µm) and plated onto 24-well, 1% Matrigel (BD Matrigel, VWR, UK)–coated plates. The plates were incubated overnight at 37°C in an atmosphere of 95% O₂ and 5% CO₂.

2.3.1.2 Human colonic crypt isolation

Ethical approval for the use of human colonic tissue biopsies was obtained from the Hammersmith & Queen Charlotte's Research Ethics Committee (2000/5795). Healthy colonic tissue was obtained from subjects undergoing diagnostic colonoscopy at Hammersmith Hospital. Common reasons for undergoing the procedure included suspicion of benign polyps or haemorrhoids, and follow up after a previous history of colitis. Eight to ten biopsies, measuring approximately 2mm², were obtained from each patient by Professor

Julian Walters or Dr Ian Johnston of Imperial College London. The colonic crypts were isolated as described above. However, as the human colonic crypts were larger in diameter, the last filtration step was omitted.

2.3.1.3 Secretion experiments

Gut hormone secretion experiments were carried out within 24 hours after plating, unless otherwise stated. The cells were washed three times with standard bathing solution referred to as secretion buffer (4.5mM KCl, 138mM NaCl, 4.2mM NaHCO₃, 1.2mM NaH₂PO₄, 2.6mM CaCl₂, 1.2mM MgCl₂, and 10mM HEPES, which was adjusted to pH 7.4 with NaOH; refer to Appendix I) supplemented with 0.1% fatty acid free bovine serum albumin (BSA, Sigma UK). The cells were then incubated in secretion buffer containing the test reagents (300µl/well) for 2 hours at 37°C in an atmosphere of 95% O₂ and 5% CO₂. Test reagents were all obtained from Sigma (UK) unless otherwise stated. The adenylyl cyclase activator forskolin and the phosphodiesterase inhibitor IBMX were prepared as 10mM stock solutions in dimethyl sulfoxide (DMSO) and used as positive controls at a final concentration of 10µM (Reimann *et al.* 2008). Test solutions were prepared on the day of the secretion experiment.

Following incubation, the cell supernatants were collected and centrifuged at 4°C to remove any dead cells (3 min, 100 × g). The resulting supernatants were then stored at -20°C pending analysis. The cells remaining in the plates were treated with 250µl/well cell lysis buffer (refer to Appendix I) and were frozen at -80°C overnight. The plates were then scraped and washed using 250µl secretion buffer and lysates were stored at -20°C pending analysis.

Gut hormone secretion is expressed as a fraction of the total hormone (secreted + extracted) measured from a well.

$$\% \text{ hormone release} = \frac{(\text{hormone measured in supernatant})}{(\text{hormone measured in supernatant} + \text{hormone measured in lysed cells})} \times 100$$

2.3.2 Radioimmunoassay

GLP-1 and PYY release was measured by radioimmunoassay. The principle of the radioimmunoassay (RIA) is based on competition between unlabelled peptide (in the sample) and a known amount of radiolabelled peptide for binding to a fixed concentration of antibody. The concentrations of radiolabelled peptide and antibody remain constant. Therefore, increasing the concentration of unlabelled peptide will decrease the binding sites available for binding of the radiolabelled peptide. Thus, the amount of radiolabelled peptide bound to the antibody is inversely proportional to the amount of unlabelled peptide in the sample being assayed. Unknown peptide concentration values were interpolated from a standard curve prepared using known concentrations of unlabelled peptide (standard).

Antibody-bound peptide was separated from unbound peptide by charcoal adsorption (GLP-1) or using a primary/secondary antibody complex (PYY). All cell supernatant samples were assayed in singlet, whereas cell lysates were assayed in duplicate.

2.3.2.1 Radioimmunoassay controls

To assess non-specific binding (NSB), tubes which do not contain antibody were included at the beginning of the assay. Tubes containing either half or twice the volume of radio-labelled peptide were included to assess the quality of the label. Tubes with no sample (zero tubes) were placed throughout the assay to monitor baseline drift. Quality control (QCs) samples containing plasma spiked with low, medium or high known concentrations of peptide were also added to the beginning and end of each assay. Freeze-dried QCs were reconstituted in GDW on the day.

2.3.2.2 GLP-1

GLP-1-like immunoreactivity was measured using an established, specific and sensitive radioimmunoassay (Kreymann *et al.* 1987). The antibody was produced in rabbits against GLP-1 coupled to BSA. The antibody shows 100% cross-reactivity with all amidated forms of GLP-1 but does not cross react with glycine extended forms (GLP-1₁₋₃₇ and GLP-1₇₋₃₇) or any other known gastrointestinal peptides. ¹²⁵I-GLP-1 was prepared by Professor Mohammad Ghatei using the iodogen method (Wood *et al.* 1981) and purified by high pressure liquid chromatography (HPLC). The assay was performed in a total volume of 350µl of 0.06M sodium barbitone buffer (pH 8) (refer to Appendix I) containing 0.3% BSA. The standard curve was constructed by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of GLP-1 at a concentration of 0.125pmol/ml. Supernatants were added at a volume of 100µl and lysates were added at a volume of 5µl, as determined by optimisation assays (refer to Results Section 2.4.2). The assay was incubated over four nights at 4°C before separation of the free from antibody-bound labelled peptide by charcoal adsorption. Free and bound radioactivity was measured using γ scintillation counters (model NE1600, Thermo Electron Corporation, Ohio, USA) (intra-assay variation 5.4%; inter-assay variation 11.7%).

2.3.2.3 PYY

PYY-like immunoreactivity was measured using a specific and sensitive radioimmunoassay (Adrian *et al.* 1985). The antiserum (Y21) was produced in rabbits against synthetic porcine PYY coupled to BSA by glutaraldehyde. The Y21 antibody cross-reacts fully with the biologically active forms of PYY: full length PYY₁₋₃₆ and the truncated fragment PYY₃₋₃₆. It does not cross-react with other gastrointestinal peptides. ¹²⁵I-PYY was prepared by Professor Mohammad Ghatei using the iodogen method (Wood *et al.* 1981) and purified by HPLC. The assay was performed in a total volume of 350µl of 0.06M phosphate buffer (pH 7.3) (refer to Appendix I) containing 0.3% BSA. The standard curve was constructed by

adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of synthetic PYY at a concentration of 0.5pmol/ml. Supernatants were added at a volume of 150µl and lysates were added at a volume of 20µl, as determined by optimisation assays (refer to Results Section 2.4.2). The assay was incubated over three nights at 4°C before separation of the free from antibody-bound label by immunoprecipitation using sheep anti-rabbit antibody. Free and bound radioactivity was measured using γ scintillation counters as above (intra-assay variation 4.2%; inter-assay variation 10.4%).

2.3.3 Statistical Analysis

Normality was determined using the D'Agostino-Pearson omnibus test where $n \geq 8$ per group. Statistical significance was calculated using either an unpaired t-test (2 groups) or one-way ANOVA (≥ 3 groups). ANOVA pairwise comparisons were carried out using the Dunnett post hoc test, unless otherwise stated. Statistical significance was accepted at $P < 0.05$ throughout. Data are presented as mean $\pm$ standard error of the mean (SEM). Analysis was carried out using Graph Pad Prism software, version 5.0.

2.4 Results

2.4.1 Primary colonic crypt isolation: murine and human

Initial experiments demonstrated that the digestion protocol was effective in isolating individual colonic crypts from mouse colonic tissue. The digestion method resulted in the isolation of largely intact crypts from both murine (Fig. 2.5) and human colonic tissue (Fig. 2.6). The human crypts were broader and approximately 2.5× the length of the mouse crypts (~250µm vs. 100µm, respectively). The crypts had a clearly defined morphology following digestion. However, the crypt structure was lost overnight, as the crypt cells began migrating out of the crypt creating a partial monolayer of cells (Fig. 2.7).

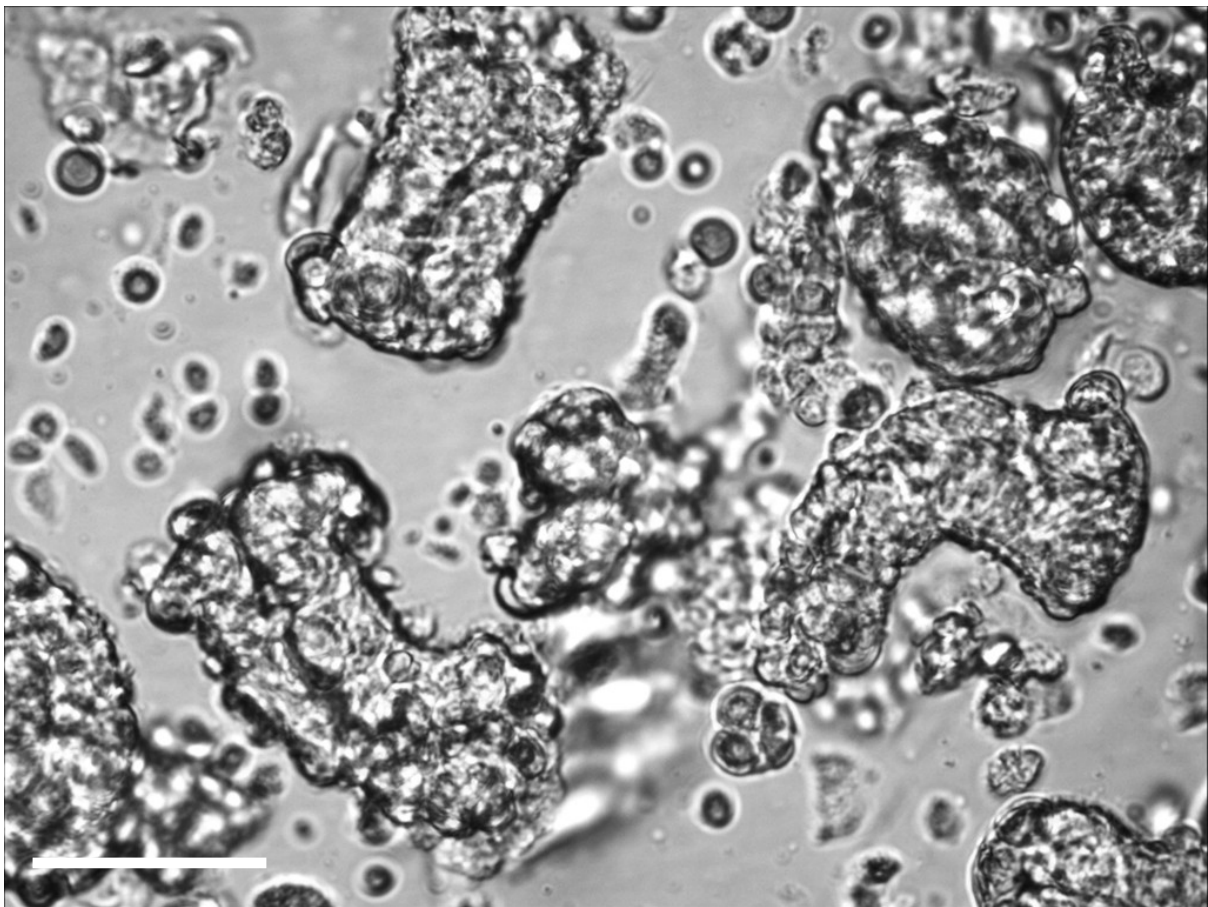


Figure 2.5 Isolated murine primary L cell-containing murine colonic crypts. Photomicrograph of mouse colonic crypts obtained following tissue digestion (×40 magnification). The scale bar represents 50µm.



Figure 2.6 **Isolated human primary L cell-containing human colonic crypts.** Photomicrograph of human colonic crypts obtained following tissue digestion ($\times 20$ magnification). The scale bar represents $100\mu\text{m}$.

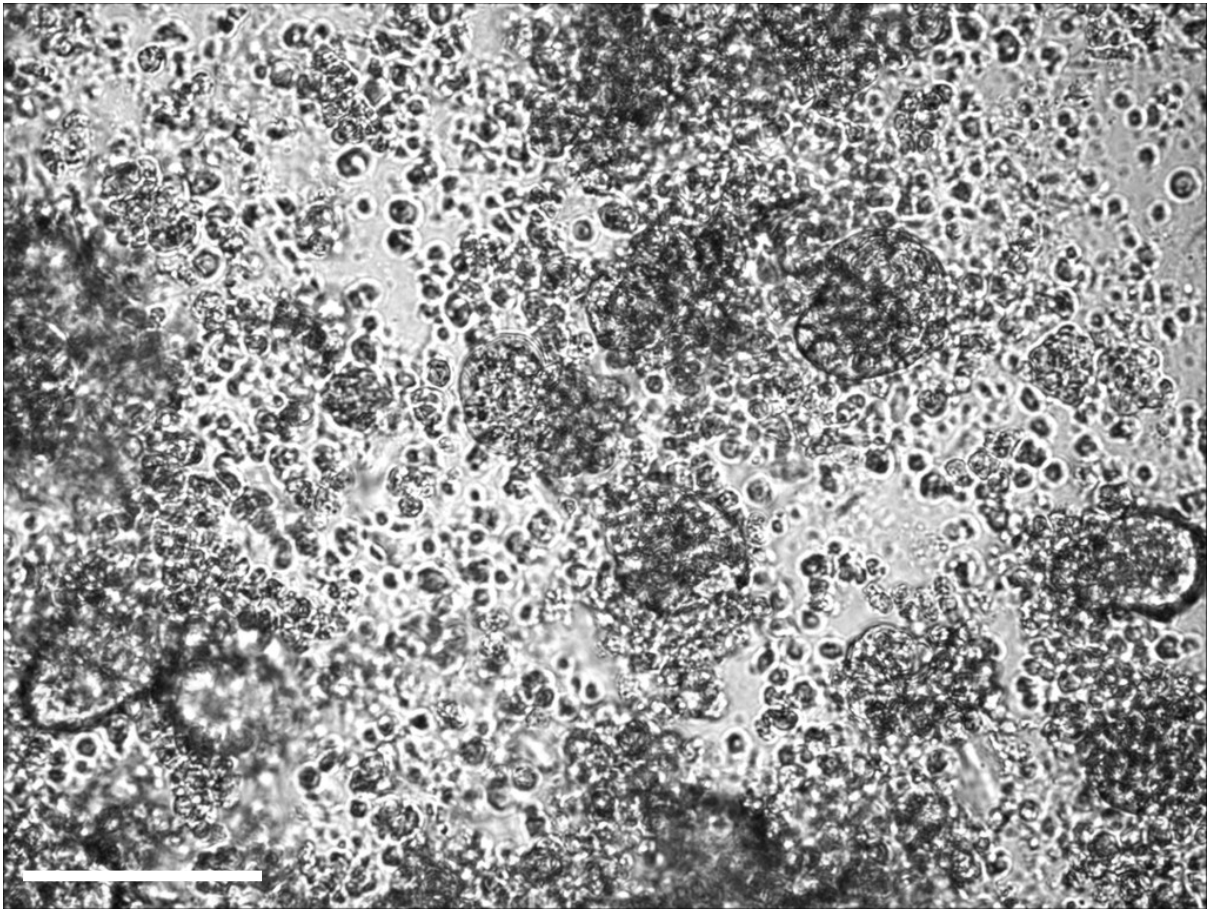


Figure 2.7 **Partial monolayer of primary colonic cells.** Photomicrograph of mouse colonic cells that have migrated out of their crypt structures overnight following tissue digestion ($\times 20$ magnification). The scale bar represents $100\mu\text{m}$.

2.4.2 Optimisation of radioimmunoassay methods

During the initial process of assaying the lysed cell samples it became apparent that the cell lysis buffer was interfering with the radioimmunoassays, thus distorting the percentage binding levels. To identify the volume of lysis buffer which does not interfere with the PYY and GLP-1 assays, test assays were constructed with either three or four standard curves containing different volumes of lysis buffer. The results showed significant distortion of the standard curve when containing 20 μ l or 5 μ l lysis buffer in the PYY and GLP-1 assay, respectively (Fig. 2.8). However, 10 μ l and 2.5 μ l lysis buffer caused minimal interference in the PYY and GLP-1 assay, respectively.

Lysates are diluted 1:1 with secretion buffer during the plate scraping process. Consequently, the lysates were added at volumes of 20 μ l and 5 μ l, containing 10 μ l and 2.5 μ l lysis buffer, respectively. Nevertheless, a standard curve containing the same volume of 1:1 lysis buffer/secretion buffer was added prior to lysate sample tubes into future assays and was used for data reduction purposes to correct for potential assay interference.

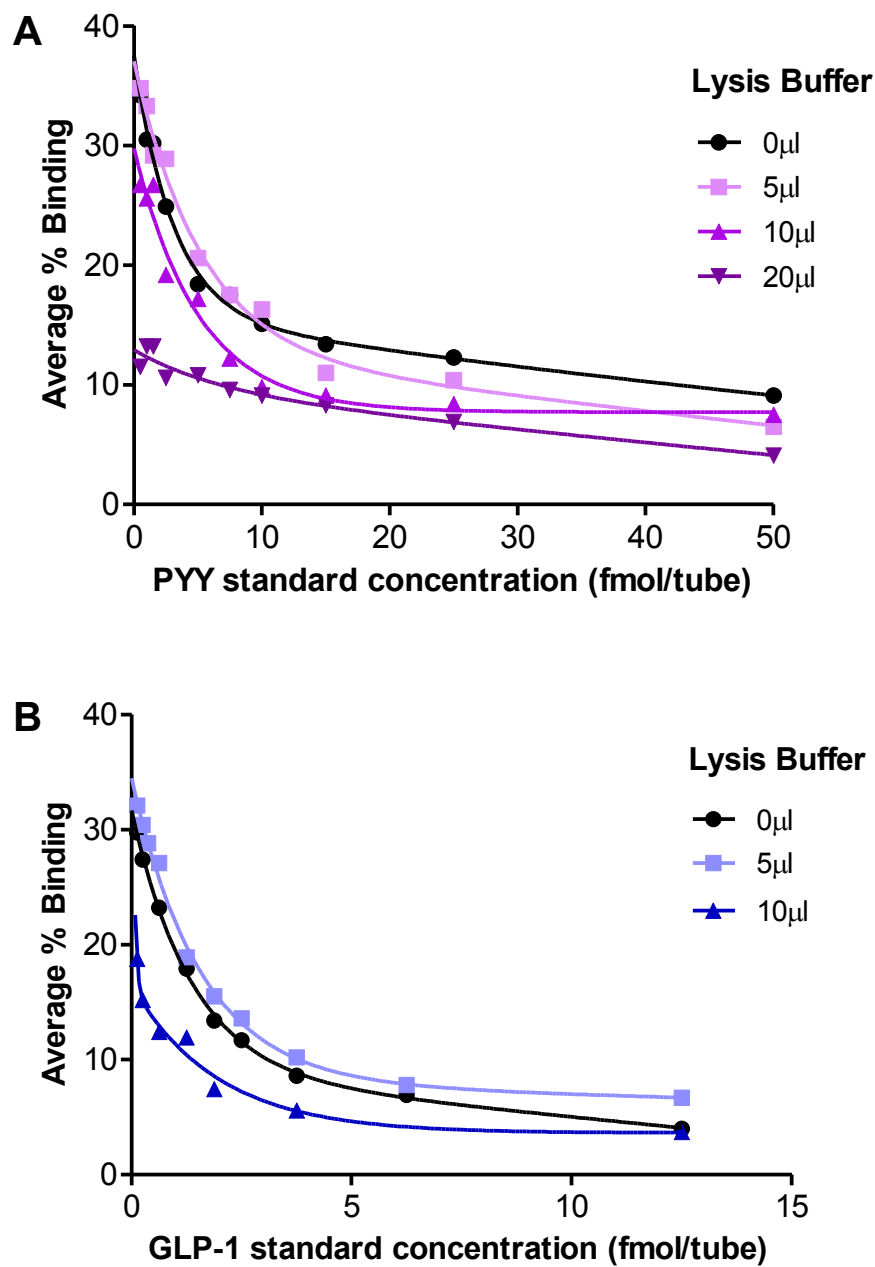


Figure 2.8 **The effect of cell lysis buffer on PYY (A) and GLP-1 (B) radioimmunoassay performance.** Average percentage binding represents the percentage of antibody-bound ^{125} I-labelled PYY or GLP-1 when competing with unlabelled PYY or GLP-1 (standard) for primary antibody binding sites.

2.4.3 Validation of primary L cell culture method

2.4.3.1 The effect of D-glucose on gut hormone release from murine primary L cells

To validate the primary L cell culture method, key experiments conducted by Reimann *et al.* were replicated to confirm that the L cells respond to certain nutrients in a manner consistent with the original publications (Reimann *et al.* 2008). The first experiment tested the effect of a 2-hour incubation of primary colonic cells with varying concentrations of D-glucose on PYY and GLP-1 release.

As shown in Figure 2.9, the primary L cells secreted both PYY and GLP-1 in response to a low (1mmol/L) and high (100mmol/L) concentration of glucose. Basal PYY secretion over the 2-hour incubation period was $5.1 \pm 0.8\%$. PYY secretion rose to $8.2 \pm 2.0\%$ following treatment with 1mmol/L glucose and was significantly increased to $8.9 \pm 3.1\%$ with the 100mmol/L dose of glucose (Fig. 2.9A). Basal GLP-1 secretion over the 2-hour incubation period was $1.2 \pm 1.0\%$. Incubation with both doses of glucose led to a significant 3-fold increase in GLP-1 secretion (Fig. 2.9B).

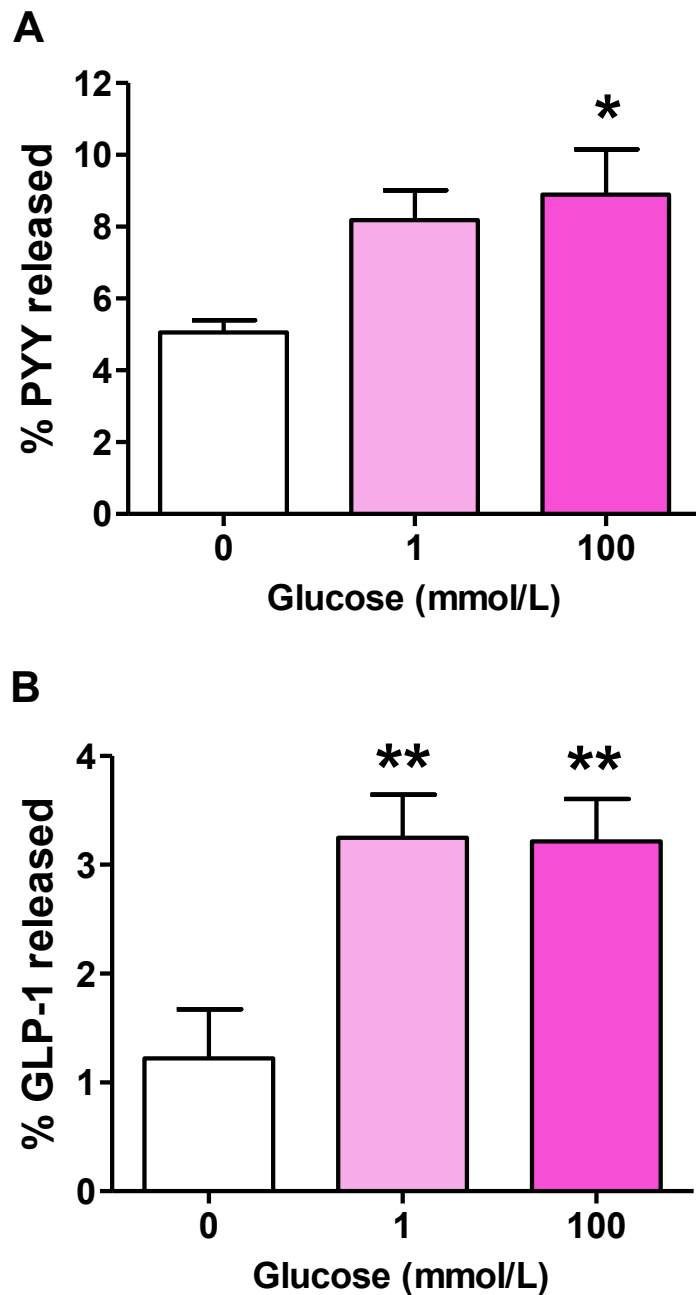


Figure 2.9 The effect of D-glucose on gut hormone secretion from murine primary L cells. Mixed primary colonic cultures were incubated in secretion buffer containing increasing glucose concentrations. PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=6 wells). Significance is shown relative to basal secretion using One-way ANOVA with a Dunnett post hoc test (* $P < 0.05$; ** $P < 0.01$).

2.4.3.2 The effect of L-glutamine on gut hormone release from murine primary L cells

The second set of experiments tested the effect of incubating murine primary colonic cells with two different concentrations of the amino acid L-glutamine on PYY and GLP-1 release. Figure 2.10 shows that the L-glutamine stimulated gut hormone secretion from murine primary L cells, with some evidence of a dose-response relationship.

Basal PYY and GLP-1 secretion over the 2-hour incubation period was $5.6 \pm 2.8\%$ and $3.2 \pm 1.4\%$, respectively. At a concentration of 1mmol/L, L-glutamine increased PYY and GLP-1 secretion to $8.6 \pm 3.0\%$ and $4.2 \pm 1.6\%$, respectively (Fig. 2.10). Furthermore, incubation of the colonic cells with 10mmol/L L-glutamine significantly increased both PYY and GLP-1 release to $11.6 \pm 2.3\%$ and $6.5 \pm 1.9\%$, respectively.

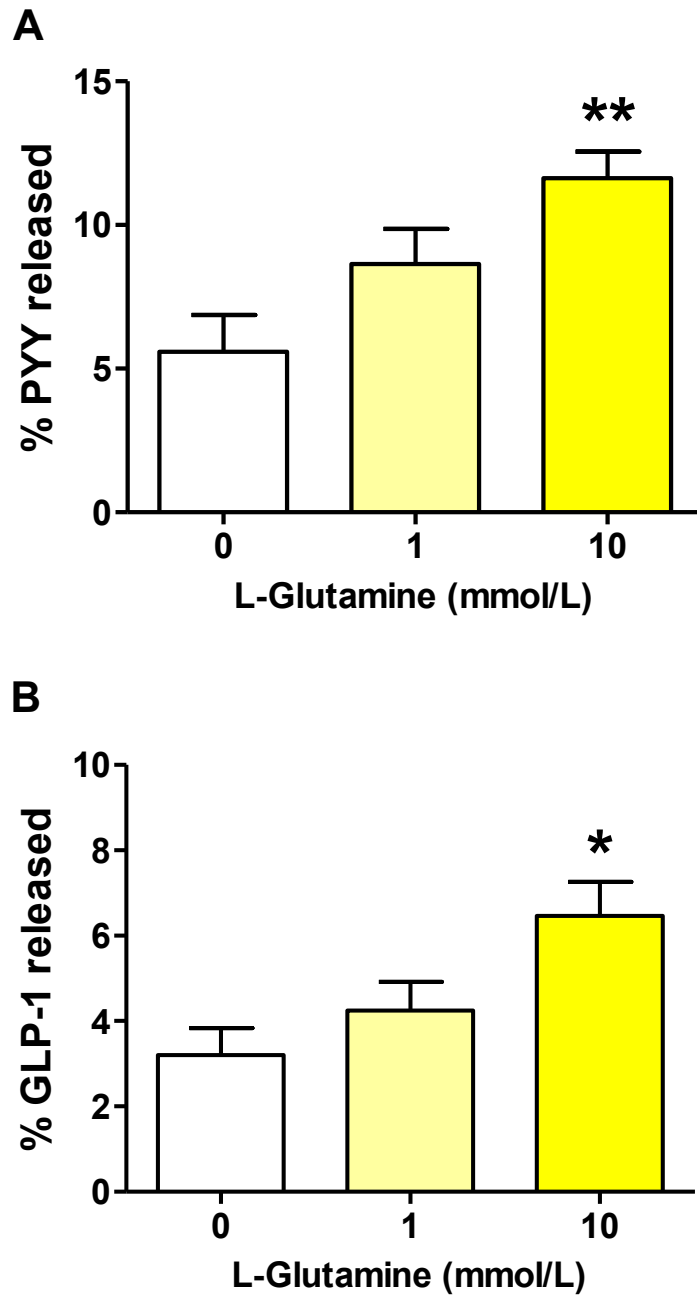


Figure 2.10 **The effect of L-glutamine on gut hormone secretion from murine primary L cells.** Mixed primary colonic cultures were incubated in secretion buffer containing increasing L-glutamine concentrations. PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=6 wells). Significance is shown relative to basal secretion using One-way ANOVA with a Dunnett post hoc test (* $P < 0.05$; ** $P < 0.01$).

