Investigation of gut hormone physiology in the regulation of appetite

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Abstract

Peptide tyrosine tyrosine (PYY) and glucagon like peptide-1 (GLP-1) are endogenous, anorectic gut hormones released from entero-endocrine L cells. The aims of this thesis were to: investigate the breakdown of PYY using peptide analogues in vitro and in vivo, determine the stimuli for release of PYY and GLP-1 from L cells in vitro and finally to attempt to stimulate the endogenous secretion of PYY and GLP-1 and so suppress appetite in man.

The breakdown of PYY was studied using specially designed PYY analogues with changes to known enzyme cleavage sites. Degradation of the analogues was studied by incubation with proteolytic enzymes. Receptor binding assays were carried out to confirm that changes to the PYY structure had not altered binding to the endogenous PYY receptors. In vivo studies in rodents previously confirmed an extended pharmacological profile of these analogues.

In order to study the various stimulants for PYY and GLP-1 release, a primary L cell model was developed. This was used to study the effect of various nutrients: glucose, amino acids and short chain fatty acids (SCFA) on PYY and GLP-1 release. In mouse and human primary L cell cultures the SCFA propionate increased PYY release significantly compared to basal levels, indicating that propionate is a potent stimulus of PYY release.

To further investigate the results of the in vitro work, a randomised, double blind, crossover study was carried out in human volunteers to evaluate the effect of propionate on appetite. Administration of propionate ester over six days reduced energy intake at a buffet meal by 18.8% compared to control (P < 0.05, n = 20). However, there was no significant change in plasma GLP-1 or PYY levels between the groups, possibly suggesting an alternative explanation for the reduction in appetite seen. This may provide an interesting avenue for future studies.

These studies of the physiological mechanisms underlying release and degradation of PYY and GLP-1 may contribute towards the development of an anti-obesity therapy based around L cell stimulation.
Declaration of contributors

All of the work in this thesis was carried out by the author unless stated otherwise.

All collaborations are listed below.

Chapter 2:

Development of the PYY analogue was by Professor Stephen Bloom. Testing of the analogues in animal studies was carried out by the PYY analogue team, Imperial College (Dr. Tricia Tan, Dr. Ben Field, Dr. James Minnion, Ms. Joyceline Shillito, Dr. Jordan Baxter, Dr. Melisande Addison, Dr. Mohammed Hankir, Dr. Samar Ghourab, Ms. Klara Hostomska, Ms. Jennifer Parker, Mr. James Plumer, Dr. Katherine Simpson and Dr. Victoria Salem). The clinical studies were carried out in collaboration with Dr. Victoria Salem, Dr. Tricia Tan and Dr. Ben Field. Dr. James Gardiner and Dr. Emily Thompson assisted in setting up the hY1 and hYS receptor over-expressing cell lines. Ms. Joyceline Shillito assisted in the receptor binding assays and enzyme degradation studies.

Chapter 3:

Dr. Fiona Gribble, Cambridge, kindly taught me the method of primary cell culture. Dr. Jelena Anastasvoska, Dr. Benjamin Jones and Ms. Arianna Psichas assisted with these studies. Human colon biopsies were obtained by Dr. Julian Walters, Dr. Jonathan Nolan and Dr. Ian Johnston. Dr. Roger White and Dr. Jimmy Bell assisted by providing primary cell culture facilities.

Chapter 4:

The human study was performed in collaboration with Ms. Norlida Matt Daud. Dr. Douglas Morrison, Glasgow, provided all of the test reagents used in the study.

All human studies were carried out at the Sir McMichael Centre, the Clinical Investigations Unit, Hammersmith Hospital.