2.4.3.3 The effect of increasing intracellular [cAMP] on gut hormone release from murine primary L cells

Experiments were carried out to test the effect of increasing intracellular cAMP levels on gut hormone release from isolated murine L cells. This was achieved using a combination of forskolin, which activates adenylyl cyclase, and IBMX, a competitive non-selective phosphodiesterase inhibitor. IBMX and forskolin were both added at a concentration of 10 μ mol/L (Ong *et al.* 2009).

Increasing intracellular cAMP concentrations, using a combination of IBMX and forskolin, potently stimulated gut hormone release from primary murine L cells. Basal secretion of PYY was $3.6 \pm 0.4\%$, which increased significantly in response to IBMX/forskolin treatment, to $23.2 \pm 3.0\%$ (Fig. 2.11A). Basal GLP-1 secretion was lower, $1.7 \pm 0.2\%$, and rose to $10.3 \pm 1.6\%$ following incubation with IBMX/forskolin (Fig. 2.11A).

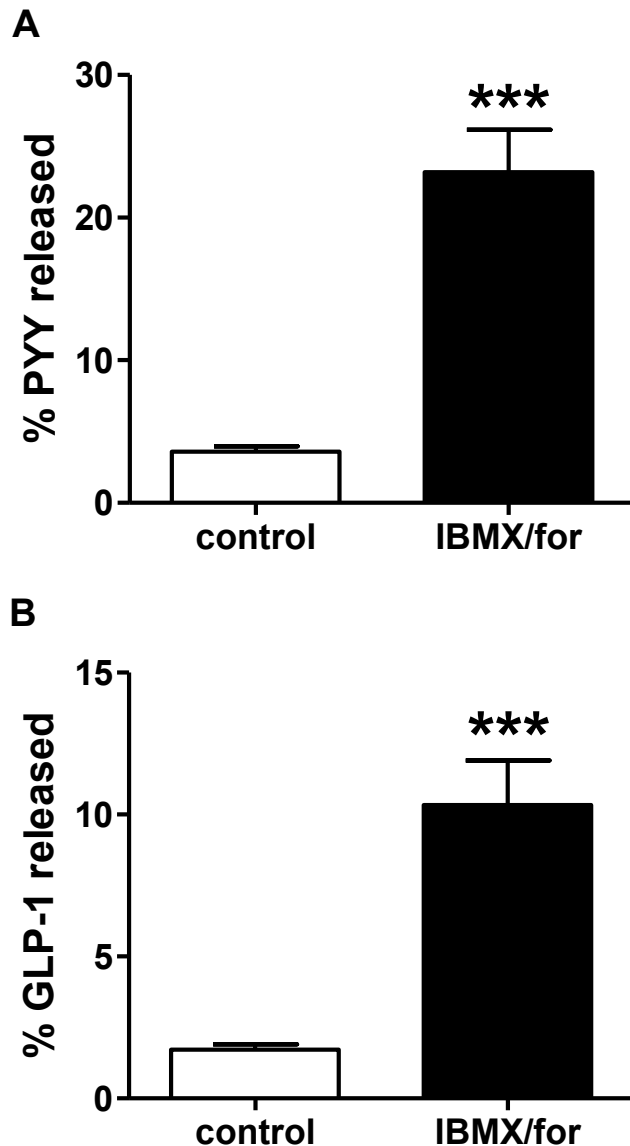


Figure 2.11 **The effect of increasing intracellular cAMP levels on gut hormone secretion from murine primary L cells.** Mixed primary colonic cultures were incubated in secretion buffer containing 10 μ mol/L IBMX and forskolin. PYY and GLP-1 secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to basal secretion (control) measured in parallel within the same experiment. PYY and GLP-1 data are from separate experiments. Data represent means $\pm$ SEM (2 separate experiments, n=2 mice, 11-14 wells). Significance is shown relative to basal secretion using an unpaired t-test (***) $P < 0.001$.

2.4.3.4 The effect of D-glucose and an increase in intracellular [cAMP] on GLP-1 release from human primary L cells

Following the successful isolation of viable human primary L cells, the effect of glucose and of IBMX/forskolin, on GLP-1 release was determined. As shown in Figure 2.12, the human L cells dose-dependently secreted GLP-1 in response to D-glucose. Basal GLP-1 secretion over the 2-hour incubation period was $0.5 \pm 0.2\%$. GLP-1 secretion rose to $1.0 \pm 0.3\%$ (2.1-fold increase) and $1.3 \pm 0.2\%$ (2.7-fold increase) following treatment with 1mmol/L and 10mmol/L glucose, respectively. The highest dose of glucose (100mmol/L) significantly increased GLP-1 secretion to $1.9 \pm 0.3\%$, representing a 3.9-fold increase. Furthermore, incubation of human primary L cells with IBMX/forskolin also significantly increased %GLP-1 secretion to $1.8 \pm 0.4\%$, reflecting a 3.8-fold increase from basal.

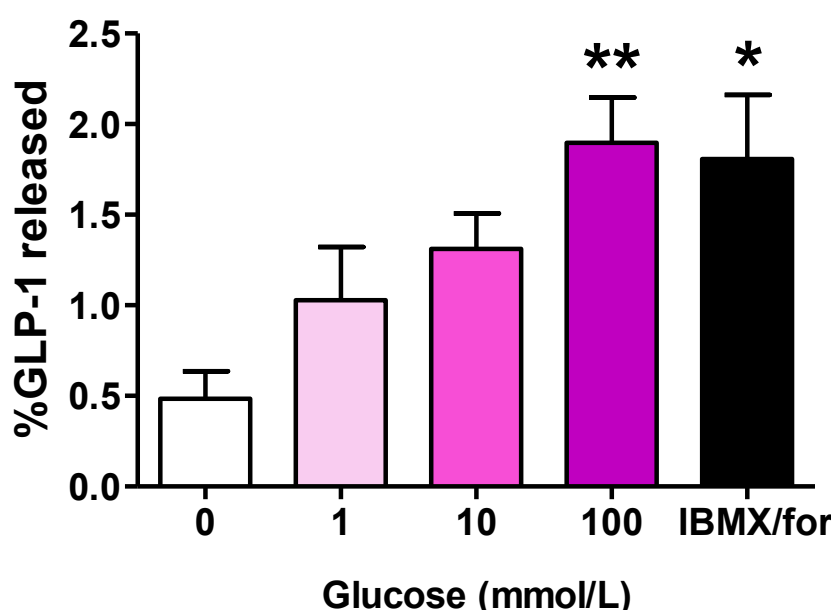


Figure 2.12 **The effect of D-glucose and increasing intracellular [cAMP] on gut hormone secretion from human primary L cells.** Mixed human primary colonic cultures were incubated in secretion buffer containing increasing glucose concentrations or IBMX and forskolin (10 μ mol/L each). GLP-1 secretion in each well is expressed as a percentage of total GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=4-5 wells). Significance is shown relative to basal secretion using One-way ANOVA with a Dunnett post hoc test (*P<0.05; **P<0.01).

2.5 Discussion

2.5.1 Summary of results

- The colonic digest method pioneered by Reimann *et al.* led to the successful isolation of colonic crypts from both mouse and human colonic tissue.
- Glucose (1 and 100mmol/L) and L-glutamine (10mmol/L) significantly stimulated the release of GLP-1 and PYY from murine primary colonic L cells.
- Increasing intracellular cAMP levels potently stimulated the release of GLP-1 and PYY from murine primary colonic L cells.
- For the first time, it was demonstrated that glucose and increasing intracellular cAMP levels induced GLP-1 secretion from human primary colonic L cells.

2.5.2 Detailed discussion

The aim of the work described in this chapter, was to establish a primary L cell model in our laboratory based on the method for isolating primary colonic crypts originally published by Reimann *et al.* (Reimann *et al.* 2008). I showed that the colonic digest method leads to the isolation of largely intact and viable colonic crypts from mouse colon, and with minor adjustments, the method was also effective for human colonic crypt isolation to create a novel human primary L cell model.

Professor Fiona Gribble's group in Cambridge has successfully used this method to study GLP-1 release from murine primary L cells using an active GLP-1 ELISA (Parker *et al.* 2012, Reimann *et al.* 2008, Rogers *et al.* 2011). Therefore, it was necessary to set up and optimise protocols for the assessment of total GLP-1 and PYY levels in supernatants and lysed cells using our in-house radioimmunoassays. Assay interference caused by the lysis buffer in the lysed cell samples was overcome by identifying and not exceeding the volumes of lysis

buffer which caused minimal interference, and by adding a lysis buffer-containing standard curve to each assay to account for any potential interference.

The third objective was to validate the primary L cell model by replicating published work that demonstrated the effect of various stimuli on active GLP-1 secretion (Reimann *et al.* 2008, Rogers *et al.* 2011). The results of the above experiments confirm that viable L cells, which release PYY and GLP-1, are present within murine and human isolated colonic crypts and are nutrient responsive.

Basal gut hormone secretion from primary L cells

Reassuringly, basal secretion of gut hormones from this primary L cell model appears relatively consistent across experiments. However, what is apparent is that basal PYY secretion (4-6%) is higher than GLP-1 secretion (1-3%). Basal secretion reflects a basal tone of electrical activity, which L cells maintain in culture, reported as infrequent Na⁺-dependent action potentials (Reimann *et al.* 2008). These action potentials serve to sustain a background level of gut hormone secretion. It is difficult to know whether the difference in basal secretion between the two gut hormones is genuine or whether it merely reflects a difference in the half-lives of the two peptides; PYY has a reported half-life of ~8-9min, whereas GLP-1 has a reported half-life of ~1-2min (Adrian *et al.* 1986, Kieffer *et al.* 1995, Lluís *et al.* 1989). Publications from Professor Gribble's laboratory routinely display GLP-1 release data as 'relative to basal secretion' and not as percentage release. However, in a recent publication they reported average basal active GLP-1 release to be approximately 1.3% (Habib *et al.* 2013), which closely resembles the values presented here. Basal GLP-1 release from human primary L cells appears slightly lower than that of murine L cells, with a mean of 0.5% compared to 2.0% (across experiments presented in this Chapter), respectively.

The effect of glucose on gut hormone release from primary L cells

The stimulatory effect of glucose on GLP-1 and PYY release *in vivo* and from whole intestinal preparations is well established in the literature (Fu-Cheng *et al.* 1995, Herrmann *et al.* 1995, Mace *et al.* 2012, Plaisancie *et al.* 1995). Glucose-induced GLP-1 secretion has also been demonstrated using GLUTag cells (Reimann & Gribble 2002). However, experiments conducted using fetal rat intestinal cultures and primary cultured canine L cells, suggested a lack of response to glucose and brought into question whether L cells are glucose-sensitive (Brubaker & Vranic 1987, Damholt *et al.* 1998).

Using a perforated patch recording set up, the original primary L cell model publication showed that L cells are electrically excitable and respond to glucose (10 mmol/L) by increasing action potential frequency (Reimann *et al.* 2008). In addition, the authors demonstrated that L cells exhibit elevations in $[Ca^{2+}]_i$ following addition of glucose (10 mmol/L). Glucose had a significantly greater stimulatory effect on intracellular calcium in L cells, compared to colonic *non*-L cells, suggesting that the response to glucose does in fact originate within the L cell.

The results presented in this chapter are in agreement with the findings of the original primary L model publication; glucose triggers the release of GLP-1 at concentrations as low as 1mmol/L, which is within the physiological range for the rodent large intestine (Ferraris *et al.* 1990). I have shown for the first time that this is also true for PYY. Furthermore, the fold increase in GLP-1 and PYY secretion in response to glucose at both concentrations is similar to that reported for active GLP-1 using the same primary L cell model (Table 2.2) (Reimann *et al.* 2008).

There is some evidence to suggest that glucose in the large intestine is able to increase plasma gut hormone levels in humans (Qualmann *et al.* 1995), though not all studies have arrived at this conclusion (Printz *et al.* 1998). The effect of glucose on GLP-1 secretion from isolated human primary L cells has not been previously demonstrated. The data presented in

this chapter suggest that human L cells are responsive to glucose, which appears to stimulate GLP-1 in a dose-dependent manner. However, despite a 2-fold and 2.7-fold increase in GLP-1 release with 1 and 10mmol/L glucose, respectively, the increase in GLP-1 release only reached statistical significance at 100 mmol/L, a highly supraphysiological concentration for the human large intestine (Ferraris *et al.* 1990). The lack of a statistically significant GLP-1 response to glucose in this instance may reflect the low n numbers used due to the limited amount of human colonic tissue available.

Table 2.2 **Comparison of observed relative PYY and GLP-1 secretion from murine primary colonic cultures, in response to different stimuli, with values reported in the literature.**

Stimulant	Relative* ↑PYY	Relative* ↑GLP-1 (total)	Reported ↑GLP-1 (active)	Reference
Glucose				
1mmol/L	1.6-fold	2.7-fold	1.8-fold	(Reimann <i>et al.</i> 2008)
100mmol/L	1.8-fold	2.6-fold	2.1-fold	
L-Glutamine				
1mmol/L	1.5-fold	1.3-fold	1.7-fold	(Tolhurst <i>et al.</i> 2011)
10mmol/L	2.1-fold	2.0-fold	1.9-fold	
IBMX/forskolin				
10μmol/L	6.5-fold	6.0-fold	9.0-fold 6.0-fold (PYY)	(Reimann <i>et al.</i> 2008)

**Relative to basal secretion measured in parallel within the same experiment*

GLP-1, glucagon-like peptide 1; IBMX, 3-isobutyl-1-methylxanthine; PYY, peptide YY

The effect of L-glutamine on gut hormone release from primary L cells

Certain individual amino acids as well as amino acid mixtures are known to stimulate GLP-1 and PYY secretion *in vivo* (Fu-Cheng *et al.* 1995, Greenfield *et al.* 2009), from primary intestinal preparations (Mace *et al.* 2012) and from GLUTag cells (Oya *et al.* 2013, Reimann *et al.* 2004). Using the primary L cell model employed in the work described in this chapter, Tolhurst *et al.* recently demonstrated that L-glutamine dose-dependently stimulates active GLP-1 secretion (0.1 – 10mmol/L) (Tolhurst *et al.* 2011).

Further studies revealed that this response to L-glutamine consisted of two components: membrane depolarisation and an increase in [cAMP]_i. The first pathway involves the Na⁺-dependent electrogenic entry of L-glutamine into the L cell which triggers membrane depolarisation and voltage-gated Ca²⁺ influx. The identity of the L-glutamine transporter(s) involved remains unclear. The amino acid transporters most highly expressed in colonic L cells are SNAT2 (sodium-coupled neutral amino acid transporter 2, Slc38a2) and B⁰AT1 (system B (0) neutral amino acid transporter 1, Slc6a19), which is specific to L cells (Tolhurst *et al.* 2011). These co-transporters are both Na⁺-dependent and electrogenic. The second component consists of an amplifying pathway, now known to be mediated by an increase in [cAMP]_i, presumed to be triggered by GPCR activation (Tolhurst *et al.* 2011).

The data presented in this chapter confirm that L-glutamine dose-dependently stimulates the secretion of GLP-1, but also show for the first time that it stimulates PYY release to a similar extent. The observed fold increases in gut hormone secretion following 1 and 10mmol/L L-glutamine were consistent with those reported by Tolhurst *et al.* using the same L cell model (Table 2.2).

The effect of elevating intracellular cAMP concentrations on gut hormone release from primary L cells

An intracellular rise in cAMP within EECs is a well-recognised stimulus for gut hormone release; studies using various model systems, such as murine and human cell lines [GLUTag (Simpson *et al.* 2007) and NCI-H716 cells (Reimer *et al.* 2001)], primary colonic cultures (Brubaker 1988, Damholt *et al.* 1998), and isolated perfused intestinal preparations (Ballantyne *et al.* 1993), consistently demonstrate this. Furthermore, a number of neurotransmitters and gut peptides found within the enteric nervous system and intestinal epithelium, such as CGRP and GIP, are known to activate adenylyl cyclase (Furness 2000) and to trigger gut hormone secretion (Brubaker 1991, Dumoulin *et al.* 1995, Plaisancie *et al.* 1994). Therefore, it is plausible that hormonal and/or enteric nervous circuits may play a role in modulating L cell secretion via a cAMP-dependent pathway.

A study by Simpson *et al.* revealed that elevated intracellular cAMP modulates ion channel activity in the plasma membrane, leading to depolarisation, action potential firing and an increase in intracellular Ca^{2+} (Simpson *et al.* 2007). Elevated cAMP was shown to specifically modulate hyperpolarisation-activated currents and the background potassium current, rendering L cells more excitable and thus also enhancing responses to other depolarising stimuli.

In the experiment investigating the effect of increasing intracellular cAMP described in this chapter, a combination of IBMX and forskolin led to a 6-fold increase in total GLP-1 and a 6.5-fold increase in PYY secretion (Table 2.2). The PYY response was similar to that reported by Reimann *et al.* (6-fold). However, despite the robust 6-fold increase in GLP-1 ($P < 0.001$) following IBMX/forskolin treatment observed in the current work, it was lower than the increase in active GLP-1 reported by Reimann *et al.* The reason for this is unclear but may reflect a difference between total and active GLP-1.

On the other hand, IBMX/forskolin-induced GLP-1 secretion from the human L cells was higher than that recently published by Habib *et al.*, who used larger surgical specimens as opposed to the mucosal biopsies used in this work, obtained during diagnostic colonoscopy. They reported a 2.5-fold increase in active GLP-1 secretion following IBMX/forskolin treatment, compared to the 3.7-fold increase in total GLP-1 observed here. Therefore, there does appear to be a degree of variation in the magnitude of gut hormone responses to certain stimuli.

Conclusion

This chapter describes the successful application of a method for the isolation of intact colonic crypts containing viable L cells. Radioimmunoassay protocols were optimised to detect the secretion of the anorectic gut hormones PYY and GLP-1 from these primary L cells. The preliminary data presented in this chapter demonstrate that glucose and L-glutamine, as well as agents which increase intracellular cAMP levels, all trigger the release of PYY and GLP-1. This suggests that the process of isolating the crypts does not significantly disrupt intracellular signalling pathways. Moreover, the observed fold increases in gut hormone secretion were broadly in line with those reported in previous publications employing the same primary L cell model, suggesting that nutrient-stimulated responses are reproducible and that our model may therefore be considered valid.

In conclusion, these results supported the further use of this L cell model for the study of the effect of SCFAs on gut hormone release.

Chapter III:

**The effect of short chain
fatty acids on gut
hormone release**

3.1 Introduction

Fermentable fibres have been proposed to at least partly exert their satiating effects via the luminal sensing of short chain fatty acids (SCFAs), triggering the release of the anorexigenic gut hormones PYY and GLP-1 from enteroendocrine L cells (Sleeth *et al.* 2010). NDCs up-regulate expression of proglucagon and PYY in the colon and caecum, and increase plasma gut hormone levels both in rodents and humans (Cani *et al.* 2009, Delzenne *et al.* 2005, Freeland *et al.* 2010, Keenan *et al.* 2006, Zhou *et al.* 2006, Zhou *et al.* 2008). However, whether SCFAs mediate this increased gut hormone secretion remains to be established.

Investigation into the effect of SCFAs on gut hormone release began with a number of studies in the 90's using various *in vivo* models. While the results of these studies, discussed below, were inconsistent; the findings largely supported a role for SCFAs in the stimulation of gut hormone release (Anini *et al.* 1999, Cherbut *et al.* 1998, Fu-Cheng *et al.* 1995, Longo *et al.* 1991, Plaisancie *et al.* 1996).

The discovery of two SCFA receptors, GPR41 and GPR43, in 2003, and demonstration of their expression in rodent and human enteroendocrine L cells in 2006 and 2008, respectively, sparked new interest in the effect of SCFAs on gut hormone release and, crucially, in their mechanism of action, which to date remains incompletely understood (Karaki *et al.* 2006, Karaki *et al.* 2008, Tazoe *et al.* 2009).

3.1.1 The effect of SCFAs on gut hormone release

Early *in vivo/ex vivo* studies in anaesthetised animals

The first investigation into the effect of SCFAs on gut hormone release was carried out by Longo *et al.* in rabbits (Longo *et al.* 1991). Infusion of acetate and butyrate (10-100mmol/L) into the isolated, vascularly-perfused left colon led to a significant increase in PYY levels in

plasma collected from the interior mesenteric vein. This work was followed by a number of studies conducted by a group from the French National Institute for Medical Research (INSERM) examining the effect of SCFAs on gut hormone release from the rat ileum and colon, employing ileal and colonic cannulas, as well as innervated isolated intestinal loops (Anini *et al.* 1999, Cherbut *et al.* 1998, Dumoulin *et al.* 1998, Fu-Cheng *et al.* 1995, Plaisancie *et al.* 1995, 1996). Colonic administration of a mixed SCFA bolus (150mmol/L) via an ileal or colonic cannula in rats led to a significant increase in circulating plasma PYY levels (Fu-Cheng *et al.* 1995). A significant and sustained rise in plasma PYY was also reported following a one hour colonic infusion of a mixture of SCFAs (500mmol/L, 2mmol/h) in rats (Cherbut *et al.* 1998).

When administered to an isolated, vascularly-infused rat colon as a bolus, butyrate and propionate (5 and 20mmol/L, respectively) stimulated PYY secretion, but not GLP-1 (Plaisancie *et al.* 1995, 1996). This finding led the authors to speculate that SCFAs may differentially induce the release of PYY and GLP-1. Interestingly, a SCFA-triggered increase in circulating PYY without a concurrent GLP-1 response has also been reported by Anini *et al.* in the rat colon and Cuche *et al.* in the porcine ileum (Anini *et al.* 1999, Cuche *et al.* 2000). However, the reasons for this discrepancy remain unclear. Despite the findings that PYY and GLP-1 are predominantly co-localised in the mouse intestine, and that both hormones are elevated postprandially, there is precedent in the literature for conditions that favour the secretion of one hormone over the other (Nilsson *et al.* 1991). However, whether this might be due to the differential secretion of the two hormones from the same L cells, or the existence of PYY-positive but GLP-1 negative L cells remains unknown.

Recent *ex vivo* studies

Surprisingly, recent work found no effect of SCFAs on PYY and GLP-1 release from porcine ileal and colonic tissue. However, it is plausible that the concentration tested (5mmol/L) was insufficient to induce a gut hormone response from whole *ex vivo* tissue (Voortman *et al.*

2012). In contrast, Mace *et al.* demonstrated that infusion of propionate into isolated loops of rat small intestine (up to the ileo-caecal valve) dose-dependently increased both GLP-1 and PYY release from the tissue at the higher concentrations of 10-50mmol/L (Mace *et al.* 2012).

Recent *in vivo* studies in non-anaesthetised rodents

Animal studies examining the effect of SCFAs on gut hormone release and appetite have largely relied upon dietary supplementation with the sodium salts of SCFAs. Vidrine *et al.* demonstrated that resistant starch (28%) but not sodium butyrate (3.2%) in the diet increased plasma GLP-1 and PYY levels in Sprague Dawley rats over a period of 12 weeks (Vidrine *et al.* 2013). In a further study, which attempted to determine the effect of SCFAs on the development of diet-induced obesity, C57BL6 mice were put on a HFD supplemented with sodium salts of butyrate (5%w/w), propionate (4.3%) or acetate (3.7%) for 4 weeks. At the end of the study, propionate and butyrate-fed mice had gained no weight and the body weight gain in the acetate group was suppressed by 40%, compared to the control group which gained >10g (Lin *et al.* 2012). However, due to the volatile nature of SCFAs, their presence in the diet gives it a distinctive odour and taste, and has a negative impact on palatability (Darzi *et al.* 2011). Therefore, it is impossible to determine whether the reduced food intake is a direct result of the putative anorexigenic effect of SCFAs or an indirect consequence of poor palatability.

In the same study, oral administration of sodium butyrate was found to acutely increase plasma GLP-1, GIP and PYY at 10 minutes, and sodium propionate significantly increased GIP. However, while it is plausible that the SCFAs reached the proximal small intestine within 10 minutes, as gastric emptying of a liquid bolus in mice is rapid and can exceed 80% within 15 minutes (Firpo *et al.* 2005), it is highly unlikely that the SCFA boluses reached the distal small intestine or colon. Nevertheless, it is possible that SCFAs stimulated gut hormone release from proximal small intestinal L cells, though such cells are relatively

sparse. This highlights another limitation of supplementing the diet with SCFAs; following oral delivery, SCFAs are absorbed in the small intestine (Schmitt *et al.* 1976, Schmitt *et al.* 1977) before reaching the large intestine, the site containing the largest density of L cells.

Human studies

Evidence for an effect of colonic SCFAs on gut hormone release from human studies is very limited. Two small studies (n=6-7) have investigated the effect of rectal infusion of SCFAs on gut hormone release in humans. Ropert *et al.* observed no change in plasma gut hormone levels of GLP-1 or oxyntomodulin following a 180ml SCFA infusion (300 or 500 mmol/L) over one hour into the proximal colon (3ml/min) (Ropert *et al.* 1996). Furthermore, while plasma PYY concentrations rose significantly over time, there was no significant difference compared to the saline infusion.

Freeland and Wolever conducted a small single-blind randomised cross-over study in hyperinsulinaemic overweight women to determine the effect of a 300ml rectal infusion of sodium acetate (200mmol/L) vs. saline over 8 minutes on plasma GLP-1 and PYY levels (Freeland & Wolever 2010). The authors reported a slight increase in plasma GLP-1 following administration of both sodium acetate and saline, suggesting a non-specific effect of rectal distension. However, in contrast to the findings of the previous study, infusion of acetate into the rectum elicited a gradual and sustained release of PYY which was significantly greater than the transient rise in PYY in response to mechanical distension. Based on this limited data, it is difficult at present to draw any conclusions regarding the utility of targeting SCFA pathways to trigger gut hormone release in humans. Colonic or rectal infusion studies in humans are fraught with challenges. A human primary L cell model would, therefore, be valuable as an intermediate step.

Recent *in vitro* investigations

Following renewed interest in the role of gut microbiota and SCFA receptors in glucose and energy homeostasis, a number of recent studies have investigated the effect of SCFAs on gut hormone release from both enteroendocrine cell lines and primary murine L cells. *In vitro* investigations using the murine STC-1 cell line, which has been demonstrated to express both SCFA receptors (Hudson *et al.* 2013), suggest that while propionate has no effect on GLP-1 or PYY release at the relatively low concentration of 100 μ mol/L (Hand *et al.* 2013, Hudson *et al.* 2013), it significantly stimulates GLP-1 release from these cells at 1 and 10mmol/L (Hudson *et al.* 2013). The findings of the limited studies that have been conducted using primary colonic L cells are inconsistent. While acetate and propionate have previously been shown to significantly increase GLP-1 release from murine primary L cells (Nohr *et al.* 2013, Tolhurst *et al.* 2012a), another recent study using an alternative digestion method was unable to show an increase in GLP-1 release from primary colonic tissue in response to propionate (0.8, 8, 24 and 48 mmol/L) and only found a significant effect with acetate at concentrations of 80 and 160mmol/L (Li *et al.* 2013).

In conclusion, there are discrepancies in the findings of studies investigating the effects of SCFAs on PYY and GLP-1 release both *in vitro* and *in vivo*. Furthermore, there is a lack of data regarding the effect of SCFAs on PYY secretion *in vitro* presumably because there are no validated PYY-secreting cell lines and work using primary L cells has focused mainly on GLP-1. In light of the differential release of GLP-1 and PYY observed under certain conditions in response to SCFAs *in vivo*, measurement of both gut hormones in parallel from primary L cells would yield valuable information. Moreover, it is important to determine the effect of SCFAs on GLP-1 and PYY release from human primary L cells, to confirm that the effects observed in rodents are likely to be applicable to humans. Lastly, despite the fact that the majority of *in vitro* work has been carried out in murine primary L cells and mouse-derived cell lines, the effect of colonic administration of SCFAs on plasma gut hormone levels in mice has not been investigated.

3.2 Aims and Hypotheses

3.2.1 Aim

To determine the effect of SCFAs on PYY and GLP-1 release from murine and human primary colonic L cells *in vitro*, and on plasma levels of PYY and GLP-1 following intra-colonic administration in rats and mice.

Objectives:

1. To determine the effect of SCFAs on PYY and GLP-1 release from murine and human primary L cells.
2. To determine the effects of propionate on cytotoxicity and the effects of osmolarity/sodium concentration on gut hormone release.
3. To develop an *in vivo* model for simultaneously sampling from the portal vein and jugular vein following intra-colonic administration of a test substance.
4. To determine the effect of intra-colonic administration of propionate on portal and systemic PYY and GLP-1 concentrations in rats.
5. To determine the effect of intra-colonic administration of propionate on portal vein plasma PYY and GLP-1 concentrations in mice.

3.2.2 Hypotheses

- i) SCFAs will stimulate the release of both GLP-1 and PYY from murine and human primary L cells.
- ii) The stimulatory effect of SCFAs on gut hormone release will be independent of any osmotic or cytotoxic effects.
- iii) Intra-colonic administration of propionate will increase portal and circulating plasma gut hormone levels *in vivo* in both rodent models.

3.3 Methods

3.3.1 *In vitro* methods

Primary cell culture: *Refer to Chapter II, Section 2.3.1*

Radioimmunoassay: *Refer to Chapter II, Section 2.3.2*

3.3.1.1 Lactate dehydrogenase (LDH) cytotoxicity assay

Materials

Cytoscan™ LDH cytotoxicity assay (Cat. #786-210, G-Biosciences, MO, USA)

Free fatty acid-free bovine serum albumin (BSA) (Sigma, UK)

Phosphate-buffered saline (PBS) 1× (Refer to Appendix I)

Glass distilled water (GDW)

Principle

The cytotoxicity of cell culture test reagents was measured using an LDH cytotoxicity assay. The assay quantitatively measures the stable cytosolic enzyme, lactate dehydrogenase (LDH), which is released upon cell lysis. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt, iodonitrotetrazolium (INT) into a red coloured agent, formazan (Fig. 3.1). The LDH activity is determined as NADH oxidation or INT reduction over a defined time period. The resulting formazan absorbs maximally at 492nm.

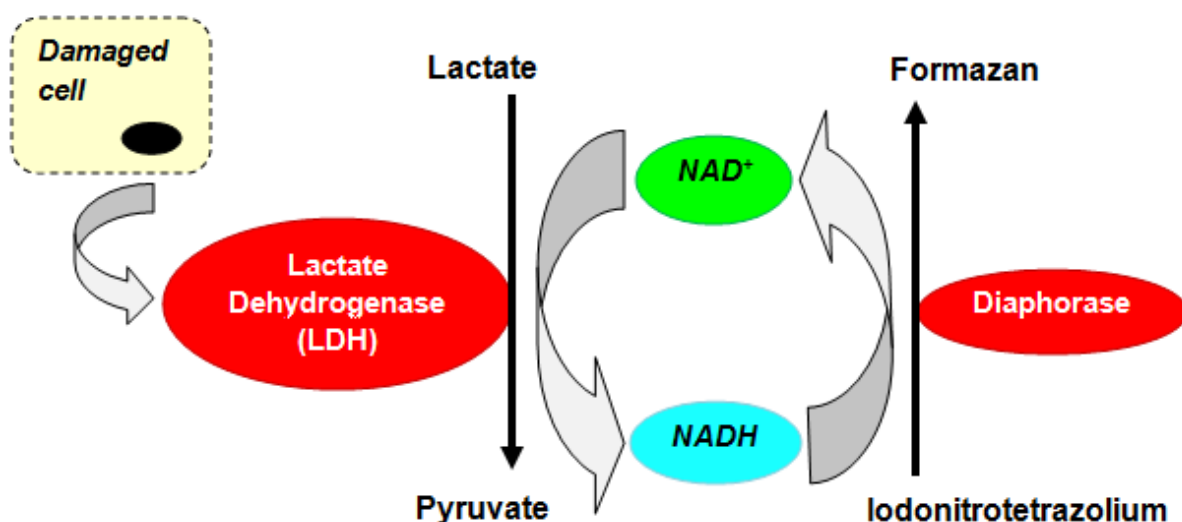


Figure 3.1. The principle of the lactate dehydrogenase (LDH) cytotoxicity assay. *NAD⁺/NADH*, nicotinamide adenine dinucleotide oxidised/reduced form

Preparation of reagents

The positive control was prepared according to the manufacturer's instructions; 1µl LDH (supplied) was added to 10ml 0.1% PBS (1x) supplemented with 1% free-fatty acid free BSA. The substrate mix was made up by dissolving one vial in 11.4ml GDW and mixing gently. The light-sensitive assay buffer (0.6ml) was allowed to warm to room temperature and was then added to the reconstituted substrate mix and was mixed thoroughly.

Experimental procedure

Murine or human primary colonic cultures were incubated with test reagents for a period of 2-4 hours to assess the cytotoxicity induced during the secretion experiments. The cytotoxicity incurred by the treatments was normalised to maximum LDH release obtained from lysed wells. Maximum LDH release was determined as the mean LDH levels in the

supernatants of 6 wells per experiment set aside as lysis wells. These wells were washed with secretion buffer three times along with all treatment wells before incubation. One hour prior to the end of the incubation period, 20% (60µl) lysis buffer (supplied) was added to each well and returned to the incubator for a further hour. At the end of the incubation period, the plates were centrifuged at 270 x g for 10 minutes.

All controls, untreated and treated cell samples, and lysed cell samples (maximum LDH) were assayed in triplicate in a 96-well plate format. Controls within the assay included: secretion buffer alone as a negative control, the spontaneous LDH release control (supernatants from cells incubated with secretion buffer only) and the LDH positive control.

A total of 50µl of control solution, supernatant or cell lysate was added per well, in triplicate. This was superseded by a 50µl addition of reconstituted substrate mix. The plates were then wrapped in foil and incubated at 37°C for 20 minutes. Following the addition of 50µl stop solution per well, absorbance was recorded at 490nm using a micro-plate reader (BioTek Model No. ELx808, VT, USA).

Percentage cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental } OD_{490} - \text{Spontaneous } OD_{490}}{\text{Maximum LDH release } OD_{490}} \times 100$$

(OD, optical density)

3.3.2 *In vivo* methods

3.3.2.1 Animals and housing

All animal procedures undertaken were carried out under the British Home Office Animals Scientific Procedures Act 1986 (Project Licence numbers: 70/7236 and 70/7596). On arrival, male Wistar rats (Charles River, UK) or male C57BL6 mice (Harlan Laboratories, UK) were housed in pairs and maintained at 21-23°C on a 12-hour light, 12-hour dark cycle (light period 07:00-19:00). During the 72-hour acclimatisation period, all rodents were given *ad libitum* access to water and RM1 standard chow (RM1 diet, Special Diet Services Ltd., Witham, Essex).

3.3.2.2 Rat intra-colonic administration study

Male Wistar rats (200-250g) were fasted overnight and anaesthetised under isoflurane. The animals were kept on a heat pad and pedal reflexes were monitored throughout the procedure. Following confirmation of anaesthesia, a jugular vein cannulation was performed [PORTEX fine bore polythene tubing, 0.28mm inner diameter and 0.61mm outer diameter (Smiths Medical ASD, USA)]. A laparotomy was then carried out to gain access to the abdominal cavity; the caecum was located and the portal vein exposed. The abdominal cavity was covered with a swab containing warm saline. Ten minutes later, the first baseline jugular vein blood sample was collected ($t = -15\text{min}$, Fig. 3.2). A second baseline sample was collected at 0min, after which 2.5ml of either saline control or propionic acid (180mmol/L, pH 5.5 using NaOH) was injected into the rat proximal colon distal to the caecum. At $t = 15\text{min}$, a portal vein sample and a jugular vein sample were collected. The last two jugular vein samples were collected at $t = 30$ and $t = 60\text{min}$.

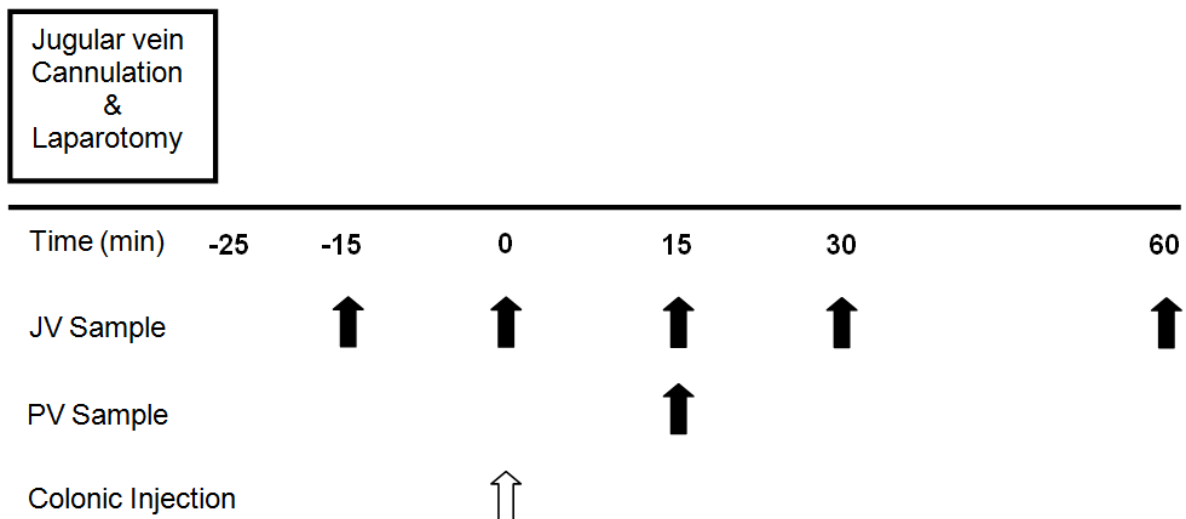


Figure 3.2. **Rat intra-colonic study design: colonic injection and blood sampling time points.** *JV, jugular vein; PV, portal vein*

Blood samples were collected into eppendorfs containing DPPIV inhibitor (Millipore, 1µl/100µl blood, 100µmol/L final concentration) and protease inhibitor cocktail (Sigma, 1µl/100µl blood, refer to Appendix II), and centrifuged for 10 minutes at 3000 x g for separation of plasma. Plasma was collected into a fresh eppendorf and placed on dry ice. Samples were stored at -20°C pending gut hormone analysis by RIA, as described previously.

3.3.2.3 Mouse intra-colonic administration studies

Male C57BL6 mice (13 weeks of age, 25-28g) were fasted overnight and anaesthetised under isoflurane. Pedal reflexes were monitored throughout. A laparotomy was performed and the colon and portal vein were located and exposed. The abdominal cavity was covered with a swab containing warm saline, as above. Ten minutes later, the mice received an intra-colonic injection (300µl) of either saline control or propionic acid (180mmol/L, pH 5.5). Portal vein blood was collected 15 minutes post-injection. Plasma was separated and stored as above. Plasma concentrations of PYY and GLP-1 were measured by RIA.

3.3.3 Statistical Analysis

Normality was assessed using the D'Agostino-Pearson omnibus test where $n \geq 8$ per group. Statistical significance of normally distributed data was calculated using either a t-test (2 groups) or one-way ANOVA (≥ 3 groups). ANOVA pairwise comparisons were carried out using either the Dunnett or Bonferroni post hoc tests. The statistical significance of data that were not normally distributed (AUC data for the rat study) was determined using the Mann-Whitney U test for non-parametric data. Statistical significance was accepted at $P < 0.05$ throughout. Data are presented as mean $\pm$ standard error of the mean (SEM). Analysis was carried out using Graph Pad Prism software, version 5.0.

3.4 Results

3.4.1 The effect of SCFAs on gut hormone release *in vitro*

3.4.1.1 The effect of acetate, butyrate and propionate on PYY and GLP-1 release from murine primary L cells

Following validation of the primary L cell model described in Chapter II, the first aim was to determine the effect of a 2-hour incubation of murine primary colonic cells with varying concentrations of the SCFAs acetate (C2), propionate (C3) and butyrate (C4) on PYY and GLP-1 release.

Acetate

As shown in Figure 3.3, PYY and GLP-1 release from murine primary L cells was significantly increased in response to treatment with acetate. Basal PYY and GLP-1 secretion over the 2-hour incubation period was $4.2 \pm 0.4\%$ and $1.8 \pm 0.2\%$, respectively. The secretion of both hormones was significantly elevated at all three concentrations tested. PYY secretion increased ~3-fold to $13.4 \pm 0.7\%$, $13.2 \pm 1.9\%$ and $11.3 \pm 0.6\%$ with 50, 100 and 200mmol/L acetate (all, $P < 0.001$), respectively (Fig. 3.3A). Furthermore, GLP-1 secretion rose to $6.0 \pm 0.6\%$ ($P < 0.001$), $5.9 \pm 0.5\%$ ($P < 0.001$) and $3.7 \pm 0.3\%$ ($P < 0.05$) following stimulation with 50, 100 and 200mmol/L acetate, respectively (Fig. 3.3B). While still significant, 200mmol/L acetate only led to a 2-fold increase in GLP-1 release compared to the 3-fold increase observed with the lower doses.

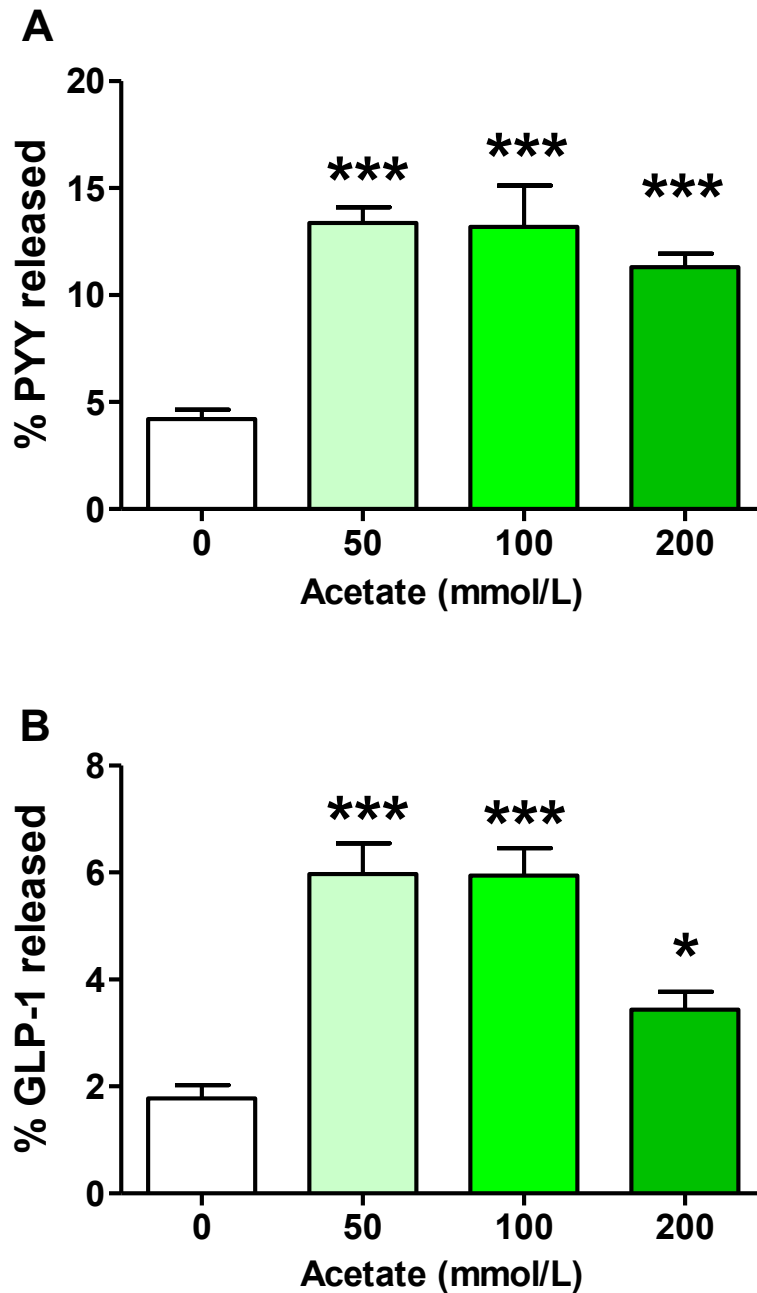


Figure 3.3 The effect of acetate on gut hormone secretion from murine primary L cells. Mixed primary colonic cultures were incubated in secretion buffer containing vehicle alone or sodium acetate (50, 100 or 200mmol/L). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=2 mice, 7-9 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Dunnett post hoc test (*P<0.05, ***P<0.001).

Butyrate

Butyrate significantly increased PYY and GLP-1 secretion from murine primary L cells (Fig. 3.4). Basal PYY and GLP-1 secretion over the 2-hour incubation period was $4.2 \pm 0.4\%$ and $1.8 \pm 0.2\%$, respectively. Interestingly, while 50mmol/L butyrate significantly increased GLP-1 release ($P < 0.05$), the higher dose of 200mmol/L was required to significantly stimulate PYY release ($P < 0.001$). However, it is worth noting that the magnitude of the increase in secretion of both PYY and GLP-1 in response to 50mmol/L butyrate was the same, approximately 2-fold (Fig. 3.4A and B). Furthermore, whereas the PYY response appeared to be dose-dependent, there was no evidence of a dose-dependent effect on the GLP-1 response, which did not increase in magnitude with concentrations above 50mmol/L.

Propionate

As displayed in Figure 3.5, treatment of primary murine L cells with propionate significantly increased PYY and GLP-1 release. Basal PYY and GLP-1 secretion over the 2-hour incubation period was $3.6 \pm 0.2\%$ and $3.6 \pm 0.5\%$, respectively. Following incubation with 25, 50, 100 and 200 mmol/L propionate, PYY secretion increased ~2-fold, to $6.7 \pm 0.7\%$, $7.5 \pm 0.6\%$ ($P < 0.001$), $7.8 \pm 0.6\%$ ($P < 0.001$) and $8.2 \pm 0.9\%$ ($P < 0.001$), respectively (Fig. 3.5A). Similarly, GLP-1 increased between 2- and 3-fold in response to increasing propionate concentrations. GLP-1 release was significantly increased following stimulation with 25 ($P < 0.05$), 50 ($P < 0.05$) and 200mmol/L propionate ($P < 0.01$) (Fig. 3.5B).

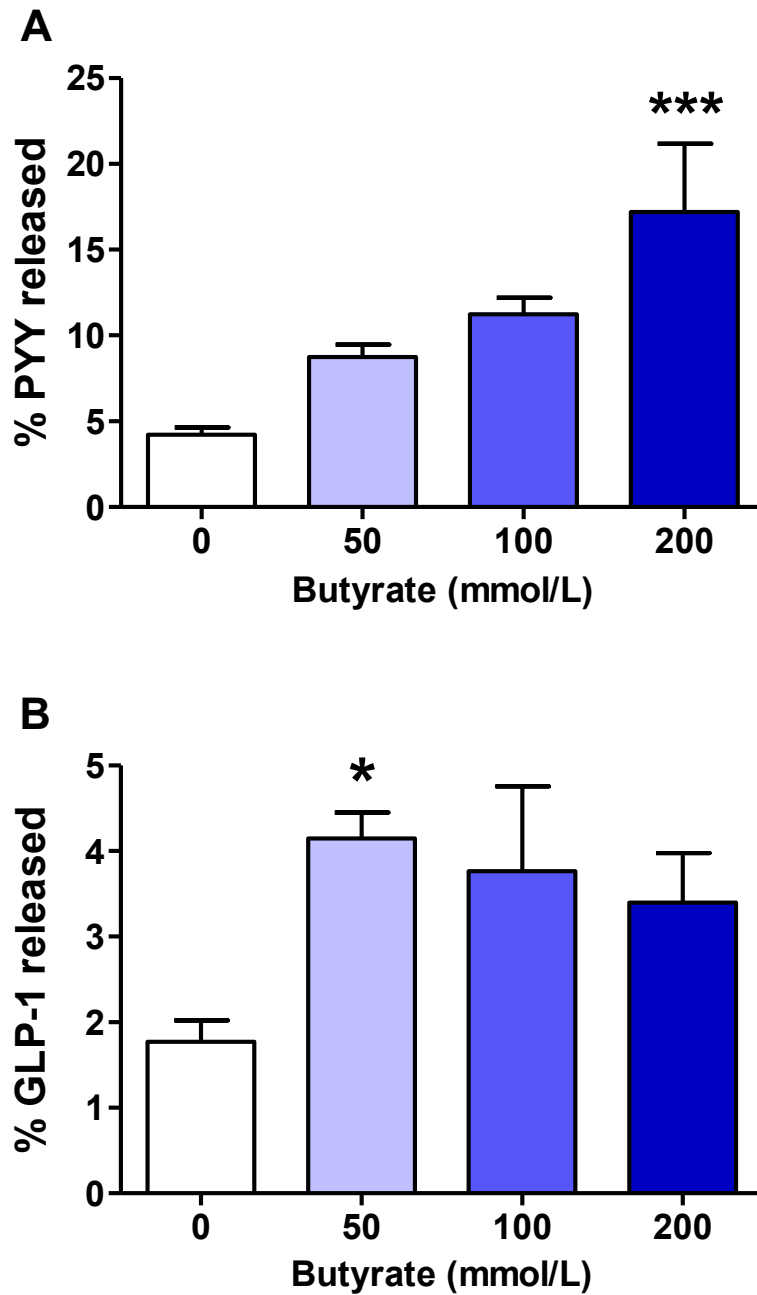


Figure 3.4. **The effect of butyrate on gut hormone secretion from murine primary L cells.** Mixed primary colonic cultures were incubated in secretion buffer containing vehicle alone or sodium butyrate (50, 100 or 200mmol/L). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=2 mice, 5-9 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Dunnett post hoc test (* P <0.05, *** P <0.001).

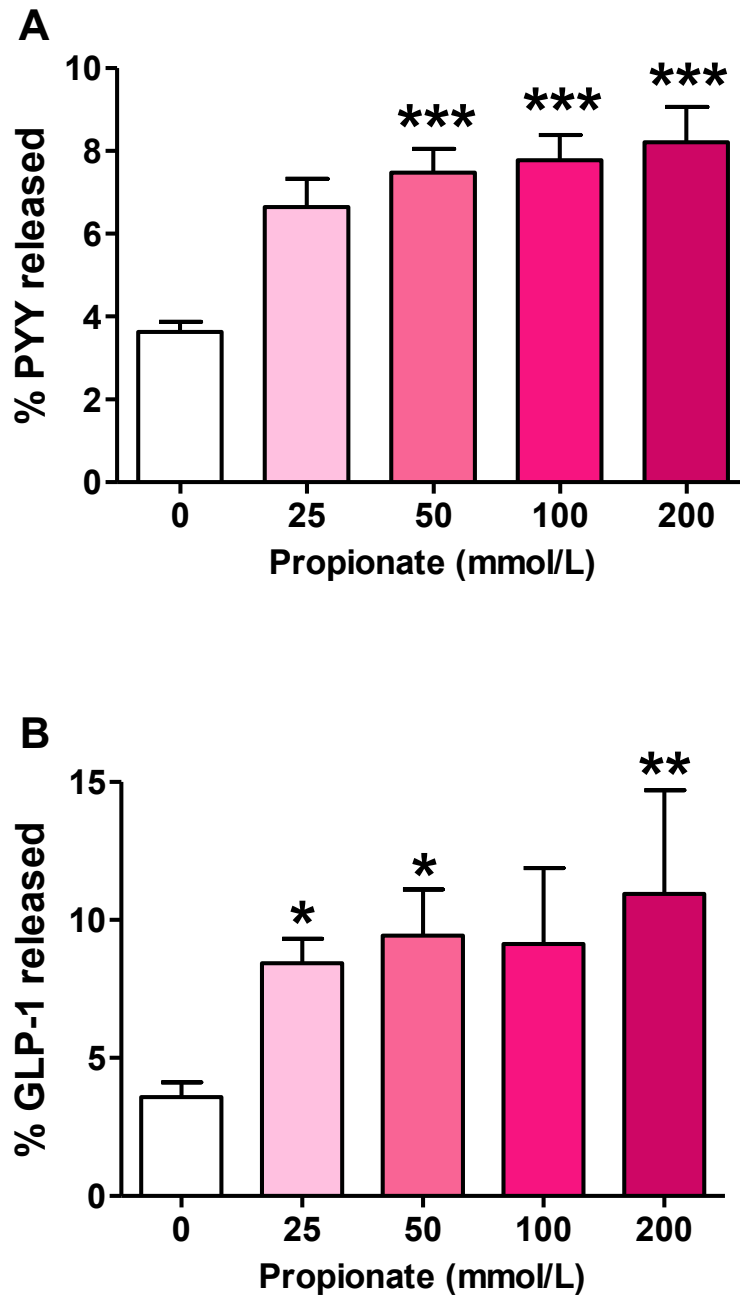


Figure 3.5. **The effect of propionate on gut hormone secretion from murine primary L cells.** Mixed primary colonic cultures were incubated in secretion buffer containing vehicle alone or sodium propionate (25, 50, 100 or 200mmol/L). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (2 separate experiments, n=2 mice, 5-19 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Dunnett post hoc test (* P <0.05, ** P <0.01, *** P <0.001).

3.4.1.2 The effect of acetate, butyrate and propionate on PYY and GLP-1 release from human primary L cells

Using the human L cell model described in Chapter II, the effect of SCFAs on the release of gut hormones from human colonic tissue was determined for the first time.

Acetate and butyrate significantly stimulated the release of PYY from human primary L cells (Fig. 3.6). PYY release was increased following incubation with both acetate and butyrate at a concentration of 200mmol/L, 1.8-fold ($3.6 \pm 1.3\%$ to $6.5 \pm 1.6\%$) and 1.7-fold ($7.3 \pm 1.1\%$ to $12.6 \pm 1.0\%$), respectively. However, the increase in PYY secretion was only statistically significant at the higher dose of 400mmol/L (acetate, 2.9-fold increase, $P < 0.05$; butyrate, 2-fold increase, $P < 0.05$). With regard to PYY release following butyrate treatment, it is possible that the results were affected by the relatively high basal release measured in that experiment.

Propionate significantly increased PYY and GLP-1 secretion from human primary L cells (Fig. 3.7). As with acetate and butyrate, GLP-1 secretion was elevated in response to 200mmol/L propionate (%GLP-1, $2.8 \pm 0.3\%$ to $5.2 \pm 0.9\%$, 1.9-fold increase) and PYY increased significantly (%PYY, 3.3 ± 0.9 to $8.1 \pm 1.0\%$, 2.4-fold increase, $P < 0.01$). Following incubation with 400mmol/L propionate, PYY and GLP-1 release were both significantly increased by 4-fold ($4.1 \pm 0.5\%$ to $13.8 \pm 1.3\%$, $P < 0.001$) and 2.9-fold ($2.8 \pm 0.3\%$ to $8.1 \pm 1.1\%$, $P < 0.001$), respectively.

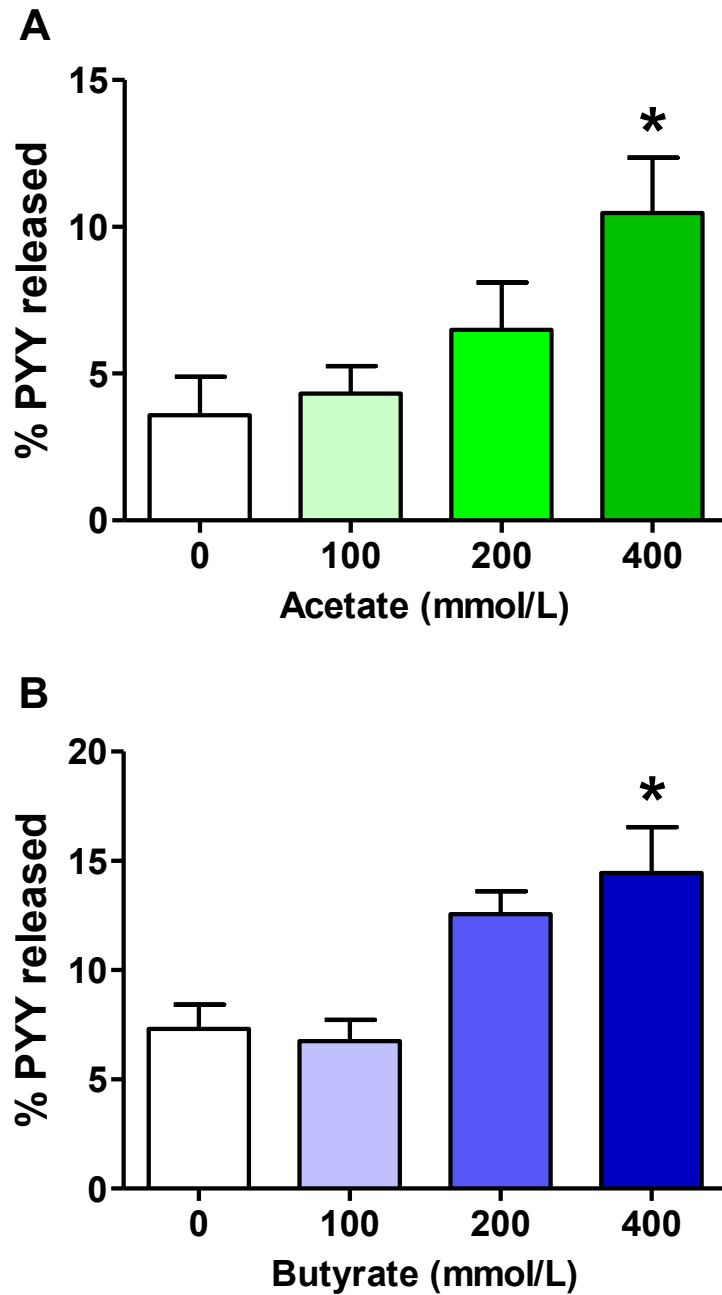


Figure 3.6. **The effect of acetate and butyrate on PYY secretion from human primary L cells.** Mixed primary colonic cultures derived from healthy human colonic biopsies were incubated in secretion buffer containing vehicle alone, sodium acetate (100, 200, 400mmol/L) or sodium butyrate (100, 200, 400mmol/L). PYY secretion in each well is expressed as a percentage of total PYY contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=2 patients' biopsies, 4-5 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Dunnett post hoc test (* $P < 0.05$).

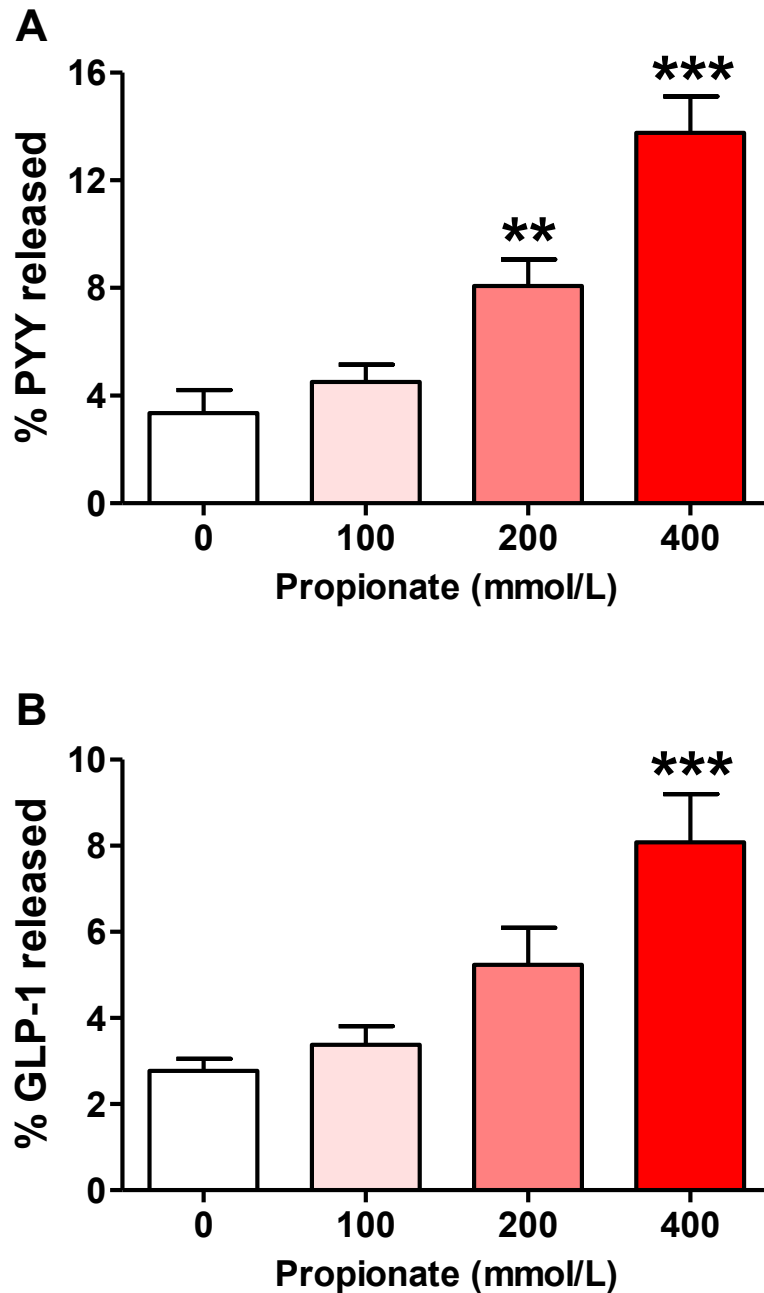


Figure 3.7. The effect of propionate on gut hormone secretion from human primary L cells. Mixed primary colonic cultures were incubated in secretion buffer containing vehicle alone or sodium propionate (100, 200, 400mmol/L). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (PYY, n=3 patients' biopsies, 3-13 wells per group; GLP-1, n=2 patients' biopsies, 3-6 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Dunnett post hoc test (**P<0.01, ***P<0.001).

3.4.1.3 The effect of propionate on cytotoxicity: LDH assay results

To confirm that the increased gut hormone release following treatment with propionate was not secondary to increased cell death, the effect of propionate on cell health was determined using a lactate dehydrogenase (LDH) cytotoxicity assay. LDH release following propionate treatment was compared to spontaneous LDH release over the same time period from cells incubated with secretion buffer alone, and was expressed as a percentage of mean maximum LDH release from lysed wells.

The results of the LDH assay suggested that incubation with 200mmol/L propionate over a 2- or 4-hour period had no significant impact on cytotoxicity in either human or murine colonic cells (Fig. 3.8).

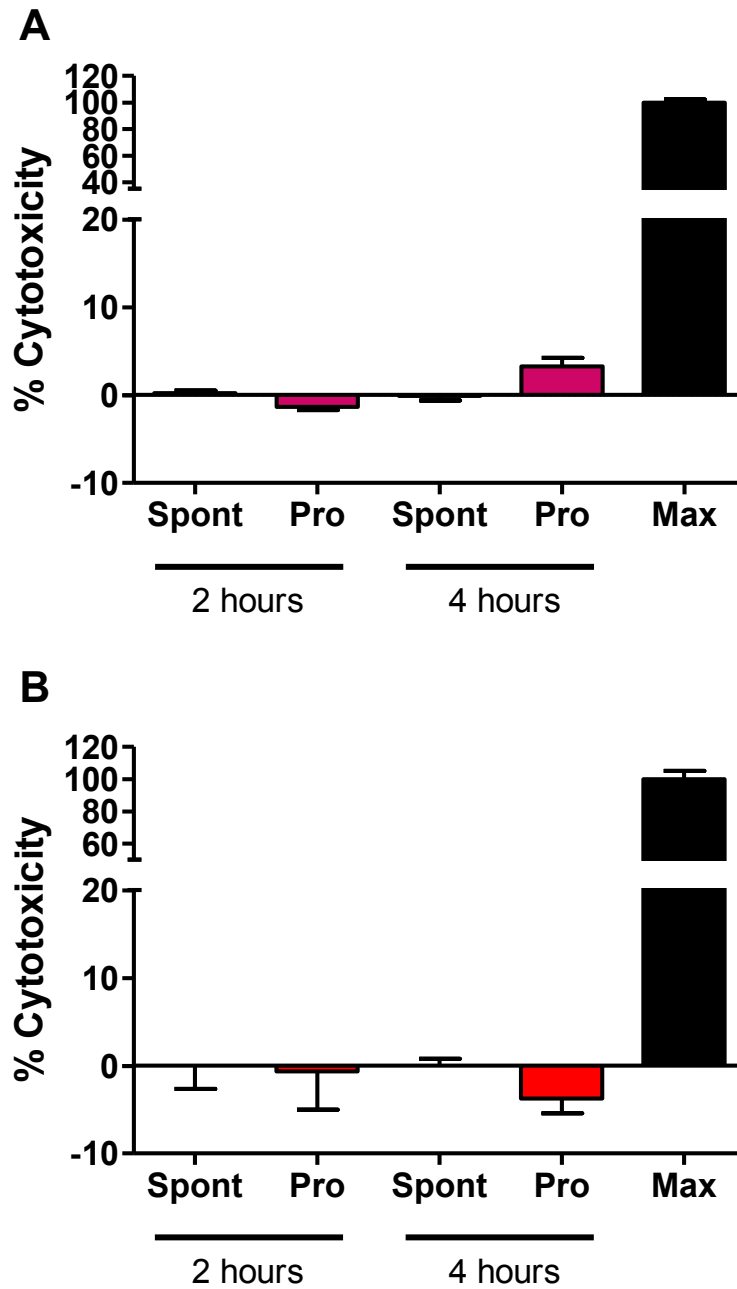


Figure 3.8. **The effect of propionate on cytotoxicity in human and murine primary colonic cultures.** Mixed murine (A) or human (B) primary colonic cultures were incubated over a 2- or 4-hour period in secretion buffer containing 200mmol/L propionate (Pro). Cytotoxicity is expressed as a percentage of mean maximum cytotoxicity from lysed wells (Max), and relative to spontaneous cytotoxicity (Spont) measured in parallel within the same experiment. Data represent means $\pm$ SEM (human, 3 patients' biopsies, n=9 wells and mouse, 2 colons, n=11 wells per group).

3.4.1.4 The effect of propionate vs. sodium chloride on gut hormone release

As all SCFAs tested as part of this work were sodium salts, it was necessary to demonstrate that propionate had a stimulatory effect on gut hormone release beyond any effects of osmolality or sodium ion content. This was achieved by comparing the effect of different concentrations of propionate with equivalent concentrations of sodium chloride, on gut hormone release from human and murine primary L cells.

Murine L cells

As shown in Figure 3.9, there was a significant effect of osmolality on gut hormone release beyond 100mmol/L. Incubation with 200mmol/L NaCl significantly increased both PYY ($P<0.001$) and GLP-1 ($P<0.05$) release above basal levels. Furthermore, at that concentration, there was no difference between the gut hormone responses elicited by NaCl and propionate. However, at concentrations of 100mmol/L and below, the effect of osmolality on gut hormone release is minimal, and there is a significant 2-fold difference in response between NaCl and propionate at 50mmol/L (PYY, $3.7\pm0.3\%$ vs. $7.5\pm0.6\%$, $P<0.001$, and GLP-1, $4.4\pm0.8\%$ vs. $9.4\pm1.7\%$, $P<0.05$) and 100mmol/L (PYY, $4.8\pm0.5\%$ vs. $7.8\pm0.6\%$, $P<0.05$).

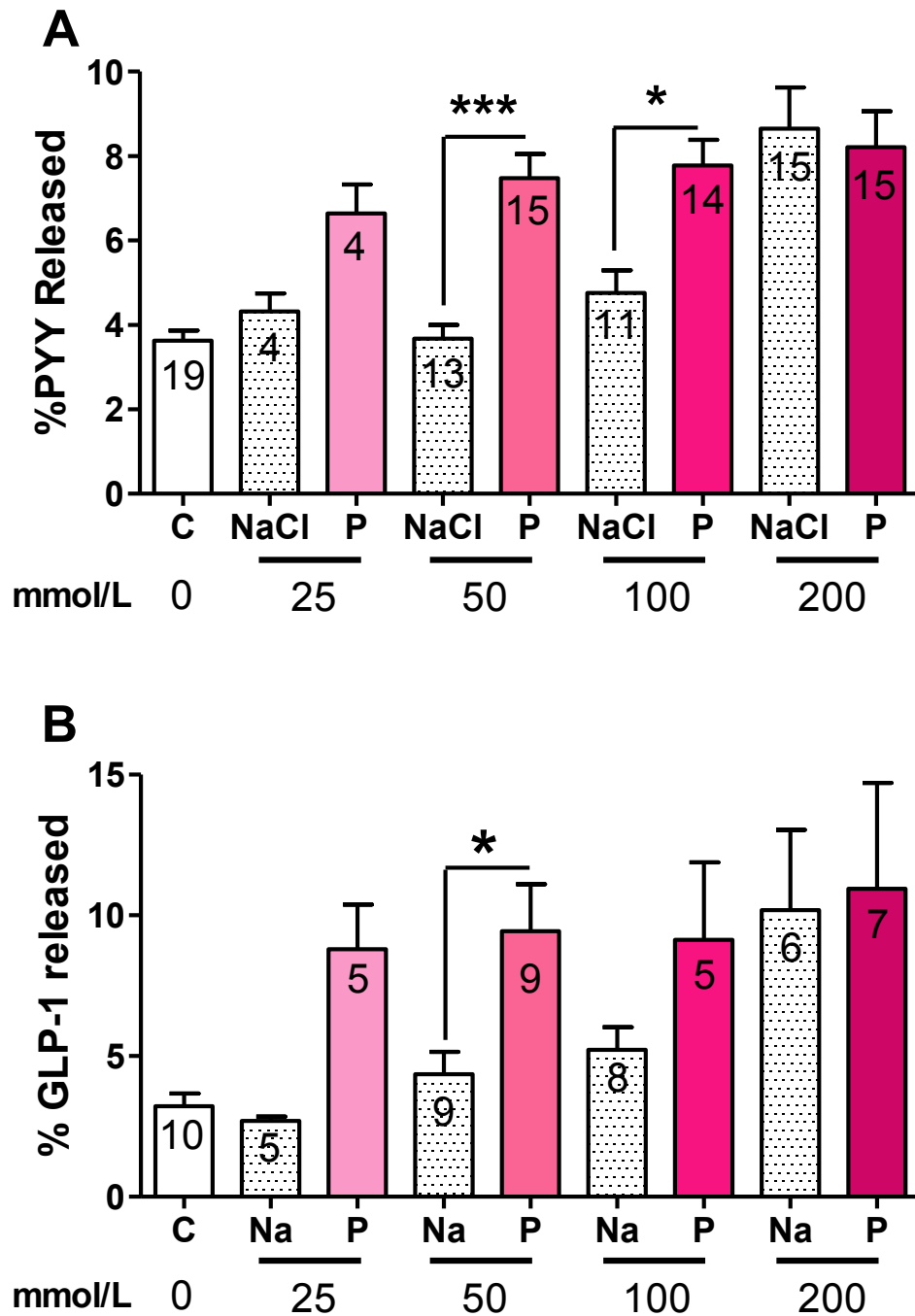


Figure 3.9. The effect of sodium chloride vs. propionate on PYY and GLP-1 secretion from primary murine L cells. Mixed primary colonic cultures were incubated in secretion buffer containing varying concentrations of either sodium propionate (P) or sodium chloride (NaCl). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion (C) measured in parallel within the same experiment. Data represent means $\pm$ SEM (2-3 separate experiments, $n=4$ mice, total wells per group displayed within columns). Significance is shown relative to basal secretion using one-way ANOVA with a Bonferroni post hoc test (* $P<0.05$; *** $P<0.001$).

Human L cells

Due to the limited amount of human tissue available, the effect of sodium chloride vs. propionate was only tested at 200mmol/L. As previously demonstrated, propionate significantly stimulated PYY release from human primary L cells (Fig. 3.10). NaCl at this concentration in human tissue did not significantly increase PYY secretion. Furthermore, there was a significant ~2-fold increase in PYY release with propionate, beyond that induced by NaCl ($8.4 \pm 1.4\%$ vs. $4.7 \pm 0.9\%$, respectively).

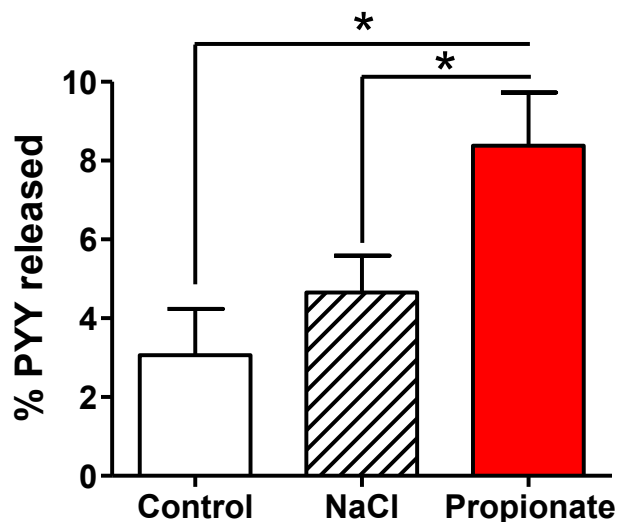


Figure 3.10. **The effect of sodium chloride vs. propionate on PYY secretion from human primary L cells.** Mixed primary colonic cultures were incubated in secretion buffer containing 200mmol/L sodium chloride (NaCl) or propionate. PYY secretion in each well is expressed as a percentage of total PYY contained within the well and compared to the basal secretion (control) measured in parallel within the same experiment. Data represent means $\pm$ SEM (2 separate experiments, n=4 patients' biopsies, 7-9 wells per group). Significance was determined using One-way ANOVA with a Bonferroni post hoc test (* $P < 0.05$, ** $P < 0.01$).

3.4.1.5 The effect of low concentrations of propionate on gut hormone release

Due to the saturated gut hormone response observed with concentrations above 50mmol/L propionate, the effect of lower concentrations of propionate was determined.

Propionate, even at 1 and 5mmol/L, significantly stimulated both PYY and GLP-1 release from murine primary L cells (Fig. 3.11). Following incubation with 1 and 5mmol/L propionate, PYY secretion increased from $4.3 \pm 0.5\%$ basal to $7.8 \pm 0.8\%$ (1.8-fold, $P < 0.05$) and $8.2 \pm 0.7\%$ (1.9-fold, $P < 0.01$), respectively. Furthermore, 1 and 5mmol/L propionate led to a GLP-1 increase from $3.3 \pm 0.3\%$ basal to $5.6 \pm 0.5\%$ (1.7-fold, $P < 0.05$) and $6.3 \pm 0.5\%$ (1.9-fold, $P < 0.01$), respectively.

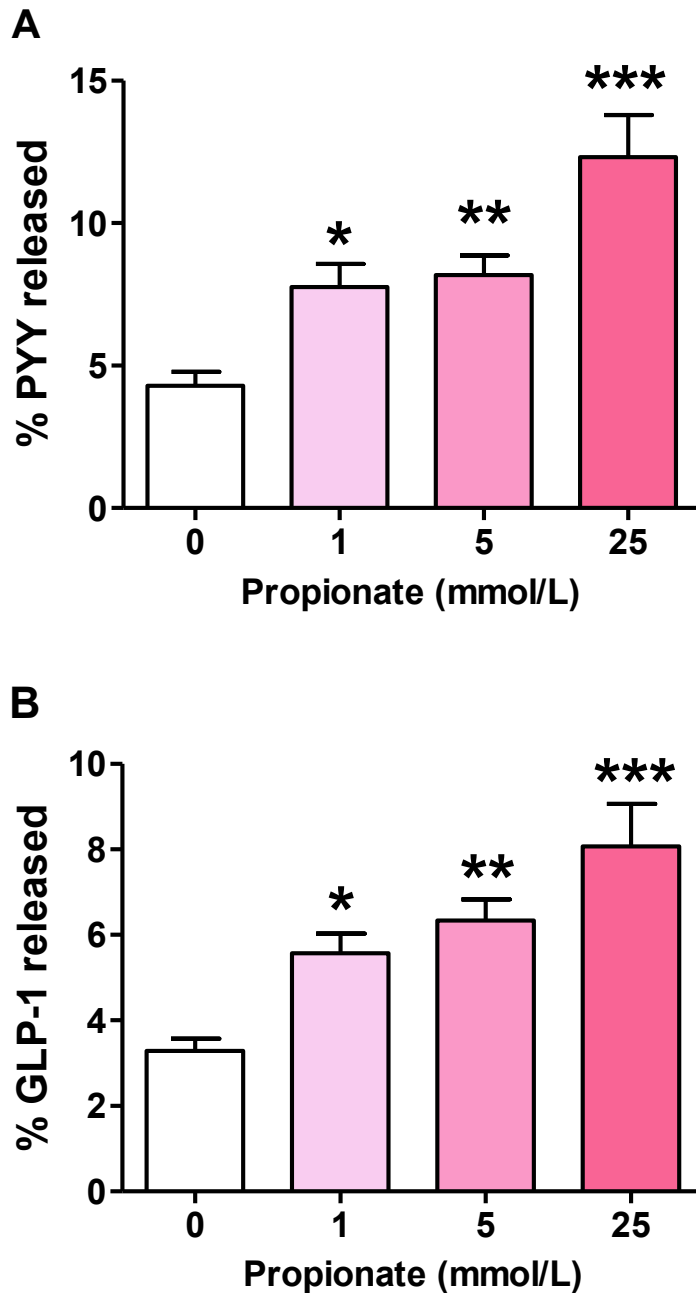


Figure 3.11. **The effect of low doses of propionate on gut hormone secretion from murine primary L cells.** Mixed primary colonic cultures were incubated in secretion buffer containing increasing concentrations of sodium propionate. PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=1 mouse, 5-7 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Dunnett post hoc test (* P <0.05, ** P <0.01, *** P <0.001).

3.4.1.6 The effect of chronic prior exposure to low doses of propionate on acute propionate-stimulated gut hormone secretion

Primary murine colonic cultures were incubated over 24 hours in media with or without a low concentration of 1mmol/L propionate, to minimise the risk of cytotoxicity, and were then stimulated acutely with 50mmol/L propionate to determine whether chronic exposure to propionate impairs subsequent gut hormone release. Following 24-hour exposure to propionate, the L cells retained their ability to respond to propionate by secreting both PYY and GLP-1 (Fig. 3.12).

The absolute values of both basal and stimulated PYY and GLP-1 measured in the supernatants were reduced following the 24-hour exposure to propionate (Fig. 3.12B+D). Absolute basal PYY and GLP-1 release was 1.9-fold lower following long term propionate exposure and stimulated release of PYY and GLP-1 was 1.4- and 1.3-fold lower, respectively. However, these differences were not statistically significant.

Furthermore, both %PYY and %GLP-1 release, basal and stimulated, were practically identical irrespective of prior propionate exposure (Fig. 3.12A+C). %PYY rose from $4.4 \pm 0.3\%$ to $13.3 \pm 1.9\%$ ($P < 0.001$) and $4.0 \pm 0.5\%$ to $13.0 \pm 1.2\%$ ($P < 0.001$) with acute stimulation, with or without prior exposure to propionate, respectively. Similarly, %GLP-1 increased from $2.0 \pm 0.3\%$ to $4.7 \pm 0.7\%$ ($P < 0.001$) and $2.7 \pm 0.2\%$ to $5.4 \pm 0.6\%$ ($P < 0.001$) with acute stimulation, with or without prior exposure to propionate, respectively.

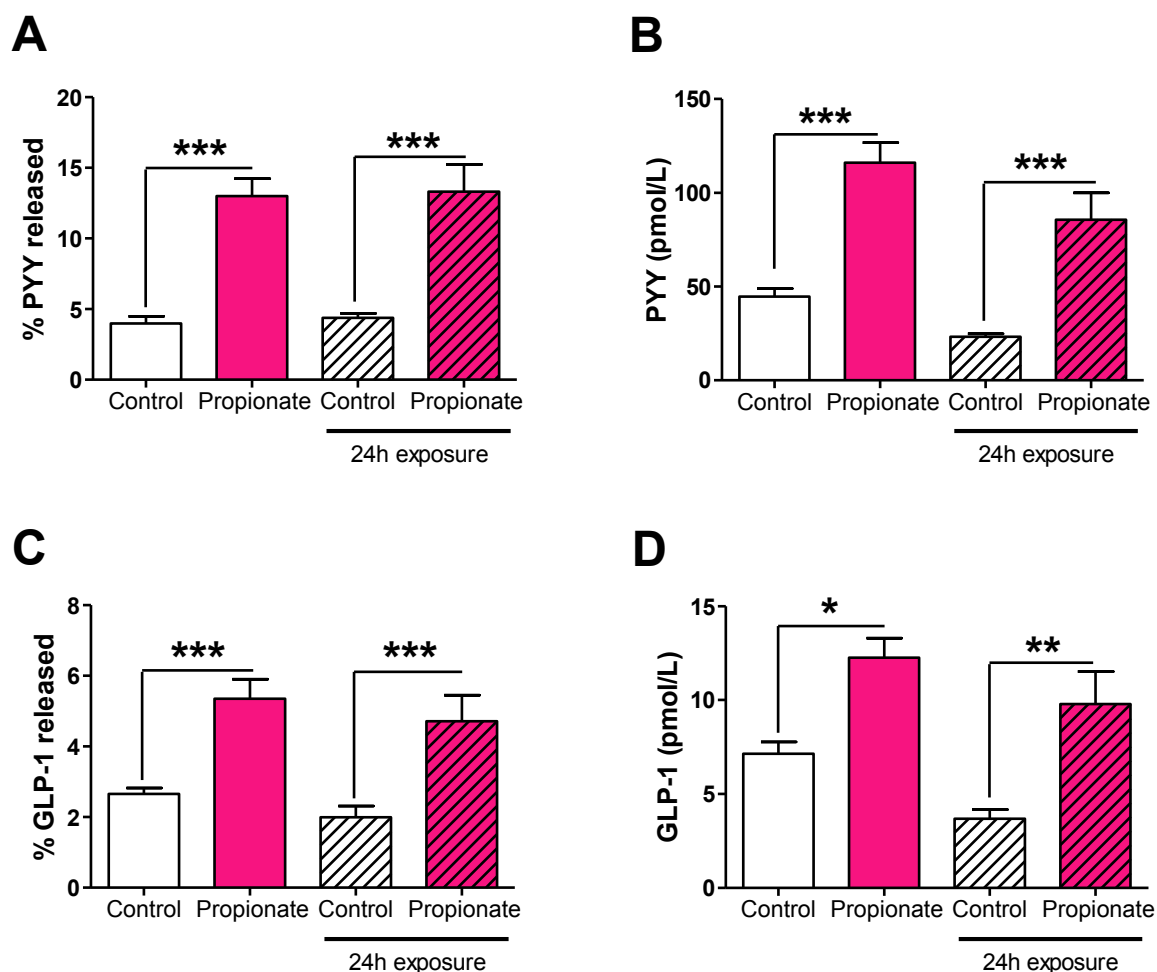


Figure 3.12. The effect of chronic prior exposure to propionate on acute propionate-stimulated gut hormone secretion from murine primary L cells. Mixed primary colonic cultures were pre-incubated in DMEM with or without 1mmol/L propionate for 24 hours. Colonic cultures were then acutely treated with sodium propionate (50mmol/L) or secretion buffer alone (control). PYY (A) and GLP-1 (C) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Absolute PYY (B) and GLP-1 (D) levels were measured in the supernatants. Data represent means $\pm$ SEM (n=2 mice, 10-12 wells per group). Significance is shown relative to basal secretion using one-way ANOVA with a Bonferroni post hoc test (*P<0.05, **P<0.01, ***P<0.001).

3.4.2 The effect of colonic propionate on gut hormone release *in vivo*

3.4.2.1 The effect of intra-colonic administration of propionate on portal and circulating PYY and GLP-1 concentrations in male Wistar rats

An *in vivo* model was developed to allow the simultaneous measurement of gut hormone levels in the portal and general circulation following intra-colonic administration of a test substance. Cannulation of the jugular vein followed by a laparotomy in isoflurane-anaesthetised male Wistar rats allowed access to the colon and portal vein and enabled sampling from both the jugular and the portal vein in parallel.

A 2.5ml intra-colonic bolus of propionate (180mmol/L, pH 5.5 using NaOH) induced a significant increase in systemic circulating plasma PYY and GLP-1 levels over a 60-minute period compared to saline (treatment effect, $P < 0.05$), though there was no significant difference in area under the curve (AUC; PYY, propionate 913 ± 363 vs. saline 204 ± 275 pmol/L, GLP-1, 167 ± 444 vs. -40 ± 46 pmol/L) (Fig. 3.13C and Fig. 3.14C).

Following intra-colonic administration, plasma PYY rose from a baseline of 28.3 ± 3.6 pmol/L, to 40.4 ± 5.8 , 47.0 ± 7.8 , and 49.3 ± 8.0 pmol/L at 15, 30 and 60 minutes, respectively (Fig. 3.13A). Plasma PYY reached a plateau at 30 minutes and remained elevated at the end of the study, representing a 21 pmol/L change from baseline. Saline, on the other hand, had a minimal effect on plasma PYY, which rose only by 4 pmol/L from baseline and remained at similar levels throughout the 60-minute period.

The effect of the propionate bolus on circulating GLP-1 levels appeared slower and more transient (Fig. 3.14A and B). Plasma GLP-1 peaked at 30 minutes, at 17.9 ± 4.5 pmol/L, reflecting a 5.7 ± 3.0 pmol/L change from baseline (12.2 ± 2.0 pmol/L). By 60 minutes, plasma

GLP-1 was markedly lower (14.1 ± 5.0 pmol/L). However, it remained elevated compared to baseline and saline (propionate, 2.0 ± 3.3 pmol/L vs. saline, -3.3 ± 0.7 pmol/L compared to baseline).

At 15 minutes following propionate administration, there was a significant increase in portal vein PYY compared to saline (76.5 ± 9.4 vs. 46.5 ± 9.5 pmol/L) (Fig. 3.15A). Portal vein PYY levels were markedly higher compared to the peripheral circulation. Portal vein GLP-1 levels, however, were either at the same level or lower and were not significantly different between propionate and saline at 15 minutes (propionate, 13.8 ± 4.1 vs. saline, 8.7 ± 1.9 pmol/L) (Fig. 3.15B).

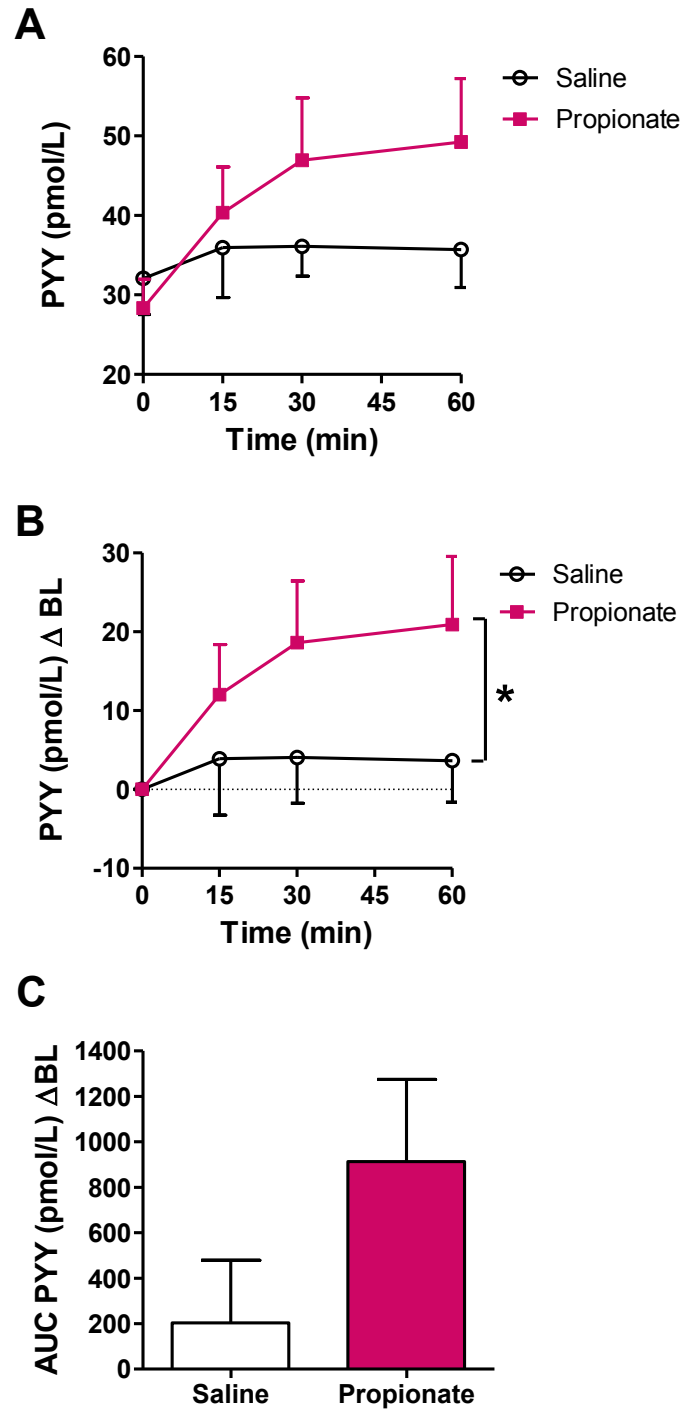


Figure 3.13. **The effect of intra-colonic administration of propionate on circulating plasma PYY concentrations in male Wistar rats.** Blood samples were collected over a 60-minute period, via a jugular vein cannula, following an intra-colonic injection (2.5ml) of 180mmol/L propionic acid or saline (matched for pH and sodium content) in isoflurane-anaesthetised rats. Absolute PYY concentrations in plasma are presented in (A), change from baseline values and AUC data are shown in (B) and (C), respectively. Data represent means $\pm$ SEM (n=14 per group). Significance was determined using two-way ANOVA (treatment effect, *P<0.05).

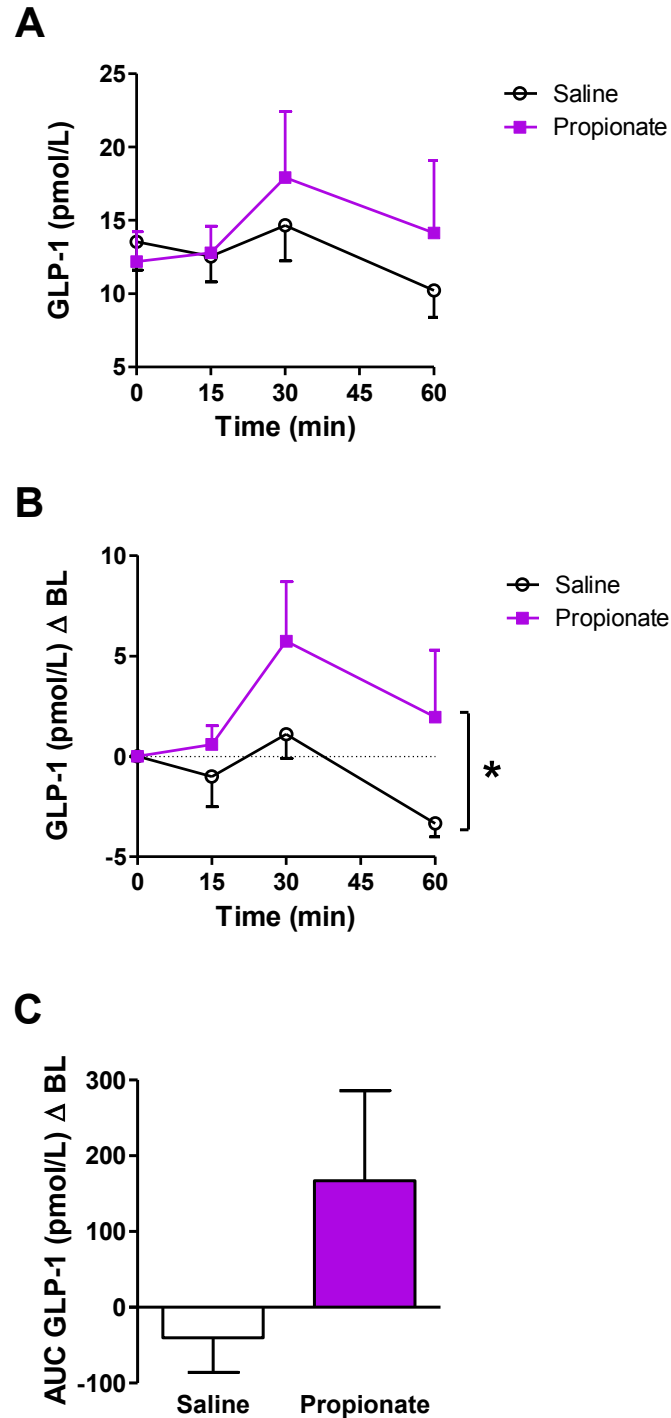


Figure 3.14. **The effect of intra-colonic administration of propionate on circulating plasma GLP-1 concentrations in male Wistar rats.** Blood samples were collected over a 60-minute period, via a jugular vein cannula, following an intra-colonic injection (2.5ml) of 180mmol/L propionic acid or saline (matched for pH and sodium content) in isoflurane-anaesthetised rats. Absolute GLP-1 concentrations in plasma are presented in (A), change from baseline values and AUC data are shown in (B) and (C), respectively. Data represent means $\pm$ SEM (n=14 per group). Significance was determined using two-way ANOVA (treatment effect, *P<0.05).

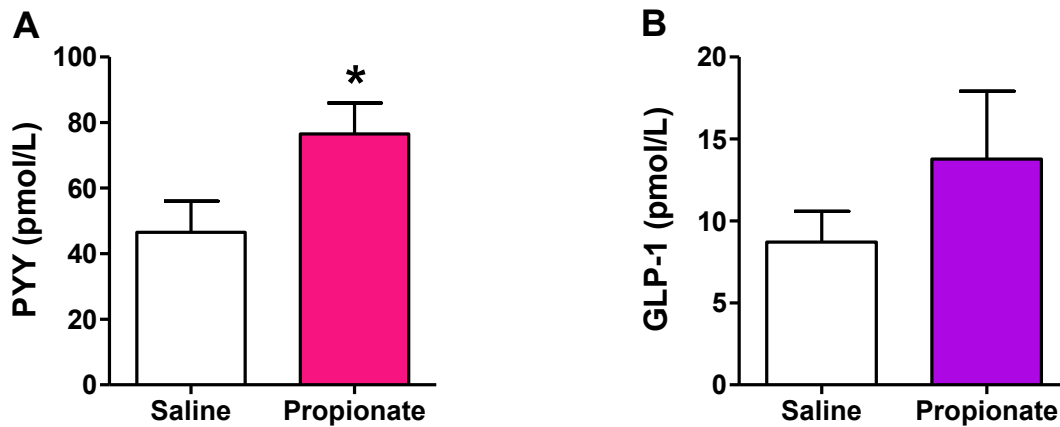


Figure 3.15. **The effect of intra-colonic administration of propionate on portal vein plasma PYY (A) and GLP-1 (B) concentrations in male Wistar rats.** Blood samples were collected from the portal vein 15 minutes following an intra-colonic injection (2.5ml) of 180mmol/L propionic acid or saline (matched for pH and sodium content) in isoflurane-anaesthetised rats. Data represent means $\pm$ SEM (n=14 per group). Significance was determined using an unpaired t-test (*P=0.0358).

3.4.2.3 The effect of intra-colonic administration of propionate on portal vein gut hormone concentrations in male C57BL6 mice

The effect of intra-colonic administration of propionate on portal vein plasma PYY and GLP-1 levels was also determined in male C57BL6 mice. Following a 300 μ l bolus injection of propionate (180mmol/L, pH 5.5 using NaOH), GLP-1 and PYY levels were markedly elevated in portal plasma at 15 minutes (Fig. 3.16). Plasma GLP-1 rose from 13.1 $\pm$ 8.5pmol/L to 21.2 $\pm$ 2.7pmol/L ($P=0.0517$) and plasma PYY increased significantly from 95.3 $\pm$ 12.2pmol/L to 134.1 $\pm$ 11.6pmol/L ($P=0.0307$).

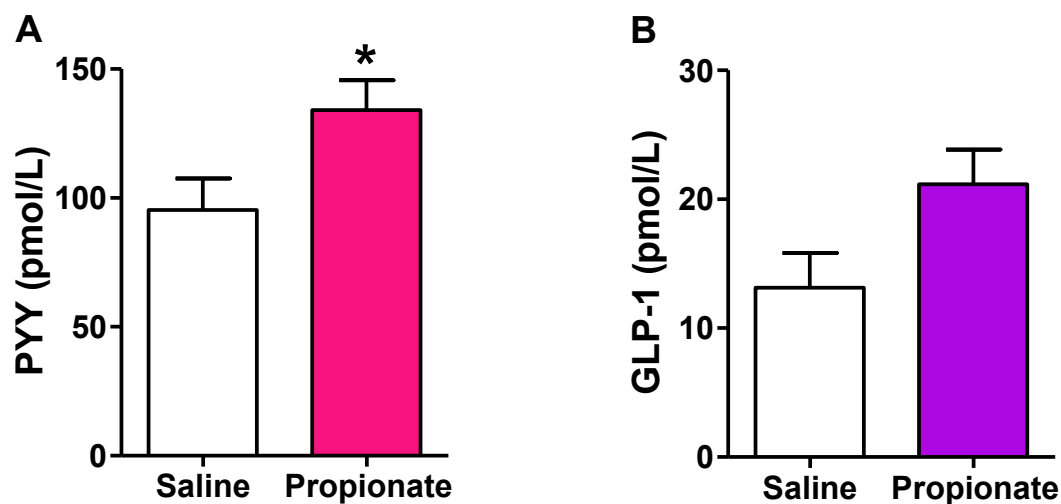


Figure 3.16. The effect of intra-colonic administration of propionate on portal vein plasma PYY and GLP-1 concentrations in male C57BL6 mice. Blood samples for measurement of plasma (A) PYY and (B) GLP-1 were collected from the portal vein 15 minutes following an intra-colonic injection (300 μ l) of 180mmol/L propionic acid or saline (matched for pH and sodium content) in isoflurane-anaesthetised mice. Data represent means $\pm$ SEM (n=11-14 per group). Significance was determined using an unpaired t-test (GLP-1, $P=0.0517$; PYY, $*P=0.0307$).

3.5 Discussion

3.5.1 Summary of findings

In vitro investigation

- SCFAs (C2-C4) stimulate the release of both PYY and GLP-1 from murine and human primary L cells.
- A 2-hour incubation with 200mmol/L propionate does not cause cytotoxicity in primary colonic cultures (murine and human).
- Above 200mmol/L, NaCl significantly stimulates gut hormone release from murine primary L cells. However, at concentrations <100mmol/L, NaCl has no effect on gut hormone release.
- At 200mmol/L, NaCl does not significantly increase PYY release from human primary L cells.
- Prior exposure (24 hours) of murine primary L cells to a low concentration of propionate does not impair the ability of the cells to secrete GLP-1 and PYY in response to acute stimulation with propionate.

In vivo investigation

- Intra-colonic propionate administration significantly increases portal vein PYY concentrations and sustains elevated circulating plasma PYY levels over a 60-minute period in rats.
- Intra-colonic propionate administration transiently, but significantly, increases circulating plasma GLP-1 levels in rats.
- Intra-colonic administration of propionate in mice elevates portal vein GLP-1 and significantly increases PYY concentrations in portal vein plasma.

3.5.2 Detailed Discussion

In vitro investigation

SCFAs and gut hormone release from murine primary L cells

All three major SCFAs (C2-C4), acetate, propionate and butyrate, significantly stimulated the release of both PYY and GLP-1 from murine colonic cultures, including at concentrations within the physiological range [70-130mmol/L (Mortensen & Clausen 1996)]. The effect of SCFAs on PYY release has not been previously investigated in primary colonic cells. The results presented here provide no evidence to suggest differential release of PYY vs. GLP-1.

Experiments using lower concentrations of propionate (1-25mmol/L) demonstrated that even at 1mmol/L, propionate was able to significantly induce both GLP-1 and PYY release from murine primary L cells. These results are in accordance with previous findings for active GLP-1 (Tolhurst *et al.* 2012a). In contrast to the high physiological concentrations of SCFAs reported in the gut lumen, these lower concentrations are more in line with the EC₅₀s of GPR43 and GPR41 for SCFAs, which are in the region of 0.5-1mmol/L (Brown *et al.* 2003, Le Poul *et al.* 2003). It is possible that luminal SCFAs are lower in the immediate vicinity of the L cells due to active uptake of SCFAs by surrounding enterocytes and/or due to the presence of the mucous layer, which acts as a diffusional barrier (Barthe *et al.* 1998, Levine *et al.* 1970, Plumb *et al.* 1987). Nohr *et al.* proposed that colonic EECs may sense the considerably lower concentration of SCFAs found at the basolateral surface (Nohr *et al.* 2013). Alternatively, Tolhurst *et al.* speculated that colonic SCFAs may play a role in providing a chronic stimulatory tone on L cells via apical or basolateral SCFA receptors (Tolhurst *et al.* 2012a), which could account for the presence of circulating GLP-1 in the fasted state. However, the chronic effect of SCFAs on primary L cells has not been investigated.

Effect of chronic exposure to propionate

To determine whether colonic L cells retain their stimulatory response to propionate following longer term exposure to a low but stimulatory concentration of propionate, murine L cells were incubated with or without 1mmol/L propionate over 24 hours (termed 'chronic') prior to acute stimulation with 50mmol/L propionate. This concentration was chosen as it was the highest concentration at which NaCl had no effect on gut hormone release. Prior chronic exposure to a low dose of propionate did not impair the L cells' ability to respond to subsequent acute stimulation with propionate. Interestingly, Hand *et al.* reported a 3.4-fold increase in cellular stores of PYY following chronic propionate exposure in STC-1 cells (0.1mmol/L over 72 hours) (Hand *et al.* 2013). The results presented here, following a shorter incubation period and in primary cells, do not support this finding in immortalised cells as absolute PYY and GLP-1 release appeared reduced, reflecting, if anything, a potential reduction in cellular content. However, it is not possible to discount a difference in L cell number between wells of different treatments as the cause of this reduced gut hormone content. Recent work has revealed that the SCFA receptor GPR43 may undergo β -arrestin 2-mediated internalisation following activation with an agonist (Lee *et al.* 2013). However, the data described here do not provide evidence for 'chronic' desensitisation under the conditions tested.

Human primary L cells and SCFA-mediated gut hormone release

A novel human L cell model was developed following adaptation of the murine colonic digest protocol (Reimann *et al.* 2008) to healthy human colonic tissue biopsies. Using this human L cell model, it was demonstrated for the first time that propionate stimulates GLP-1 secretion and that all three SCFAs (C2-C4) stimulate PYY release from human primary L cells, albeit at higher concentrations than in murine primary L cells (≥ 200 mmol/L propionate). However, it is possible that a stimulatory effect may have been observed at lower concentrations if more

human tissue had been available to increase the n numbers investigated. Furthermore, the effect of acetate and butyrate on GLP-1 release from human primary L cells now merits investigation.

There is a suggestion in the literature that human L cells are ~4-fold less responsive to stimuli including the LCFA linoleic acid than murine cultures, despite comparable basal rates of secretion (Habib *et al.* 2013). However, as previously alluded to, it is unknown whether this is a genuine difference in responsiveness between human and murine L cells or whether it reflects a difference in response to primary cell culture conditions.

Nevertheless, the fact that human L cells appear to respond to SCFAs is an important finding, though the experiments described in this chapter require repetition and confirmation. It demonstrates that SCFA-stimulated gut hormone release is relevant to humans and supports further investigation in this area. Furthermore, it supports the use of the murine colonic crypts as a valid L cell model. On the other hand, it also suggests that there may be some differences between the two species' cellular responses, which require further elucidation. Nevertheless, this human L cell model could prove a useful tool for investigating differences between murine and human L cells and for screening stimulants to evaluate their translational potential.

Cell health and Osmolarity

Additional experiments were carried out to eliminate potential effects of cytotoxicity or osmolarity as a cause of gut hormone release from primary colonic cultures. Cell health was assessed following a 2- or 4-hour incubation with propionate. LDH assay results revealed that there was no increase in cytotoxicity following the 2-hour incubation with 200mmol/L propionate in either murine or human colonic cells. These results support and extend the findings by Hand *et al.*, which demonstrated no effect on cytotoxicity following treatment of STC-1 cells with SCFAs over a 30-minute period (Hand *et al.* 2013). However, their study

reported a 2-fold increase in cytotoxicity in response to treatment with SCFAs over 72 hours. There was a slight, non-significant increase in cytotoxicity observed in the murine crypt cultures after 4 hours, however there was no impact on cell viability in the human colonic crypts. The slight increase in cytotoxicity observed in the murine cells is not detrimental as 200mmol/L was the maximal dose tested and all incubations lasted 2 hours. Furthermore, subsequent *in vitro* murine L cell experiments described in this thesis only use concentrations of 1-50mmol/L propionate. Ideally, an additional test for the assessment of cell viability would have also been employed, such as an MTT test (Sato & Clevers 2013). Crucially, it should be noted that it is the viability of the entire mixed cell population within a given well that is being assessed and may not be representative of the L cell population.

The effect of osmolarity on gut hormone release was also examined; primary murine cultures were incubated with different concentrations of sodium chloride (NaCl) alongside equivalent concentrations of sodium propionate. The data derived from these experiments indicate that while 200mmol/L NaCl has a potent stimulatory effect on gut hormone release, at concentrations <100mmol/L, the effect on gut hormone release is negligible. Therefore, at 50mmol/L for example, propionate significantly increases GLP-1 and PYY secretion from murine primary L cells compared to 50mmol/L NaCl (~2-fold difference). Furthermore, investigation of the effect of 200mmol/L propionate vs. 200mmol/L NaCl on PYY release from human colonic crypts indicated that NaCl *per se* did not significantly stimulate PYY release and that there was a significant difference between the %PYY released following propionate and NaCl treatment.

These experiments suggest that the effect of propionate on gut hormone release is specific, and is not the result of either increased osmolarity or cytotoxicity.

***In vivo* investigation**

Colonic propionate and gut hormone release in rats

Development of a novel *in vivo* model enabled the measurement of gut hormone levels in both the portal and peripheral circulation following intra-colonic administration of propionate in anaesthetised rats. The observed 18.6- and 20.9pmol/L rise in circulating plasma PYY levels at 30 and 60 minutes was similar in magnitude to that reported following intra-colonic administration of a mixture of SCFAs in the same strain of rats (18pmol/L rise above baseline at 30min) (Fu-Cheng *et al.* 1995). The SCFA mixture used in this previous study was at a similar concentration (150mmol/L) and was administered as a 3ml bolus, similar to the 2.5ml bolus used in this study. The results of this work also mirror those of the Fu-Cheng *et al.* study in that the elevated plasma PYY levels were sustained over the duration of the study, 60min and 120min. Furthermore, a 20pmol/L rise in PYY was also reported by Cherbut *et al.* in the same strain of rats following a one-hour colonic infusion with a 500mmol/L SCFA mixture (4ml/h) (Cherbut *et al.* 1998). In both studies there was a marked increase in plasma PYY from 30 minutes onwards.

However, in contrast to previous studies that failed to show an effect on GLP-1 (Anini *et al.* 1999, Cuche *et al.* 2000, Plaisancie *et al.* 1995), the data presented here suggest that both PYY and GLP-1 were elevated in parallel following intra-colonic administration of propionate. One difference between the studies described in this chapter and those which did not show a GLP-1 response is the use of protease inhibitors including a DPP-IV inhibitor, which in this study were added to whole blood upon collection (Cherbut *et al.* 1998, Plaisancie *et al.* 1995). However, the GLP-1 response was more transient in nature; GLP-1 peaked at 30min but the levels were not maintained and were reduced at 60min.

Furthermore, while there was a significant increase in portal vein PYY levels at 15min, the increase in portal vein GLP-1 was not significant, despite the fact that both gut hormones were increased 1.6-fold compared to saline. It is possible that the time point chosen was too

delayed to detect the peak in portal vein GLP-1 or it may also reflect the greater inter-individual variation observed. Control portal vein GLP-1 concentrations recorded in this study ($8.7 \pm 1.9 \text{ pmol/L}$) were in line with those reported in the literature [$7.8 \pm 0.7 \text{ pmol/L}$ (Cani *et al.* 2004a), $9.0 \pm 0.7 \text{ pmol/L}$ (Cani *et al.* 2004b)]. Portal vein PYY concentrations at $t=15 \text{ min}$ were higher compared to circulating concentrations at $t=15 \text{ min}$ (saline, 1.3-fold higher, stimulated, 1.9-fold higher) and remained higher at $t=30$ and 60 min (saline, 1.3-fold higher, stimulated 1.6-fold higher). Portal vein GLP-1 concentrations at $t=15 \text{ min}$, on the other hand, were lower than the circulating levels measured at the same time point. The reason for this is unclear, but may be due to an earlier peak in portal vein GLP-1 (Sato *et al.* 2009).

Conflicting results in the literature may derive from the use of different species/strains and experimental designs (mode of administration e.g. bolus [volume] vs. infusion [volume, duration], site of administration, sampling site and time points), the level of anaesthesia, the feeding state of the animal and the faecal content of the large intestine, as well as the form and concentration of SCFAs supplied; i.e. acids vs. sodium salts, low vs. (supra)physiological concentrations and pH values (mildly acidic to neutral). In the set of *in vivo* experiments described in this chapter, the propionate solution used was mildly acidic (pH 5.5) to match the pH of normal saline, which also closely resembles rodent caecal and proximal colonic pH (Lin *et al.* 2012).

Colonic propionate and portal vein gut hormone levels in mice

The effect of intra-colonic administration of SCFAs on gut hormone secretion *in vivo* in mice has not been previously investigated. The rat portal vein sampling procedure described above was adapted for use in mice, to enable the measurement of portal vein plasma gut hormone levels following intra-colonic administration of propionate. Basal GLP-1 values ($13.1 \pm 8.5 \text{ pmol/L}$) were similar to those reported in the literature [$\sim 16 \text{ pmol/L}$ (Delmee *et al.* 2006)]. Notably, mouse portal vein gut hormone levels, basal and stimulated, were higher

than those in rats (basal PYY, 2-fold, GLP-1, 1.5-fold). The results obtained demonstrate that colonic propionate increases the portal vein levels of both PYY (1.4-fold, $P<0.05$) and GLP-1 (1.6-fold) in mice, as in rats, to a similar extent. However, the increase in portal vein GLP-1 only approached significance ($P=0.0517$). Demonstrating that SCFAs have the same stimulatory effect on gut hormone release *in vivo* in mice is critical as it enables investigation of this system in genetically modified animals.

Conclusion

The work described in this chapter demonstrates that propionate stimulates the release of both PYY and GLP-1 *in vitro* from murine and human primary L cells and also *in vivo* in rodents. However, the mechanisms underlying SCFA/propionate-stimulated gut hormone release remain poorly characterised. The next chapter investigates these mechanisms, with a focus on the role of Gpr43 in propionate-mediated gut hormone release, *in vitro* and *in vivo*.

Chapter IV:

**The mechanisms
underlying propionate-
mediated gut hormone
release**

4.1 Introduction

A large number of G-protein coupled receptors (GPCRs) have been identified as nutrient sensors in the gut (Wellendorph *et al.* 2010). These include the SCFA receptors, GPR41 and GPR43, which are expressed in rodent (Karaki *et al.* 2006) and human colonic tissue (Karaki *et al.* 2008, Tazoe *et al.* 2009), and which are enriched in enteroendocrine L cells (Tolhurst *et al.* 2012a).

GPCRs are seven-transmembrane receptors, which transduce external stimuli by generating intracellular signalling cascades. GPCRs are subdivided into three classes (A-C) based on their sequence homology. SCFA receptors belong to the largest class, the rhodopsin-like family A (Stoddart *et al.* 2008). Extracellular binding of ligands to a GPCR induces conformational changes that modify the receptor's interface with cytosolic effectors, predominantly the membrane-bound heterotrimeric guanine nucleotide-binding G-proteins (α , β , γ) which dissociate and activate specific downstream pathways (Reimann *et al.* 2012). GPCRs are primarily classified based on their α -subunits and the respective intracellular signalling pathways they recruit.

4.1.1 SCFA receptors: activation and signal transduction

Activation of the human orthologs of GPR41 and GPR43 by SCFAs has been demonstrated in several recombinant cellular systems, including yeast-based reporter gene assays and transiently transfected human embryonic kidney cell (HEK-293) and chinese-hamster ovary K1 (CHO-K1) cell assays (Brown *et al.* 2003, Le Poul *et al.* 2003, Xiong *et al.* 2004). Rank order potencies (Table 4.1) derived from these different cell systems and assay formats have generally been consistent ($EC_{50}s \approx 0.5$ -1 mmol/L). Propionate (C3) is the most potent agonist at both receptors. All three SCFAs (C2-C4) activate GPR43 with similar potency. However, acetate displays greater selectivity for GPR43, whereas butyrate is more potent at

GPR41. This discrepancy enables functional discrimination between the two human receptors, as acetate is 100-fold less potent at GPR41 compared with propionate and butyrate (Brown *et al.* 2003, Le Poul *et al.* 2003).

Structure-activity relationships of the receptor ligands revealed that the carboxylic moiety is essential for receptor activation. The carboxylic moiety must be present at the end of an aliphatic chain containing one to six carbon atoms, linear or branched (Le Poul *et al.* 2003). However, compounds comprising two or more carboxyl groups were not tolerated, irrespectively of carbon chain length.

Table 4.1 Rank order of short chain fatty acid potencies at human GPR41 and GPR43 based on published data (Brown *et al.* 2003, Le Poul *et al.* 2003).

Receptor	Rank order of short chain fatty acid potencies
GPR41	propionate (C3) = pentanoate (C5) = butyrate (C4) > acetate (C2) > formate (C1)
GPR43	propionate (C3) = acetate (C2) ≥ butyrate (C4) > pentanoate (C5) > formate (C1)

During the original investigations, both receptors were found to be associated with inositol 1,4,5-triphosphate (IP₃) formation, elevation of intracellular Ca²⁺, activation of extracellular signal-related kinase 1 and 2 (ERK1/2) and inhibition of cAMP accumulation (Brown *et al.* 2003, Le Poul *et al.* 2003). However, they exhibited different coupling to G proteins; GPR41 exclusively activates the G_i family, as evidenced by the absence of a signal following treatment with pertussis toxin (PTX), whereas GPR43 is capable of dual coupling through both G_i and the PTX-insensitive G_q proteins (Brown *et al.* 2003, Le Poul *et al.* 2003). Gα_i is largely associated with inhibition of adenylyl cyclase and a consequent reduction in intracellular cAMP. Gα_q activates phospholipase C (PLC), leading to the generation of diacylglycerol (DAG) and IP₃, which activate protein kinase C (PKC) and trigger Ca²⁺ release from intracellular stores, respectively. The putative roles of SCFA receptor-associated Gα-proteins in secretion and exocytosis are illustrated in Figure 4.1.

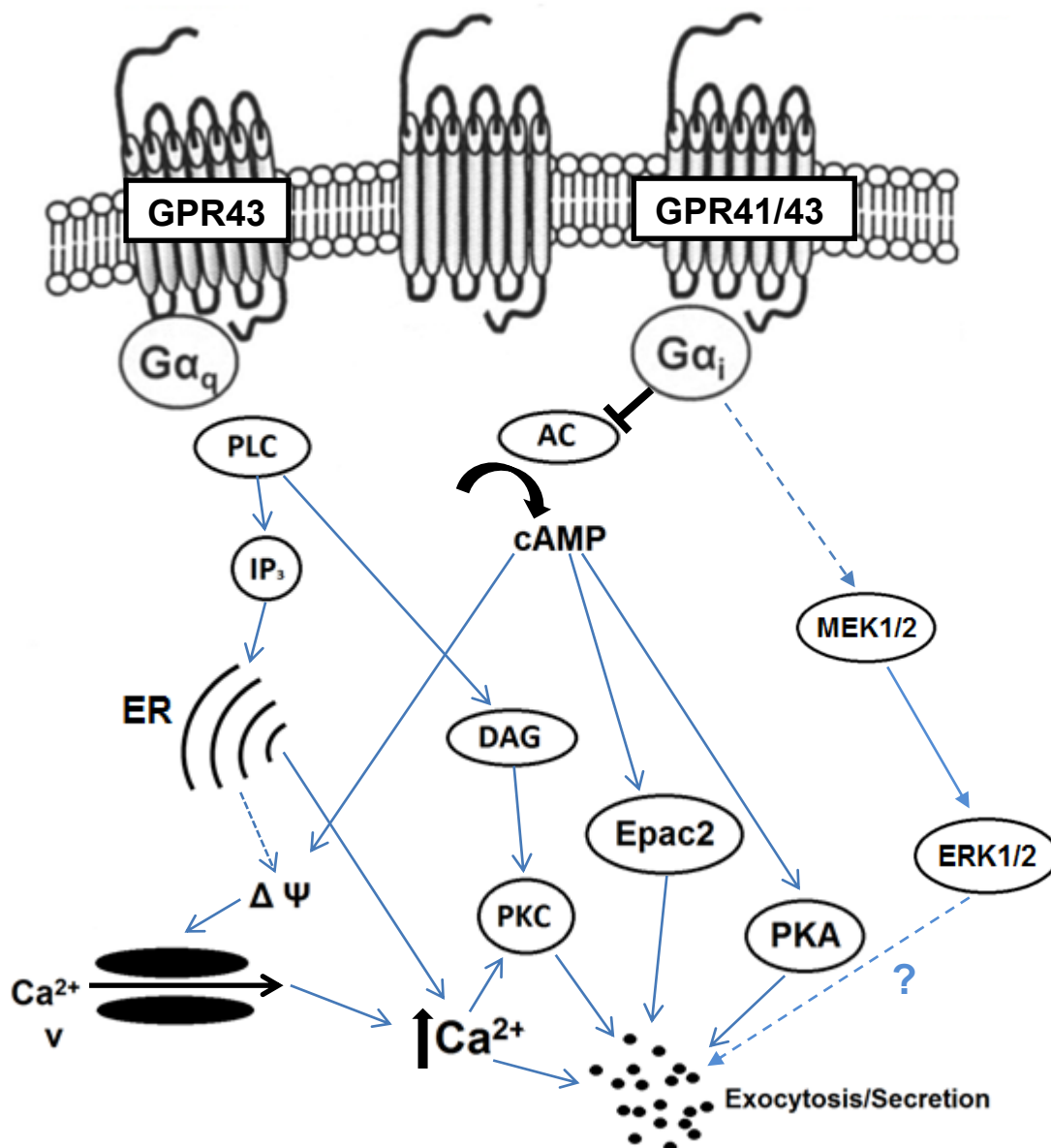


Figure 4.1 Short chain fatty acid receptor-activated intracellular signalling pathways associated with exocytosis. GPR41 couples exclusively to the G_i signalling pathway, which inhibits adenylyl cyclase (AC) thus reducing intracellular cAMP. The downstream effectors of cAMP, Epac2 (exchange protein activated by cAMP) and protein kinase A (PKA), have been implicated in exocytosis mechanisms. G_i , and potentially G_q , proteins can also indirectly regulate MEK1/2 and in turn activate ERK1/2, which is associated with exocytosis and secretion in several cell types. GPR43 is thought to couple to both G_i and G_q -coupled signalling pathways. Activation of the G_q pathway triggers the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER) and stimulation of protein kinase C (PKC), both of which are potent secretagogues.

PLC, phospholipase C, DAG, diacylglycerol; ERK1/2, extracellular-signal-regulated kinase 1 and 2; IP₃, inositol 1,4,5-triphosphate; MEK1/2, mitogen-activated protein kinase 1 and 2.

It is now well established that GPCRs can also produce G α -independent signalling. Propionate-mediated Gpr41 activation was recently demonstrated to trigger noradrenaline (NA) release from primary murine sympathetic cervical ganglion (SCG) neurons independently of G α_i signalling and the cAMP pathway. The Gpr41-dependent release of NA was mediated instead by G $\beta\gamma$ signalling and activation of PLC β and ERK1/2 (Inoue et al. 2012).

Both GPR41 and GPR43 activate ERK1/2, also referred to as p44-ERK and p42-ERK, or mitogen-activated kinase (MAPK) (Le Poul et al. 2003, Seljeset & Siehler 2012). ERK1/2 is activated upon dual phosphorylation of critical threonine and tyrosine residues by the upstream kinases MEK1/2. Interestingly, there is emerging evidence in the literature to suggest that ERK signalling is directly involved in GLP-1 release from enteroendocrine cell lines (Flock et al. 2013, Lim et al. 2009a, Lim et al. 2009b) and exocytosis in other cell types (Bieger et al. 2002, Cortez et al. 2012, Fierro et al. 2004, Ren & Guo 2012).

Lastly, GPCRs can also signal independently of G-proteins altogether, through additional pathways such as those mediated by the β -arrestin adaptor proteins (Shenoy & Lefkowitz 2005). Interestingly, there has been a recent suggestion in the literature that GPR43 interacts with β -arrestin 2 to modulate Nuclear Factor- κ B (NF κ B) signalling (Lee et al. 2013).

4.1.2 Synthetic ligands for SCFA receptors

The identification of selective synthetic ligands is an important step towards elucidating the pharmacology and therapeutic potential of GPCRs. Consequently, considerable effort has been expended to produce novel selective agonists and antagonists of the SCFA receptors. However, at present, only a limited number of these compounds are available. Although several synthetic ligands have been reported with selectivity for either GPR43 or GPR41, they have been associated with limitations, namely poor potency, low selectivity, allosteric

binding, or a lack of function at non-human orthologs of the receptors (Hudson *et al.* 2012, Lee *et al.* 2008, Schmidt *et al.* 2011, Smith *et al.* 2011). The development of novel selective ligands for the two SCFA receptors has been complicated further by recent observations describing discrepancies between the rodent and human orthologs of GPR41 and GPR43 (Hudson *et al.* 2012). Acetate was reported to be non-selective at mouse Gpr41 and Gpr43, compared to the 100-fold difference in potency at the human GPR41 receptor vs. GPR43. Furthermore, propionate which was equipotent at human GPR41 and GPR43, was reported to be ~12-fold more selective for mouse Gpr41 over Gpr43 (Hudson *et al.* 2012). These findings highlight the need for caution when designing agonists for use in animal work but also when extrapolating findings between species.

4.1.3 Evidence for a role for SCFA receptors in gut hormone release

The signalling pathways underlying SCFA-stimulated gut hormone release are poorly understood. However, a recent publication demonstrated that propionate-triggered active GLP-1 secretion from colonic cultures derived from Gpr43-deficient mice was reduced by 70% compared to wild type (WT) and GLP-1 secretion in response to acetate was abolished (Tolhurst *et al.* 2012a). Furthermore, acetate- and propionate-mediated active GLP-1 release was also significantly reduced in Gpr41^{-/-} colonic cultures. GPR43^{-/-} animals were also demonstrated to have reduced circulating levels of active GLP-1 and both GPR41^{-/-} and GPR43^{-/-} mice exhibited impaired glucose tolerance *in vivo* (Tolhurst *et al.* 2012a).

Acetate and propionate transiently increased intracellular Ca²⁺ in primary L cells, an effect also observed with the synthetic phenylacetamide GPR43 agonist (S)-2-(4-chlorophenyl)-N-(5-fluorothiazol-2-yl)-3-methylbutanamide (CFMB) (Tolhurst *et al.* 2012a). However, the elevation in intracellular Ca²⁺ was not directly linked to GLP-1 release in these experiments and indeed Nohr *et al.* did not find the same GPR43 agonist to be as effective as a GPR41 agonist at stimulating GLP-1 release from primary colonic cultures (Nohr *et al.* 2013).

Interestingly, propionate-stimulated GLP-1 release was not significantly affected by the presence of the G_i inhibitor, PTX (Tolhurst *et al.* 2012a). Taken together, these results led the authors to suggest that SCFA signalling through G_i -coupled pathways is not critical and that SCFAs are likely to signal predominantly through G_q -coupled pathways to release GLP-1. However, this does not explain the significantly reduced GLP-1 secretion in response to SCFAs in the $Gpr41^{-/-}$ tissue.

The previous chapter convincingly demonstrated that SCFAs trigger the release of gut hormones both *in vitro* from primary colonic cultures and *in vivo* following intra-colonic administration. Despite recent progress in elucidating the mechanisms underlying SCFA-mediated gut hormone release, the intracellular signalling pathways remain poorly characterised.

4.2 Aims and Hypotheses

4.2.1 Aim

To elucidate the mechanisms underlying propionate-mediated gut hormone release.

Objectives

Using the primary L cell model, to determine the role in propionate-mediated gut hormone release of:

- G_i signalling
- Gpr43 signalling
- ERK1/2 activation

Using a mouse model of intra-colonic administration and portal vein sampling, to determine:

- The role of Gpr43 signalling in propionate-induced gut hormone release *in vivo* using global Gpr43^{-/-} mice

4.2.2 Hypotheses

i) Propionate stimulates the release of gut hormones via a Gpr43-dependent mechanism. Therefore, propionate-induced gut hormone secretion will be impaired in Gpr43-deficient primary L cells *in vitro* and in Gpr43^{-/-} mice *in vivo*.

lii) Inhibition of G_i signalling and ERK1/2 activation will reduce propionate-induced gut hormone release.

4.3 Methods

Primary colonic cell culture: *Refer to Chapter II, Section 2.3.1*

Radioimmunoassay: *Refer to Chapter II, Section 2.3.2*

The *in vivo* study involving intra-colonic administration of propionate followed by portal vein sampling described in this Chapter was conducted as in Chapter III, **Section 3.3.2.3** with the only difference that portal vein blood was collected at t=5 min instead of t=15 min.

4.3.1 Generation of global Gpr43 knockout (GPR43^{-/-}) mice

Gpr43 global knockout (Gpr43^{-/-}) mice were originally acquired from Deltagen (www.deltagen.com). Figure 4.2 shows the strategy by which Gpr43 was knocked out. Mice were bred for five generations on a C57BL6 background. Mice were maintained as heterozygote breeding pairs, allowing use of littermate WT mice to compare with Gpr43^{-/-} mice. There was no difference in body weight between the GPR43^{-/-} and WT littermates on a normal chow diet.

4.3.2 Preparation of ear DNA

Ear tissue (2mm²) was placed in sterile eppendorf tubes. DNA was extracted by digestion in sodium hydroxide (50mmol/L, 30µl/tube) at 90°C for 10 minutes. A volume of 5µl 1M Tris-HCL (pH 8) was then added to each tube to neutralise the solution. Polymerase chain reaction (PCR) was then carried out.

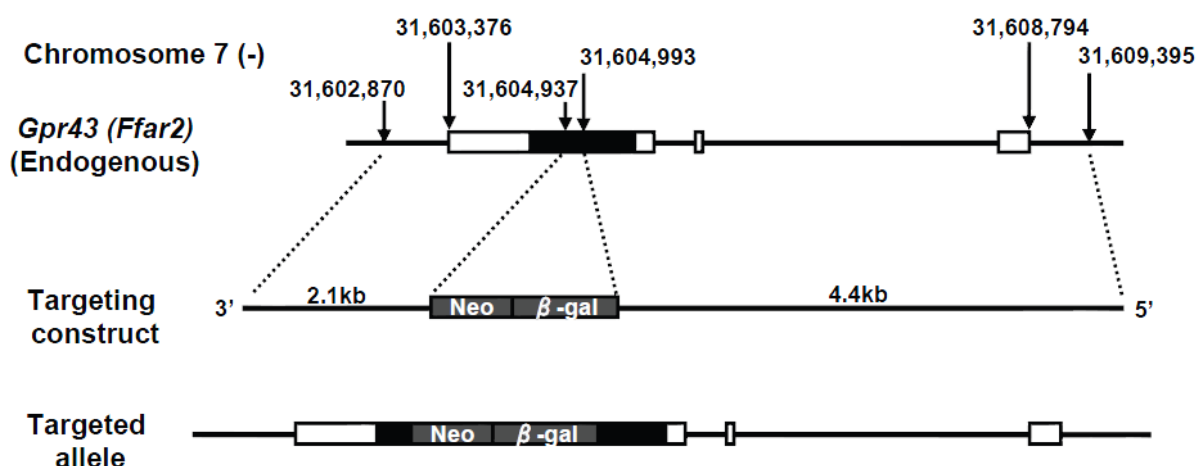


Figure 4.2 **Strategy for *Gpr43* gene targeted deletion.** Murine *Gpr43* on chromosome 7 consists of 3 exons (black box: coding sequence; empty boxes: untranslated sequences) and 2 introns. The targeting construct contains a 4.4 kb 5' arm, a β -gal-neo cassette and a 2.1 Kb 3'arm. The homologous recombination replaces a 55bp of *Gpr43* exon 1 by the β -gal-neo cassette and shifts the downstream amino acid sequence out of the reading frame. Adapted from: (Maslowski *et al.* 2009).

4.3.3 Genotyping by PCR

Detection of WT and KO alleles utilised a 3-primer set (Table 4.2): one forward primer, targeted at the endogenous sequence that was not intended to be knocked out (E1), and two reverse primers, one in an endogenous area targeted to be knocked out (ET) and the other targeted to a section of the knock-in construct (Neo). Each primer (0.5 μ l) was added to 12.5 μ l MasterMix (ThermoScientific, UK), to which 1 μ l sample DNA was then added. After an initial denaturation for 5 min at 95°C, the reaction mixes were run for 35 cycles at 95°C (45s), 68°C (45s), and 72°C (1min). All reactions were performed on the Peltier Thermal Cycler PTC-200 (MJ Research). PCR products were analysed by electrophoresis on a 1% agarose gel in 1x TAE buffer (refer to Appendix I) containing 500 ng/ml ethidium bromide (VWR, UK). Single bands at 200bp or 400bp represent the WT and KO genotype, respectively. Bands at both 200bp and 400bp represent a heterozygous genotype (Fig. 4.3).

Table 4.2 **Primer sequences for Gpr43^{-/-} genotyping**

Name	Primer Sequence	PCR product (bp)
F: E1	gcg-gaa-gtt-gga-tgc-tgc-ttc-cac-g	200
R: ET	gca-cag-ttc-ctt-gat-cct-cac-ggc-c	
F: E1	gcg-gaa-gtt-gga-tgc-tgc-ttc-cac-g	400
R: Neo	ggg-cca-gct-cat-tcc-tcc-cac-tca-t	

F, forward; R, reverse; bp, base pairs

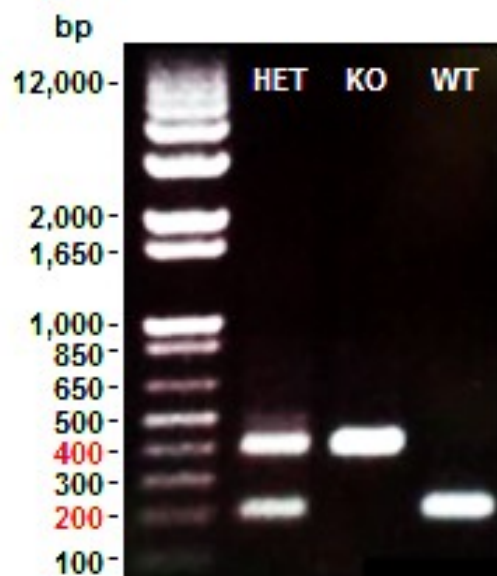


Figure 4.3 **Example of genotyping results.** Bands at 200bp or 400bp represent wild type (WT) and knockout (KO) genotype, respectively. Bands at both 200 and 400bp represent a heterozygous (HET) genotype. *Ladder, 1 Kb Plus DNA Ladder™ (Life Technologies)*

4.4 Results

4.4.1 Investigation into the mechanisms underlying propionate-mediated gut hormone release *in vitro*

4.4.1.1 The role of G_i signalling in propionate-mediated gut hormone release

To determine the role of G_i signalling in mediating the stimulatory effect of propionate on GLP-1 and PYY secretion, primary colonic cultures were pre-treated overnight with the G_i inhibitor, pertussis toxin (PTX, 200ng/ml) (Saifia *et al.* 1998, Tolhurst *et al.* 2012a).

Murine primary L cells

PTX treatment reduced the magnitude of the effect of propionate on gut hormone secretion from murine primary L cells (Fig. 4.4). The 2-fold increase in PYY observed with propionate ($P < 0.001$) was reduced to a 1.4-fold increase in the presence of PTX. However, basal secretion was also elevated following PTX treatment ($3.0 \pm 0.3\%$ vs. $2.3 \pm 0.3\%$ without PTX), though this difference was not statistically significant. The GLP-1 response to propionate was significantly attenuated following PTX treatment ($P < 0.05$). In the absence of G_i inhibition, propionate significantly increased %GLP-1 secretion from $1.7 \pm 0.2\%$ to $2.9 \pm 0.3\%$ (1.7-fold, $P < 0.001$). However, following pre-treatment with PTX, only a 1.2-fold increase in GLP-1 release was observed (1.8 ± 0.2 to $2.1 \pm 0.2\%$).

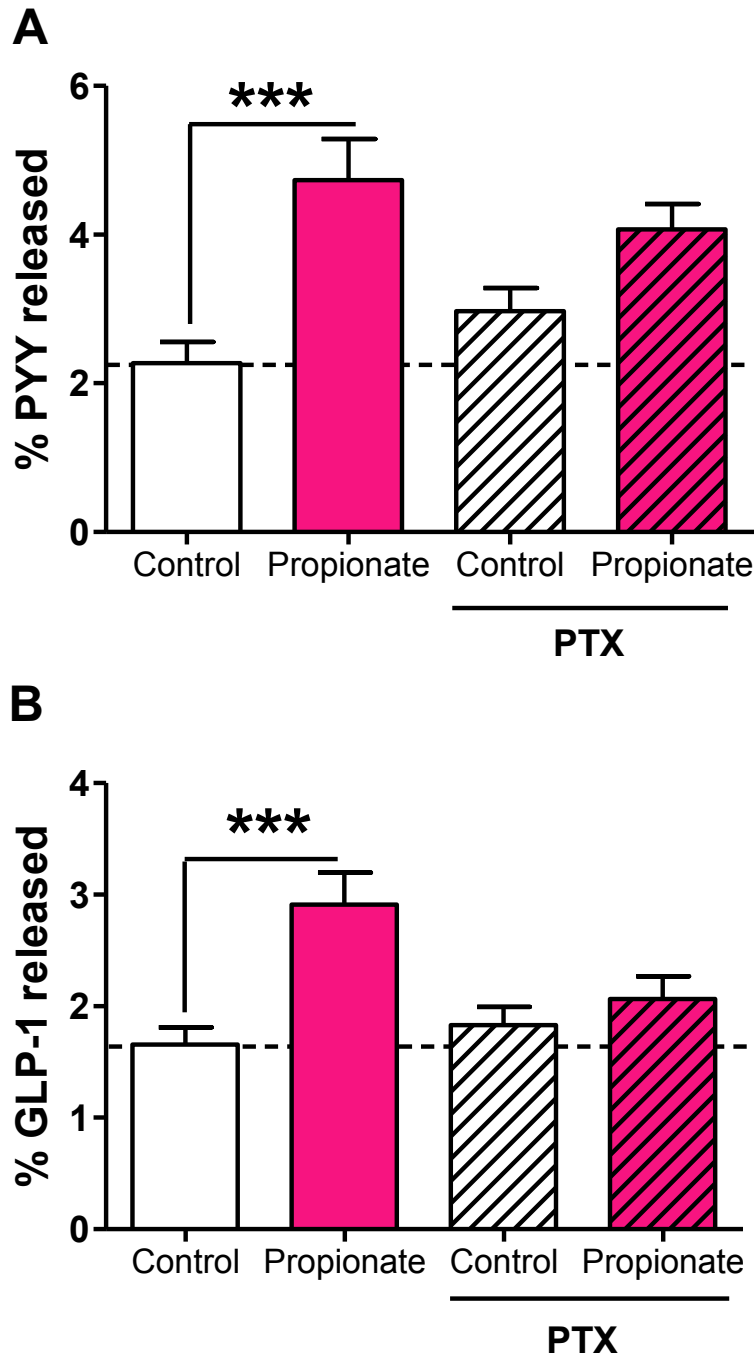


Figure 4.4 The effect of inhibiting G_i signalling on propionate-stimulated gut hormone secretion from murine primary L cells. Mixed primary colonic cultures were incubated $\pm$ sodium propionate (50mmol/L) following pre-treatment overnight in DMEM $\pm$ 200ng/ml pertussis toxin (PTX). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM ($n=2$ mice, 8-11 wells per group). Significance was determined using one-way ANOVA with a Bonferroni post hoc test (** $P<0.001$).

Human primary L cells

Inhibiting G_i signalling in human primary colonic cultures did not abolish PYY secretion in response to propionate (Fig. 4.5). Propionate-stimulated PYY secretion was $4.5 \pm 1.6\%$ and $4.2 \pm 1.0\%$, with and without PTX pre-treatment, respectively. However, there was an increase in basal %PYY release with PTX ($1.7 \pm 0.6\%$ vs. $0.9 \pm 0.6\%$ without PTX). Furthermore, while PYY secretion was increased in response to propionate under control conditions, this increase was not statistically significant.

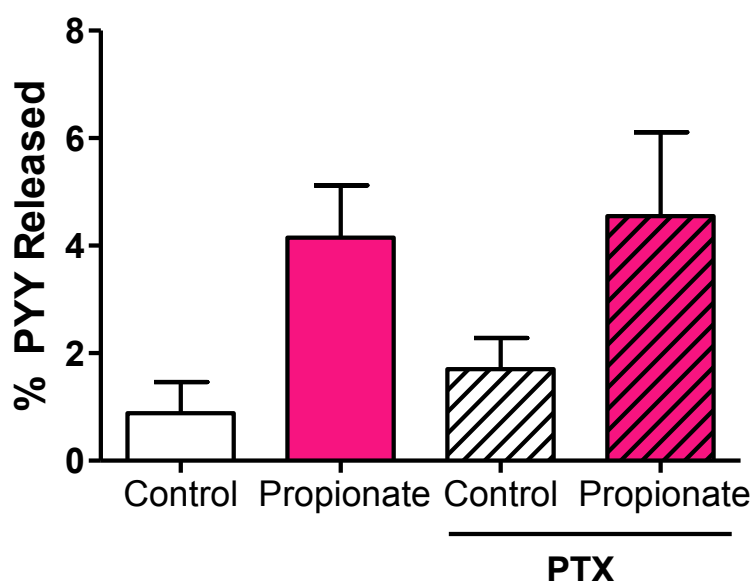


Figure 4.5 **The effect of inhibiting G_i signalling on propionate-stimulated PYY secretion from human primary L cells.** Mixed primary colonic cultures were incubated $\pm$ propionate (200mmol/L) following pre-treatment overnight in DMEM $\pm$ 200ng/ml pertussis toxin (PTX). PYY secretion in each well is expressed as a percentage of total PYY contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=2 patients' biopsies, 2-6 wells per group).

4.4.1.2 The role of Gpr43 signalling: The effect of propionate on gut hormone release from Gpr43^{-/-} vs. WT murine primary L cells

To determine the role of Gpr43 in propionate-induced gut hormone release, primary murine colonic cultures were isolated from global Gpr43^{-/-} mice and WT littermates. As expected, in the WT colonic cultures, incubation with propionate led to a significant increase in both PYY and GLP-1 secretion compared to basal secretion (%PYY, 2-fold increase, $P < 0.001$; %GLP-1, 2.2-fold increase, $P < 0.01$) (Fig. 4.6). In contrast, the propionate-induced gut hormone response from Gpr43^{-/-} L cells was significantly attenuated compared to WT L cells (%PYY, $P < 0.001$ and %GLP-1, $P < 0.05$). Incubation of Gpr43^{-/-} colonic cultures with propionate only led to 1.3-fold and 1.5-fold increases in %PYY and %GLP-1, respectively, which were not statistically significant compared to basal secretion.

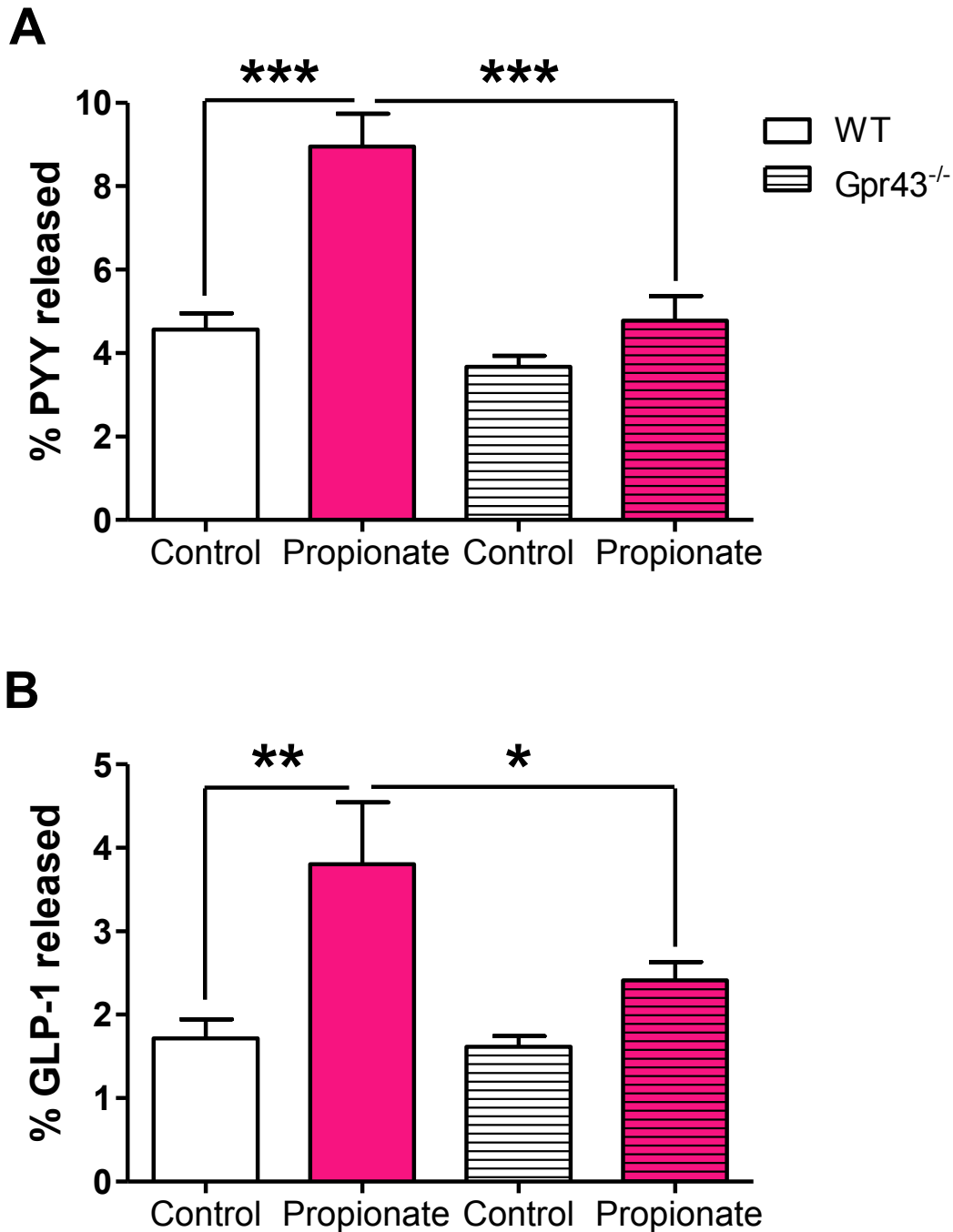


Figure 4.6. **The effect of propionate on gut hormone secretion from Gpr43^{-/-} murine primary L cells.** Mixed primary colonic cultures isolated from wild type (WT) or Gpr43^{-/-} mice were incubated $\pm$ propionate (50mmol/L). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (3 separate experiments, n=4-5 mice; PYY, 22-27 and GLP-1, 18-29 wells per group). Significance was determined using one-way ANOVA with a Bonferroni post hoc test (*P<0.05, **P<0.01, ***P<0.001).

4.4.1.2.1 The effect of L-glutamine or increasing intracellular [cAMP] on gut hormone release in Gpr43^{-/-} vs. WT murine primary L cells

To determine whether the reduced propionate-stimulated gut hormone response of Gpr43^{-/-} L cells was specific, Gpr43^{-/-} and WT primary colonic cultures were incubated either with L-glutamine, or with a combination of IBMX and forskolin to investigate gut hormone release in response to elevated intracellular cAMP concentrations. At a concentration previously shown to significantly induce gut hormone release (*refer to Chapter II, Section 2.4.3.2*), L-glutamine led to a significant increase in PYY and GLP-1 release from both WT and Gpr43^{-/-} cultures compared to basal ($P < 0.05$) (Fig. 4.7). Increasing [cAMP]_i in Gpr43^{-/-} colonic cultures also led to a significant increase in both PYY and GLP-1 secretion compared to basal (%PYY, $P < 0.01$; %GLP-1, $P < 0.001$), which was similar in magnitude to the response produced in the WT cultures (Fig. 4.8).

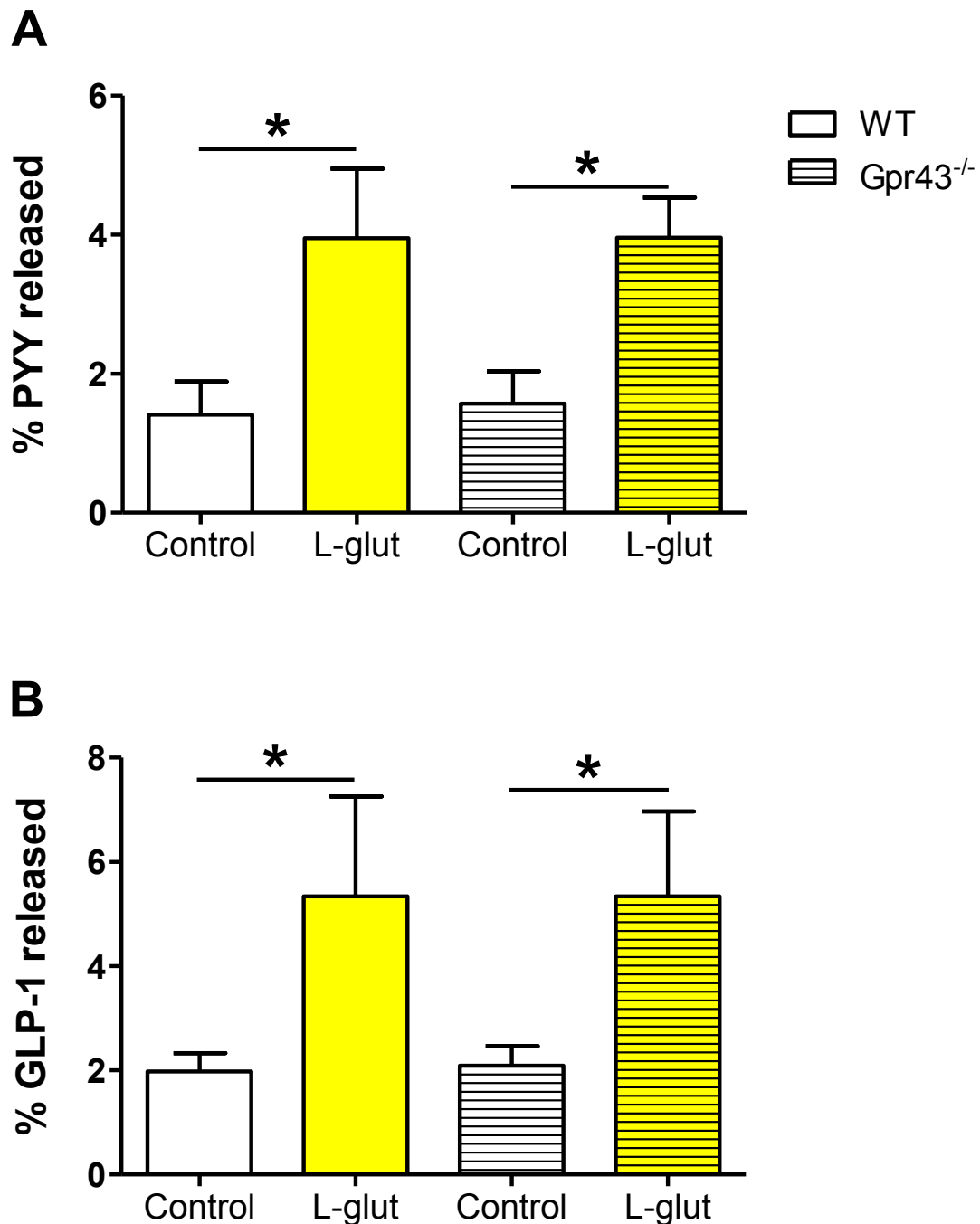


Figure 4.7. **The effect of L-glutamine on gut hormone secretion from Gpr43^{-/-} and WT murine primary L cells.** Mixed primary colonic cultures isolated from wild type (WT) or Gpr43^{-/-} mice were incubated in the presence or absence of L-glutamine (10 mmol/L). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (n=2 mice, 3-6 wells per group). Significance was determined using one-way ANOVA with a Bonferroni post hoc test (*P<0.05).

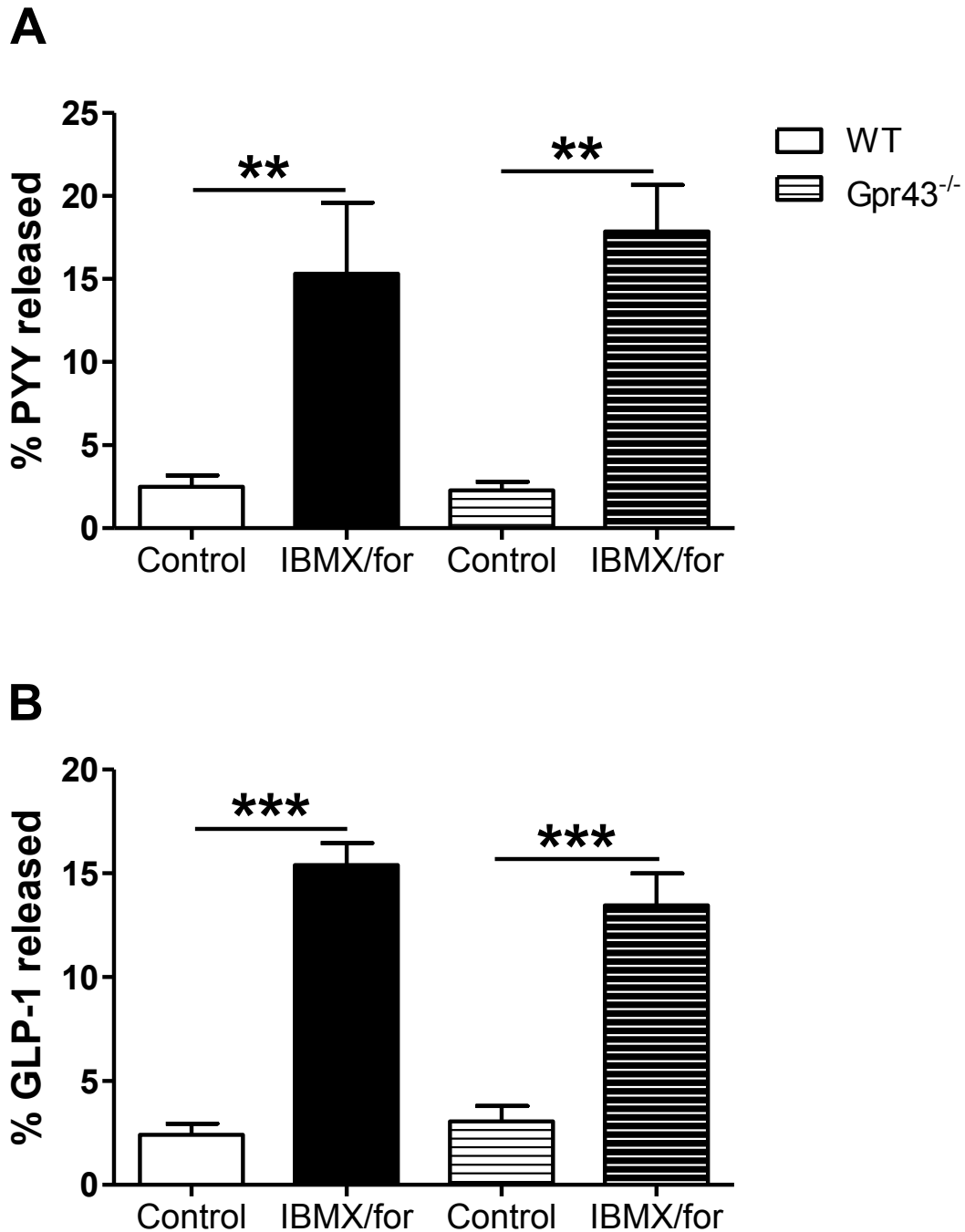


Figure 4.8. **The effect of increasing intracellular cAMP on gut hormone secretion from Gpr43^{-/-} and WT murine primary L cells.** Mixed primary colonic cultures isolated from wild type (WT) or Gpr43^{-/-} mice were incubated in the presence or absence of IBMX and forskolin (10 μ mol/L each). PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (2 separate experiments, n=2 mice, 4-7 wells per group). Significance was determined using one-way ANOVA with a Bonferroni post hoc test (**P<0.01, ***P<0.001).

4.4.1.3 The role of ERK1/2 activation in propionate-mediated gut hormone release

To investigate the role of ERK1/2 activation in propionate-mediated gut hormone release, primary murine colonic cultures were pre-treated for 2 hours in DMEM containing the MEK1/2 inhibitor, U0126 (10 μ mol/L) or 0.1% DMSO (vehicle control). They were then incubated with either 50mmol/L propionate, IBMX/forskolin (10 μ mol/L) or secretion buffer alone. No differences in basal PYY or GLP-1 release were observed following incubation with U0126 compared to control (Fig. 4.9).

As shown in Figure 4.9A, pre-treating the colonic cultures with U0126 did not prevent propionate from significantly increasing PYY release, from $2.3 \pm 0.2\%$ to $4.5 \pm 0.4\%$ ($P < 0.05$). However, the magnitude of the effect appeared to be reduced compared to control conditions, 2-fold vs. 2.4-fold increase ($2.6 \pm 0.3\%$ to $6.3 \pm 0.8\%$, $P < 0.001$), respectively. As expected, elevating intracellular cAMP effectively stimulated the release of PYY despite MEK1/2 inhibition.

In contrast, pre-treatment of the murine colonic cultures with U0126 abolished the stimulatory effect of propionate on GLP-1 release (Fig. 4.9B). Under control conditions, propionate led to a 1.9-fold increase in %GLP-1 secretion ($3.0 \pm 0.3\%$ to $5.6 \pm 0.4\%$, $P < 0.001$). However, following MEK1/2 inhibition, there was no GLP-1 release in response to stimulation with propionate. Elevating intracellular cAMP, on the other hand, retained its ability to trigger GLP-1 release ($P < 0.001$), despite MEK1/2 inhibition, to levels identical to those observed under control conditions ($7.0 \pm 1.6\%$ vs. $7.0 \pm 1.2\%$, respectively).

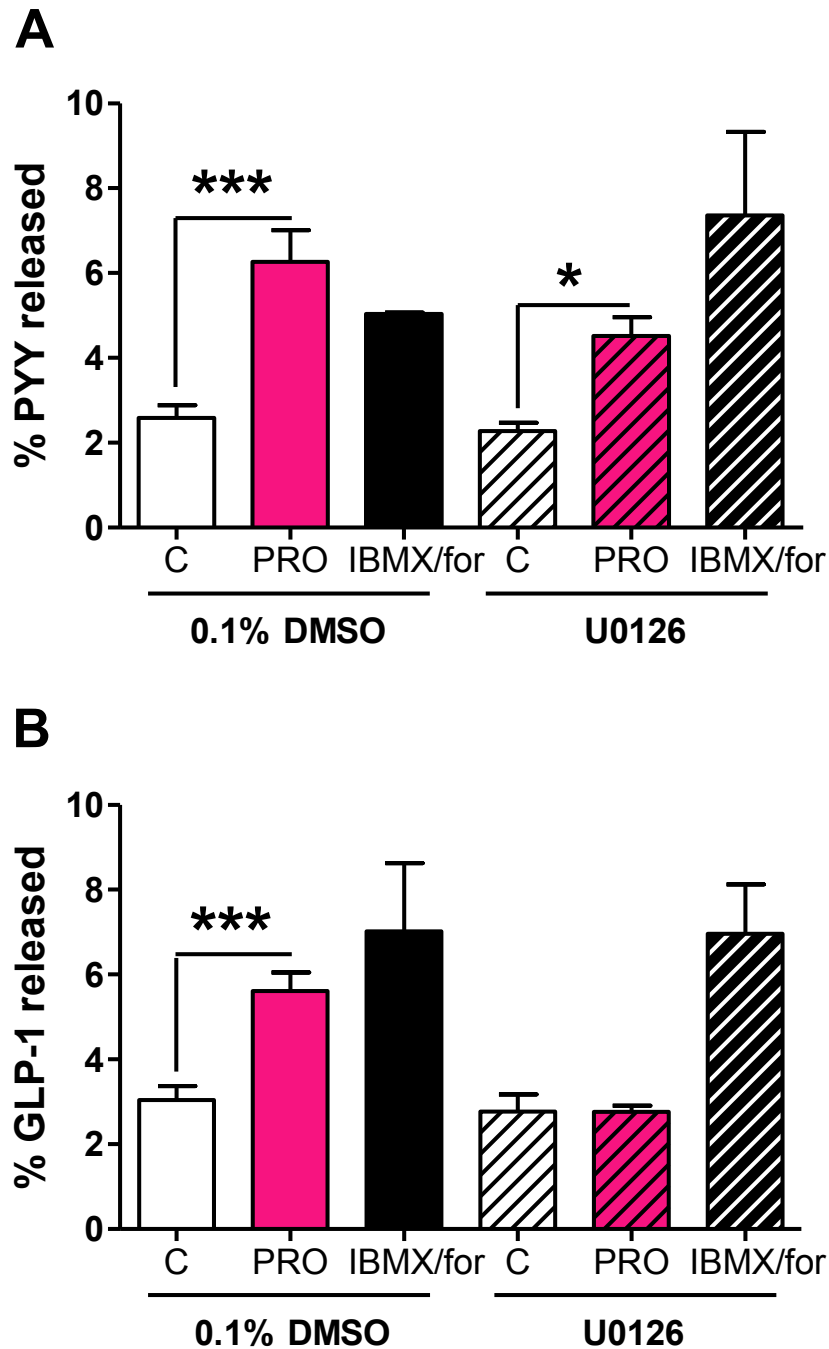


Figure 4.9. The effect of inhibiting ERK1/2 on propionate-induced gut hormone secretion from murine primary L cells. Mixed primary colonic cultures were incubated $\pm$ propionate (50mmol/L) or IBMX/forskolin (10 μ mol/L each) following a 2-hour pre-treatment with the MEK1/2 inhibitor U0126 (10 μ mol/L) or 0.1% DMSO control. PYY (A) and GLP-1 (B) secretion in each well is expressed as a percentage of total PYY or GLP-1 contained within the well and compared to the basal secretion measured in parallel within the same experiment. Data represent means $\pm$ SEM (2 separate experiments, n=2 mice, 2-10 wells per group). Significance was determined using one-way ANOVA with a Bonferroni post hoc test (* P <0.05, *** P <0.001).

4.4.2 The role of Gpr43 *in vivo*: the effect of intra-colonic administration of propionate on portal vein gut hormone concentrations in Gpr43^{-/-} vs. WT mice

The primary objective of this work was to elucidate the role of Gpr43 in propionate-induced gut hormone release. To assess the role of Gpr43 *in vivo*, Gpr43^{-/-} mice or WT littermates received an intra-colonic injection of either 180mmol/L propionic acid (pH 5.5) or saline. At t=5min, portal vein blood was collected for assessment of plasma PYY and GLP-1 levels.

Propionate significantly increased portal vein plasma PYY and GLP-1 in WT animals (Fig. 4.10, both P<0.05). In contrast, propionate-mediated gut hormone secretion was significantly impaired in the Gpr43^{-/-} mice; no increase in plasma PYY and only a slight, non-significant increase in GLP-1 were observed following intra-colonic propionate administration.

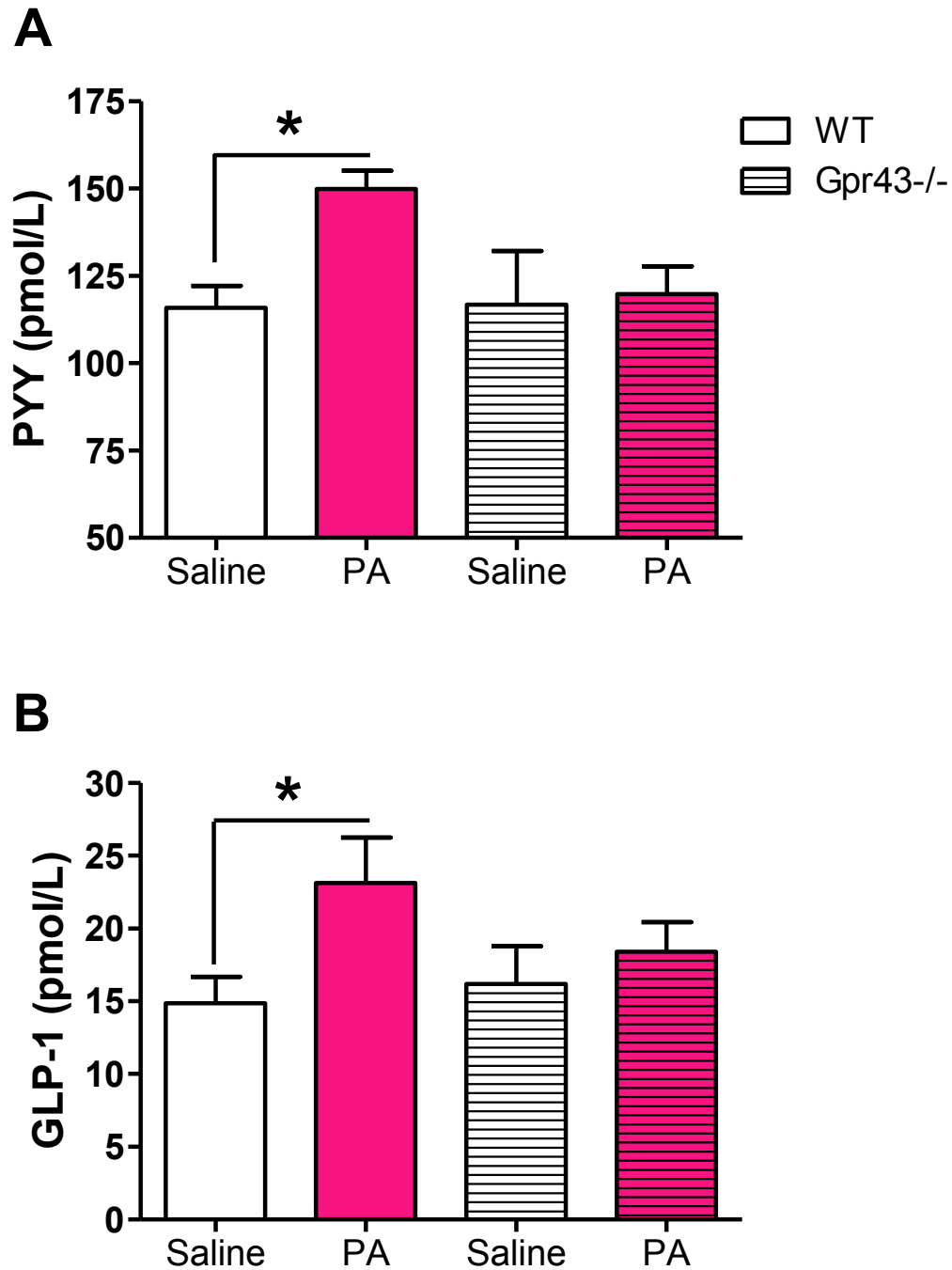


Figure 4.10 The effect of intra-colonic administration of propionate on portal vein plasma PYY and GLP-1 concentrations in male Gpr43^{-/-} and WT mice. Blood samples were collected from the portal vein for measurement of plasma (A) PYY and (B) GLP-1, 5 minutes following an intra-colonic injection (300 μ l) of 180mmol/L propionic acid or saline (matched for pH and sodium content) in isoflurane-anaesthetised Gpr43^{-/-} or wild type (WT) mice. Data represent means $\pm$ SEM (n=5-7 per group). Significance was determined using one-way ANOVA with a Bonferonni post hoc test (*P<0.05).

4.5 Discussion

4.5.1 Summary of findings

- Inhibition of G_i signalling reduced, but did not abolish, the increase in PYY release induced by propionate in murine and human primary L cells. However, inhibition of G_i signalling reduced propionate-mediated GLP-1 secretion to a greater extent than PYY in murine colonic cultures.
- Inhibition of ERK1/2 activation abolished the increase in GLP-1 secretion, but not PYY secretion, observed in response to propionate in murine primary colonic cultures.
- Propionate-stimulated secretion of both GLP-1 and PYY was significantly impaired in $GPR43^{-/-}$ murine primary colonic cultures compared to WT.
- $GPR43^{-/-}$ murine primary colonic cultures retained their response to an increase in intracellular cAMP levels, suggesting that their intracellular signalling pathways are intact.
- *In vivo*, intra-colonic administration of propionate did not significantly increase portal vein plasma GLP-1 and PYY concentrations in $GPR43^{-/-}$ mice, but did in WT mice.

4.5.2 Detailed discussion

The role of G_i signalling in propionate-induced gut hormone release

To ascertain the contribution of G_i signalling to the gut hormone release triggered by propionate, murine and human primary colonic cultures were pre-treated overnight with PTX at a concentration previously found to be effective in murine colonic tissue (Tolhurst *et al.* 2012a). The effect of PTX treatment on PYY release from murine tissue and on gut hormone release from human colonic tissue has not been previously investigated.

In murine and human colonic cultures, propionate appeared to maintain its ability to stimulate PYY release in the presence of PTX. However, the magnitude of the response was reduced, in part due to an increase in basal PYY release. Similarly, basal GLP-1 secretion was also slightly elevated in the presence of PTX and the response to propionate was impaired to a greater extent (1.2-fold vs. 1.7-fold). It is also difficult to draw conclusions from the human colonic culture experiment because of the small n number and the lack of a statistically significant difference in PYY secretion between propionate and control in the absence of PTX. In addition, as NaCl at 200mmol/L was previously demonstrated to have a slight effect on gut hormone release, a NaCl control would be useful in future experiments. Experiments with both human and murine colonic cultures would require independent repetition to confirm the results (minimum of n=3 independent experiments). However, a similar pattern, i.e. the increased basal gut hormone release and the attenuated response to propionate following PTX treatment were also observed by Tolhurst *et al.* in murine cultures (1.3-fold increase in active GLP-1 with PTX vs. 1.8-fold increase without PTX) (Tolhurst *et al.* 2012a). However, the reduction reported by Tolhurst *et al.* was not statistically significant.

Ideally, the effect of overnight pre-treatment with PTX on cell viability would have been examined prior to the experiment. The increase in basal release following PTX treatment observed in the work presented here and in the literature, may indicate cytotoxicity. This work would have also benefited from a positive control and an experiment to verify that G_i signalling had indeed been effectively knocked down under these conditions. This could have been achieved using IBMX and somatostatin, a potent inhibitor of gut hormone secretion, which activates a G_i-coupled pathway to reduce intracellular cAMP levels (Moss *et al.* 2012). IBMX should stimulate gut hormone secretion, via an increase in intracellular cAMP, irrespectively of PTX. In the absence of PTX, somatostatin should significantly reduce IBMX-stimulated gut hormone secretion, whereas in the presence of PTX, somatostatin should not be able to inhibit gut hormone release and thus IBMX should retain its stimulatory effect (Tolhurst *et al.* 2012a).

The role of ERK1/2 activation in propionate-mediated gut hormone release

ERK1/2 signalling has previously been associated with GLP-1 release from enteroendocrine cell lines in response to a number of stimuli (Flock *et al.* 2013, Lim *et al.* 2009a, Lim *et al.* 2009b). The effect of ERK1/2 inhibition on PYY release has not been investigated. To determine whether ERK1/2 also plays a role in propionate-mediated gut hormone release, primary murine colonic cultures were pre-treated with U0126, an inhibitor of MEK1/2, the upstream activator of ERK1/2. Surprisingly, in the presence of U0126, the stimulatory effect of propionate on PYY release was reduced (2.4-fold to 2-fold), whereas the GLP-1 response appeared to be completely abolished. If confirmed, these results would suggest that ERK1/2 plays a role in propionate-mediated GLP-1 secretion.

The mechanisms by which ERK1/2 regulates exocytosis and gut hormone release are largely unknown. Interestingly, recent evidence suggests that ERK1/2 directly affects exocyst function (Ren & Guo 2012). The exocyst, present in murine intestinal stem cells (Sakamori *et al.* 2012), is an evolutionarily conserved octameric protein complex that mediates the initial interaction between secretory vesicles and the plasma membrane, a process known as ‘vesicle tethering’, which precedes membrane fusion (He & Guo 2009). Ren and Guo recently identified Exo70, one of the exocyst proteins thought to associate with the plasma membrane and interact with the rest of the exocyst components on the arriving vesicles, as a direct substrate of ERK1/2 (He & Guo 2009, Ren & Guo 2012). It has also been postulated that the exocyst interacts with and regulates the assembly of the SNARE protein complex, which catalyses membrane fusion and exocytosis. Experiments using U0126 and Exo70 mutants suggest that inhibition of ERK1/2 phosphorylation of Exo70 prevents exocytosis by affecting the interaction of Exo70 with other exocyst subunits (Ren & Guo 2012). Further work to elucidate the potential roles of ERK1/2 and the exocyst in SCFA-mediated gut hormone release is warranted.

The reason why ERK1/2 inhibition did not impair PYY secretion to the same extent as GLP-1 secretion, should this be confirmed to be a true finding, is a matter of speculation. A recent study investigating the receptor-specific regulation of ERK1/2 activation by GPR41 and GPR43 reported that G_i signalling is involved in the activation of ERK1/2 by both receptors. ERK1/2 activation mediated by GPR41 stimulation was fully dependent on G_i and MEK1/2, whereas ERK1/2 phosphorylation in response to GPR43 activation was fully dependent on MEK1/2 and reduced by 75% in the presence of PTX (Seljeset & Siehler 2012). Consequently, while there is some G_q -mediated activation of ERK1/2, it appears to be predominantly linked to G_i signalling. Therefore, it is conceivable that the impaired GLP-1 release in the presence of U0126 may be related to the reduced GLP-1 secretion following G_i inhibition. While both sets of results require confirmation, they warrant further examination of the role of G_i signalling and ERK1/2 activation in SCFA-mediated gut hormone release.

This line of investigation would be strengthened by demonstrating that propionate increases ERK1/2 activation in L cells, which could be achieved by quantifying phosphorylated ERK1/2 (p-ERK), as a proportion of total ERK1/2 (t-ERK) by immunoblotting. This approach would require the use of an immortalised cell line, such as the GLUTag cell line. Furthermore, this set of experiments would have benefited from a positive control for ERK1/2 inhibition. Following optimisation, this could perhaps have been achieved using insulin. Insulin-stimulated GLP-1 secretion was abolished in the presence of a MEK1/2 inhibitor in the enteroendocrine cell line NCI-H716 (Lim *et al.* 2009a).

The concept of differential PYY and GLP-1 release is controversial. GLP-1 and PYY are predominantly thought to be co-localised both at the cellular and vesicular level in colonic L cells (Habib *et al.* 2013, Nilsson *et al.* 1991), which would suggest that they are simultaneously released. Indeed, in the work presented in this thesis, all stimuli that have triggered PYY release have also triggered GLP-1 release to a similar extent. However, there are several examples in the literature which would suggest that under certain conditions, there is selective release of only one of the two hormones (Anini *et al.* 1999, Aponte *et al.*

1988, Ballantyne *et al.* 1989, Cuche *et al.* 2000). Several hypotheses have been proposed in the literature, as previously discussed, including the differential release of the two gut hormones from the same L cell, or the existence of PYY-positive but GLP-1 negative L cells (Anini *et al.* 1999, Habib *et al.* 2013).

Given the reported presence of secretory granules within L cells containing PYY only (~15%) (Nilsson *et al.* 1991), it could be speculated that spatial organisation of signalling components may be arranged in such a way as to lead to release of discrete pools of individual hormones from an L cell that expresses both. Alternatively, it is possible to speculate that there are different subsets of colonic L cells, including ones which may not produce both hormones, and which may be differentially regulated. This would be much less surprising in light of the recent findings by Habib *et al.*, who reported marked differences between small intestinal (SI) and colonic proglucagon-expressing L cells (Habib *et al.* 2012). Furthermore, this study did not only find differences between SI and colonic L cells, but also between proximal and distal SI L cells, including a higher percentage of cells co-expressing PYY in the distal SI. Therefore, it is possible that there are also differences between L cell populations along the length of the large intestine, where L cell numbers are higher (Eissele *et al.* 1992, Larsson *et al.* 1975). Nevertheless, these recent findings by Habib *et al.* revealed the diverse nature of the L cell population across different regions of the proximal and distal gut, highlighting the complexity of the enteroendocrine system and the current limits to our understanding of it.

The role of Gpr43 in propionate-induced gut hormone release

While the two published studies that have investigated energy homeostasis in Gpr43^{-/-} mice to date led to different conclusions, both reported hyperphagia on a HFD compared to WT (Bjursell *et al.* 2011, Kimura *et al.* 2013). One of the mechanisms potentially underlying this increase in food intake in Gpr43-deficient mice is a reduced release of anorectic gut hormones from Gpr43-expressing enteroendocrine L cells. In support of this hypothesis, Gpr43^{-/-} animals were also reported to have lower basal circulating levels of GLP-1 (Tolhurst *et al.* 2012a).

The results presented in this chapter, demonstrate *in vitro*, and for the first time *in vivo*, that Gpr43 deficiency impairs SCFA-induced PYY and GLP-1 secretion. Incubation of primary colonic cultures derived from Gpr43^{-/-} mice with propionate led to significantly reduced PYY and GLP-1 release compared to WT cultures. An obvious question which arose from these results, and which is not addressed by the current literature, is whether the secretory function of Gpr43^{-/-} L cells was somehow impaired so that the reduced secretion observed in response to propionate was non-specific. However, the Gpr43^{-/-} colonic cultures maintained a robust gut hormone response to both L-glutamine and elevated intracellular cAMP concentrations, suggesting that their intracellular machinery is intact.

It is tempting to speculate that the remaining non-significant increase in gut hormone secretion observed following treatment of the Gpr43^{-/-} colonic cultures with propionate may be a consequence of Gpr41 activation. However, an effect of propionate transport across the cell membrane or metabolism within the cell cannot be ruled out. A limitation of this work was that Gpr41 expression in Gpr43^{-/-} vs. WT colonic tissue has not yet been assessed. This information is important, particularly in light of the previously reported reduced expression of Gpr41 in Gpr43-deficient mice (Tolhurst *et al.* 2012a).

Intra-colonic administration of propionate significantly increased portal vein plasma levels of both PYY and GLP-1 at t=5 min in WT mice. The increase in portal vein plasma GLP-1 in response to propionate described in Chapter III only bordered statistical significance at t=15 min post-administration (P=0.0517). However, portal vein sampling at 5 minutes resulted in higher plasma GLP-1 concentrations (~10%), presumably as a result of reduced degradation.

In $Gpr43^{-/-}$ mice, intra-colonic administration of propionate led to a significantly reduced portal vein plasma PYY and GLP-1 response. Although this might be expected, it has not been previously demonstrated. In contrast to the reduced basal circulating GLP-1 concentrations observed by Tolhurst *et al.*, there was no difference in portal vein GLP-1 or PYY between $Gpr43^{-/-}$ and WT animals following intra-colonic administration of saline.

It is critical to demonstrate that findings *in vitro* also translate into the *in vivo* setting and are of physiological relevance. In this context, given that the precise cellular location of Gpr43 is unknown, it was important to demonstrate that propionate was able to stimulate gut hormone release when administered into the gut lumen.

4.5.3 Conclusions and novel insight

The role of G_i signalling and ERK1/2 in SCFA-mediated gut hormone release is not clear. Preliminary data suggest that they may play a role in GLP-1 secretion from primary murine cultures. However, further work is required to confirm these results and to investigate the effect of G_i signalling on GLP-1 release, and ERK1/2 inhibition on GLP-1 and PYY release, from human primary L cells. Nevertheless, the findings presented here highlight the importance of measuring the release of both PYY and GLP-1 in parallel in response to different stimuli and inhibitors.

The work described in this chapter demonstrated for the first time that propionate-stimulated PYY release from primary $Gpr43^{-/-}$ colonic cultures is significantly attenuated. Furthermore, this work also demonstrated for the first time that $Gpr43^{-/-}$ mice have significantly lower portal vein PYY and GLP-1 levels following intra-colonic administration of propionate compared to their WT littermates. These findings support the concept of SCFAs, derived from the fermentation of dietary fibre, acting as signalling molecules via GPR43 to stimulate anorectic gut hormone release and, by extension, reduce appetite (Sleeth *et al.* 2010).

Chapter V:

General Discussion

5.1 Introduction

Obesity and its associated co-morbidities pose a daunting challenge to public health. Despite signs of stabilisation (Sperrin *et al.* 2013), the consequences of the dramatic rise in obesity experienced over the past few decades remain extensive. In England, approximately 25% of adults (aged 16 years and over) are currently classified as obese (HSE 2011). Therefore, there is an urgent need for strategies to improve appetite regulation and aid weight loss which can be applied at a population level.

Bariatric surgery remains the most effective treatment for the very obese (Sjostrom *et al.* 2004). However, due to its associated mortality risk, bariatric surgery is largely limited to treating morbid obesity (Flancbaum & Belsley 2007). Nevertheless, a better understanding of the mechanisms by which surgical operations such as the RYGB induce such dramatic weight loss, may enable us to exploit them therapeutically. One of the factors contributing to enhanced satiety following gastric bypass is the increased delivery of nutrients to distal regions of the small intestine, thereby increasing the secretion of the appetite-suppressing gut hormones GLP-1 and PYY (Beckman *et al.* 2011, Korner *et al.* 2005, le Roux *et al.* 2006, le Roux *et al.* 2007). However, it is increasingly evident that additional mechanisms are also involved (Pournaras *et al.* 2012, Saeidi *et al.* 2013). Intriguingly, recent evidence suggests that alterations in the relative abundance of certain gut microbial populations following RYGB may play a role in mediating the RYGB-associated weight loss (Liou *et al.* 2013), an effect which was also associated with increased caecal SCFAs and a shift in the microbial production of SCFAs favouring propionate. In keeping with these findings, another recent study reported a significant negative correlation between adiposity and caecal butyrate and propionate concentrations in germ-free mice receiving faecal transplants from human twin donors discordant for obesity (Ridaura *et al.* 2013). SCFAs may, therefore, play a role in influencing host energy homeostasis.

Given the present success of GLP-1 mimetics in reducing body weight as well as improving glucose control, targeting L cell secretion is an exciting prospect (Tong & Sandoval 2011). There is also evidence to suggest that co-administration of two gut hormones may be a more effective strategy for suppressing appetite and reducing energy intake (Field *et al.* 2010b). However, exogenous administration of gut hormones has several drawbacks: it can result in adverse effects, such as nausea and vomiting (Degen *et al.* 2005, Kanoski *et al.* 2012, le Roux *et al.* 2008), and may face difficulties with compliance, as it usually requires a needle-based delivery system which can cause discomfort. Furthermore, the injection of gut hormones is associated with a risk of eliciting an immune reaction and antibody formation. Consequently, there is considerable interest in evaluating the therapeutic potential of promoting the secretion of endogenous GLP-1 and PYY.

SCFAs have been proposed to account for the reduced food intake and the increased circulating levels of GLP-1 and PYY observed following a high intake of NDCs (Cani *et al.* 2009, Delzenne *et al.* 2005, Keenan *et al.* 2006, Zhou *et al.* 2008); a hypothesis fuelled by the discovery that the SCFA receptors, GPR41 and GPR43, are enriched in enteroendocrine L cells (Karaki *et al.* 2006, Tolhurst *et al.* 2012a). However, experimental evidence demonstrating a direct link between SCFAs, GPR43 activation and L cell secretion is limited.

The main hypotheses examined in this thesis, and discussed below, were i) that SCFAs would stimulate gut hormone release and ii) that this would occur via a GPR43-dependent mechanism.

5.2 The role of SCFAs in gut hormone release

Existing immortalised enteroendocrine 'L cell' lines do not accurately represent colonic physiology, in part due to their lack of PYY production (Reimann *et al.* 2008). Chapter II of this thesis described the development, optimisation and validation, of a murine primary L cell model for the assessment of GLP-1 and PYY release. These primary colonic cultures

contained a mixed cell population. This was necessary as isolated L cells do not survive in culture (Reimann *et al.* 2008) and indeed, may be advantageous, as the L cells remain in close proximity to neighbouring cells and may retain a degree of exposure to paracrine signals. The methodology was adapted to permit the isolation of L cell-containing human primary colonic cultures. Human L cells in culture were demonstrated for the first time to secrete both GLP-1 and PYY in response to glucose and to an increase in intracellular cAMP.

The primary L cell model has limitations. Firstly, L cells are polarised cells, with an apical surface in contact with the gut lumen and a basolateral surface typically in contact with the microvasculature of the gut (Eissele *et al.* 1992). *In vitro*, L cell polarity is disrupted and both surfaces are exposed to the culture medium and to subsequent experimental treatments targeted at the luminal surface. Therefore, assuming that the test substances are acting solely via interaction with the luminal surface could be misleading.

There are two main, alternative primary *in vitro* intestinal cell models which attempt to maintain the polarity of intestinal cells. The first is the *ex vivo* 'everted gut sac' technique, which briefly consists of gently everting an isolated rat intestine, filling it with oxygenated tissue culture medium at 37°C, and dividing into 'sacs' (~2.5cm length) using braided suture silk. Individual sacs are then placed in flasks containing oxygenated culture medium at 37°C (Barthe *et al.* 1998). However, this technique relies on the integrity of the sacs and on the viability of the intestinal tissue, which may be very poor as disruption of the epithelium and severe interstitial oedema have been described to occur within 30-60 min (Levine *et al.* 1970, Plumb *et al.* 1987). A new technique, which has been gaining momentum, is that of the three-dimensional (3D) intestinal epithelial organoids also referred to as 'epithelial mini-guts' (Sato & Clevers 2013). These intestinal organoids grow from isolated Lgr5-expressing intestinal stem cells embedded in Matrigel, a 3D laminin- and collagen-rich matrix, in the presence of culture medium containing a cocktail of growth factors, Noggin and R-spondin (Wnt signal enhancer and Lgr5 ligand). These organoids occur as 'cysts' with a central

lumen flanked by a simple, highly polarised villus epithelium with multiple crypt-like structures projecting outwards (Sato *et al.* 2009). Therefore, to a certain extent, crypt architecture and cellular polarity are maintained. Additional advantages of this technique include the ability to maintain these cultures long term, enabling the study of epithelial cell fate determination and identification of factors which increase L cell numbers *in vitro* (Petersen *et al.* 2013).

A disadvantage of all three models is that L cells *ex vivo* lose their connection to the GI neuroendocrine network, which may be fundamental in the normal regulation of L cell function (Reimann *et al.* 2012). Despite these limitations, the data presented suggest that primary colonic cultures serve as a useful and valid model for studying gut hormone release from colonic L cells.

The primary L cell model was employed in Chapter III to determine the effect of SCFAs on GLP-1 and PYY release. This work demonstrated that, as hypothesised, SCFAs trigger the release of both gut hormones in parallel from murine primary L cells. SCFA-stimulated PYY release from primary colonic cultures had not been previously investigated. Moreover, these findings were demonstrated for the first time in human primary L cells. The stimulatory effect of propionate on both PYY and GLP-1 release was also reproduced *in vivo*, as described in Chapter III, supporting the validity of the murine L cell model. The *in vivo* data derived from both rat and mouse studies support the simultaneous secretion of both gut hormones in response to propionate, in contrast to previous findings in rats and pigs, which showed an increase in circulating PYY but not GLP-1 in response to SCFAs (Anini *et al.* 1999, Cuche *et al.* 2000). However, the results of the rat study discussed in this thesis revealed that the effect on GLP-1 was transient in nature compared to the more sustained increase in circulating PYY observed. Based on the results presented, it may also be useful to measure secreted GLP-1 in portal vein blood where concentrations are higher.

Chapter IV describes the use of the primary L cell model to investigate components of the stimulus-secretion coupling mechanisms underlying propionate-stimulated gut hormone release. The preliminary experiments described in this thesis may suggest a potential role for G_i signalling and ERK1/2 activation in SCFA-mediated gut hormone release. However, these results require confirmation and the involvement of GPR41 and intracellular signalling components downstream of the cell surface receptors requires further investigation.

In accordance with hypothesis ii, it was demonstrated for the first time that PYY release, in addition to GLP-1 release, from primary Gpr43^{-/-} colonic cultures was significantly attenuated in response to propionate. Furthermore, this thesis presented the novel finding that Gpr43^{-/-} mice also have significantly lower plasma PYY and GLP-1 levels following intra-colonic administration of propionate compared to their wild type littermates. These findings are in keeping with, and further extend the published literature (Tolhurst *et al.* 2012a). Taken together, the results support the concept that SCFAs, derived from the fermentation of dietary fibre, signal via GPR43 to stimulate gut hormone release and, by extension, reduce appetite (Fig. 5.1). However, our current understanding of the complex interrelationship between diet, gut microbiota and host physiology is at best rudimentary. This thesis has taken a reductionist approach and focused primarily on just one bacterial metabolite and end-product of NDC fermentation. While this work demonstrated that all three major SCFAs stimulate gut hormone release, further work is required to dissect the signalling pathways activated by each SCFA and to identify potential differences. Crucially, it is perhaps naïve to think that these three SCFAs (C2-C4), despite being the major anions in the colonic lumen, are the only bioactive signals derived from gut bacterial fermentation.

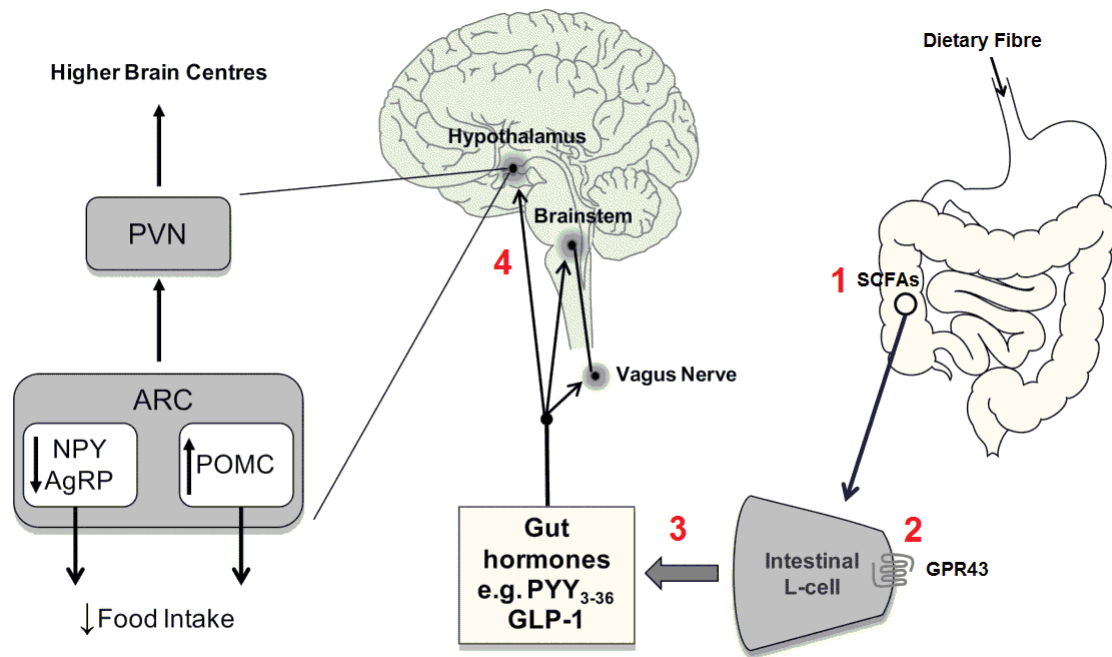


Figure 5.1 **The role of short chain fatty acids and GPR43 in appetite regulation.**

Short chain fatty acids (SCFAs), produced by the fermentation of dietary fibre by gut bacteria in the colon (1), are proposed to activate GPR43 on enteroendocrine L cells (2) stimulating the release of gut hormones (3) which influence food intake at three sites: the vagus nerve, the brainstem and the hypothalamus (4). Within the arcuate nucleus of the hypothalamus two neuronal populations are thought to be critical conduits through which peripheral signals are integrated to alter the drive to eat: orexigenic NPY/AgRP neurons and anorexigenic POMC neurons. The release of GLP-1 and PYY activates anorexigenic neurons and inhibits orexigenic neurons, ultimately resulting in a reduction in food intake. Connections between hypothalamic nuclei and higher brain centres influence the hedonic aspects of food ingestion. Adapted from: (Bewick 2012)

ARC, arcuate nucleus; AgRP, agouti related peptide; GLP-1, glucagon like peptide-1; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; PYY, peptide YY.

5.3 Colonic SCFAs and appetite regulation

Epidemiological evidence suggests that there is an inverse association between NDC intake and weight gain (Liu *et al.* 2003, Ludwig *et al.* 1999). This is supported by experimental studies which demonstrate that increasing the consumption of NDCs can reduce appetite and body weight in humans (Cani *et al.* 2006, Howarth *et al.* 2001, Rigaud *et al.* 1990, Rytting *et al.* 1989). However, these beneficial effects are not consistently observed, in part due to the large quantities of NDCs required to elicit them, which can cause gastrointestinal side effects and contribute to poor adherence in the long term (Chapman 2010, Grabitske & Slavin 2009). It is tempting to speculate that exploiting the mechanisms by which fermentable fibre suppresses appetite may offer a more effective strategy for weight management. The findings presented in this thesis support the hypothesis that increasing colonic propionate levels *in vivo* will suppress appetite by elevating circulating levels of GLP-1 and PYY.

The main challenge associated with testing this hypothesis is the delivery of SCFAs to the colon; apart from being unpalatable (Darzi *et al.* 2011), orally administered SCFAs are absorbed in the small intestine prior to reaching the colon (Schmitt *et al.* 1976, Schmitt *et al.* 1977). One strategy to address this is to develop novel delivery systems which when administered orally, are protected from digestion and absorption in the stomach and small intestine, and which are designed to ensure targeted release in the proximal colon (Kosaraju 2005). This could be achieved by encapsulating SCFAs or attaching them to NDC 'carrier molecules' via ester bonds for example, ensuring that the SCFAs are only liberated upon bacterial fermentation in the colon (Hartzell & Rose 2011). Strategies to achieve targeted delivery of SCFAs to the colon could provide a way to stimulate the release of endogenous gut hormones and thus improve appetite regulation and energy balance.

5.4 Future directions

The primary L cell model described in this thesis is a useful system for interrogating stimulus-secretion coupling mechanisms. There are numerous pharmacological tools available which can be employed within this system, and in conjunction with enteroendocrine cell lines, to further characterise the intracellular signalling mechanisms involved downstream of the receptors. My data demonstrated that SCFA-mediated gut hormone release is significantly impaired in *Gpr43^{-/-}* mice. GPR43 signals partly via a G_q -coupled pathway, which is associated with an increase in intracellular Ca^{2+} , a potent effector of exocytosis (Burgoyne & Morgan 2003). In support of this, Tolhurst *et al.* recently reported that acetate and propionate elevate $[Ca^{2+}]_i$, though a direct link to gut hormone secretion was not demonstrated (Tolhurst *et al.* 2012a). It would therefore be interesting to investigate SCFA-induced gut hormone secretion in the presence of a novel commercial G_q inhibitor, UBO-QIC. It would also be possible to investigate the role of i) intracellular $[Ca^{2+}]_i$ stores, using thapsigargin, a potent, cell-permeable, non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (Treiman *et al.* 1998) and ii) Ca^{2+} influx, using the extracellular Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA) (Chen *et al.* 2006) or specific voltage-sensitive Ca^{2+} channel blockers such as nifedipine (Nemoz-Gaillard *et al.* 1998). Inhibitors of downstream signalling components associated with exocytosis such as PKA and PKC could also be used. Understanding the intricate intracellular mechanisms underlying gut hormone secretion in both murine and human L cells in response to physiological stimuli is of fundamental importance and may facilitate the harnessing of these pathways therapeutically.

A critical advance in this field would also be the determination of the subcellular localisation of the SCFA receptors within L cells. It is currently unknown whether the receptors are localised to the apical or basolateral plasma membrane, or both. It is also possible that the receptors reside intracellularly, pending recruitment to the cell surface. The location of the receptors would attest to their physiological role and would also influence the feasibility of

pharmacologically targeting SCFA receptors *in vivo*. Identifying the subcellular localisation of SCFA receptors is currently hampered by the lack of high affinity antibodies. An alternative strategy could be perhaps to image a fluorescently conjugated GPR43-selective ligand (Hudson *et al.* 2013).

The recent development of these novel selective GPR43 agonists, including 'compound 1', which was shown to stimulate GLP-1 release from STC-1 cells (Hudson *et al.* 2013), also offers the exciting opportunity of being able to pharmacologically activate Gpr43 *in vivo* in rodents and consequently to evaluate its therapeutic potential.

It is also imperative to further characterise this system in the context of obesity. Obesity is associated with fundamental changes in the gut microbiota and SCFA production (Kimura *et al.* 2013, Ley *et al.* 2005, Ley *et al.* 2006, Ridaura *et al.* 2013, Turnbaugh *et al.* 2006). Furthermore, HFD feeding has been reported to up-regulate Gpr43 expression in mouse adipose tissue, skeletal muscle and liver (Cornall *et al.* 2011, Dewulf *et al.* 2011, Hong *et al.* 2005, Kimura *et al.* 2013). Paradoxically, supplementation of the HFD with fermentable inulin-type fructans in mice counteracted the HFD-induced increase in Gpr43 expression in adipose tissue, an effect associated with protection from HFD-induced body weight gain and fat mass accumulation (Dewulf *et al.* 2011). In contrast, overexpression of Gpr43 in adipose tissue *in vivo* was recently shown to protect against HFD-induced body weight gain in mice (Kimura *et al.* 2013). Only two studies have been carried out so far to investigate the energy homeostasis phenotype of Gpr43^{-/-} mice on a HFD background, and have reached different conclusions regarding whether the global absence of Gpr43 is protective or detrimental, potentially as a consequence of using different genetic backgrounds or due to the variation in gut microbial composition under different environments (Bjursell *et al.* 2011, Kimura *et al.* 2013). These discrepancies highlight the urgent need for further studies to address the tissue-specific and wider roles of SCFAs and GPR43 in energy homeostasis in the context of obesity.

The enteroendocrine system is instrumental in the regulation of post-prandial physiology and plays a central role in appetite regulation. The work presented in this thesis demonstrates that SCFAs can influence L cell function to trigger the release of the anorectic gut hormones GLP-1 and PYY. Propionate-induced gut hormone secretion was shown to be mediated via a Gpr43-dependent mechanism *in vitro* and *in vivo* using Gpr43^{-/-} mice. This mechanism may underpin, at least in part, the beneficial effect of fermentable fibre on appetite suppression and prevention of weight gain. Further work is required to elucidate the intracellular signalling mechanisms underlying GPR43-mediated gut hormone release as well as the wider role of GPR43 in energy homeostasis. This field opens up the exciting possibility of harnessing these pathways, pharmacologically or with novel functional foods, to activate the enteroendocrine system and suppress appetite via the release of endogenous gut hormones.

Appendix I: List of solutions

Cell lysis buffer: Add 0.25g $C_{24}H_{39}NaO_4 \cdot H_2O$ (sodium deoxycholate monohydrate), 0.88g NaCl, 0.5ml Igepal and 2.5ml Tris HCl (1M, pH 8) to 30ml GDW. Adjust volume to 40ml and add 1 tablet complete EDTA-free protease cocktail inhibitor (Roche, UK) and store at 4°C.

Dextran-coated charcoal: Add 2.4g charcoal and 0.24g dextran to 100ml phosphate buffer with gelatin and stir for 20 minutes.

Phosphate buffer: Dissolve 48g $Na_2HPO_4 \cdot 2H_2O$, 4.13g $KHPO_4$, 18.61g $C_{10}H_{14}H_2ONa_2 \cdot 2H_2O$ (EDTA) and 2.5g NaN_3 (sodium azide) in 5 litres of GDW that has been boiled and allowed to cool. Adjust pH to 7.4 and store at 4°C.

Phosphate buffer with gelatin: The buffer is produced as above, except that 12.5g gelatine is added to the boiled water prior to cooling, and before addition of the other reagents.

Phosphate buffered saline (PBS) (0.1M, 10x): Add 80g NaCl, 2g KCl, 14.4g Na_2HPO_4 and 2.4g KH_2PO_4 to 800ml GDW. Adjust volume to 1L and pH to 7.4.

Secretion buffer: Dissolve 0.335g KCl, 8.065g NaCl, 0.353g $NaHCO_3$, 0.187g $NaH_2PO_4 \cdot 2H_2O$, 2.6ml 1M $CaCl_2$, 1.2ml 1M $MgCl_2$ and 2.383g HEPES in 800ml GDW. Make up to 1L and adjust pH to 7.4.

0.06M sodium barbitone buffer: Dissolve 5.15g $C_8H_{11}N_2NaO_3$ and 0.15g NaN_3 in 500ml GDW that has been boiled and allowed to cool. Adjust pH to 8.0 and store at 4°C.

50 x TAE buffer: Dissolve 242g Trizma base in 843ml water and add 57ml glacial acetic acid and 100ml 0.5M $C_{10}H_{14}H_2ONa_2 \cdot 2H_2O$ (EDTA) pH 8.

Appendix II: Composition of protease inhibitor cocktail

Table A1. **Composition of protease inhibitor cocktail** (Sigma, UK).

Inhibitor	Concentration (neat) mmol/L	Proteases inhibited
AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride	104	Serine proteases e.g. trypsin, chymotrypsin, plasmin, kallikrein and thrombin
Aprotinin	0.08	Serine proteases e.g. trypsin, chymotrypsin, plasmin, and kallikrein
Bestatin hydrochloride	4	Aminopeptidases e.g. leucine aminopeptidase and alanyl aminopeptidase
E-64 N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide	1.4	Cysteine proteases e.g. calpain, papain, cathepsin B, and cathepsin L.
Leupeptin hemisulfate salt	2	Serine and cysteine proteases e.g. plasmin, trypsin, papain and cathepsin B.
Pepstatin A	1.5	Acid proteases e.g. pepsin, renin, and cathepsin D, and many microbial aspartic proteases

Appendix III: Publications and Presentations

Publications

Weight gain and insulin sensitivity: a role for the glycaemic index and dietary fibre?
Sleeth, M.L., **Psichas, A.**, Frost, G. (2012) *Br J Nutr* **27**: 1-3

Oral presentations

‘Short chain fatty acids stimulate the release of the anorectic gut hormone peptide YY from enteroendocrine L cells *in vitro* and *in vivo*’

A. Psichas

Rank Prize Funds Mini-Symposium on “Non-Digestible Polysaccharides in the Human Diet: From Farm to Pharmacy”, Cumbria, July 2012

‘The mechanisms underlying the beneficial effects of short chain fatty acids’

A. Psichas

BBSRC Diet and Health Research Industry Club (DRINC) 7th Dissemination Event, Manchester, October 2011

Poster presentations

‘Short chain fatty acids stimulate the release of gut hormones *in vitro* and *in vivo* via Gpr43.’

A. Psichas, M. Sleeth, K.G. Murphy, L. Brooks, G. Bewick, M. Ghatei, S.R. Bloom, G. Frost

Imperial College Graduate School Research Symposium, London, July 2013

‘Short chain fatty acids stimulate the release of gut hormone peptide YY from human primary L cells’ [Physiological Society Poster Prize]

A. Psichas, S.E.K. Zac-Varghese, K.G. Murphy, M.A. Ghatei, S.R. Bloom, G. Frost

Physiological Society Annual Meeting, Edinburgh, July 2012

‘Stimulating gut hormone release using propionate: from cells to humans’ [Poster Prize]

A. Psichas, E. Chambers, A. Viardot, D. Morrison, S. Zac-Varghese, K.G. Murphy, G. Bewick, G. Frost

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