Dr. Michael Patterson provided assistance and advice for all of the in house radioimmunoassays carried out. Professor Mohammad Ghatei established and maintained all of the radioimmunoassays.
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for my father and mother
### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti related protein</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DEBQ</td>
<td>Dutch Eating Behaviour Questionnaire</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPP IV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Ex-4</td>
<td>Exendin 4</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenly Methoxy-Carbonyl</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>GCG</td>
<td>Glucagon</td>
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GDW  Glass distilled water
GI    Gastrointestinal
GIP   Glucose-dependent insulinotropic peptide
GLP-1 Glucagon-like peptide-1
GLP-1R Glucagon-like peptide-1 receptor
GLP-2 Glucagon-like peptide-2
GRPP  Glicentin-related pancreatic polypeptide
GTE   Glucose tris EDTA
HEPES N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC  High performance liquid chromatography
HPT   Hypothalmo-pituitary-thyroid
ICV   Intracerebroventricular
IP    Intraperitoneal
KBB   Kidney brush borders
LB    Lysogeny broth
LHA   Lateral hypthalamic area
MALDI-ToF Matrix-assisted laser desorption/ionization-time of flight
MCH   Melanin-concentrating hormone
MCR   Melanocortin receptor
MCR2  Melanocortin-2 receptor
MCR3  Melanocortin-3 receptor
MCR4  Melanocortin-4 receptor
MPGH  Major proglucagon fragment
MSH   Melanocyte-stimulating hormone
NDC   Non digestible carbohydrate
NEP   Neprilysin
NHS   National health service
NPY  Neuropeptide Y
NTS  Nucleus of the solitary tract
OXM  Oxyntomodulin
PBS  Phosphate buffered saline
PEG  Polyethylene glycol
PEI  Polyethylenimine
PMSF Phenylmethylsulphonylfluoride
POMC Proopiomelanocortin
PP  Pancreatic polypeptide
PVN  Paraventricular nucleus
PYY  Peptide tyrosine tyrosine
RBA  Receptor binding assay
RIA  Radioimmunoassay
RNA  Ribonucleic acid
SC  Subcutaneous
SCFA  Short chain fatty acid
SDS  Sodium dodecyl sulphate
SP  Spacer peptide
SPPS  Solid phase peptide synthesis
TAE  Tris, acetic acid, EDTA
TFA  Trifluoroacetic acid
TRH  Thyrotrophin releasing hormone
TSH  Thyroid stimulating hormone
VAS  Visual analogue scales
VMH  Ventromedial hypothalamus
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Chapter 1

1. Introduction

1.1 Obesity

Claude Bernard and Walter Cannon described control of the internal milieu, homeostasis, as an essential condition of life (Cannon, 1932; Bernard et al., 1949). In terms of energy homeostasis, food intake must precisely match energy output in order for weight to remain stable. However, food intake and energy output do not precisely correlate causing weight to fluctuate. Excess energy is stored, mainly in adipose tissue, and this energy store may be relied upon in times of emergency or food deprivation. In current Western lifestyles, demand for physical exertion is low and food supply is abundant. In this environment, chronic, excess energy storage leads to increasing adipose tissue mass and obesity. Obesity, the excessive accumulation of adipose tissue, is an important risk factor for a number of common metabolic diseases, such as type 2 diabetes mellitus and ischaemic heart disease (World Health Organisation, 2006). In adult populations, obesity is categorised by body mass index (BMI); weight (kg) divided by height squared (m$^2$) (Table 1).

<table>
<thead>
<tr>
<th>BMI (kg/m$^2$)</th>
<th>WHO classification</th>
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<tbody>
<tr>
<td>&lt; 18.5</td>
<td>Underweight</td>
</tr>
<tr>
<td>18.5-24.9</td>
<td>Healthy</td>
</tr>
<tr>
<td>25-29.9</td>
<td>Overweight</td>
</tr>
<tr>
<td>30-39.9</td>
<td>Obese</td>
</tr>
<tr>
<td>≥ 40</td>
<td>Morbidly obese</td>
</tr>
</tbody>
</table>

Table 1: The World Health Organisation (WHO) classification of overweight and obese (World Health Organisation 2006)
1.2 Prevalence and economic cost of obesity

In the United Kingdom (UK) one in three adults are obese (Zaninott and Wardle, 2006). This figure is predicted to rise to 60% by the year 2050 (Kopelman, 2010). Globally by 2015, the WHO estimates that approximately 700 million adults will be obese (World Health Organisation, 2006). The cost of obesity to the UK is enormous. The National Health Service spends approximately £991 million per year on obesity related morbidity and the economic cost related to obesity has been estimated at around £7 billion (Butland et al., 2008).

![Global obesity map](image)

**Figure 1.1:** Global obesity map. Figure adapted from World Health Organisation, 2005
1.3 Aetiology of obesity

The causes of obesity are multifactorial (Figure 1.2). The thrifty genotype hypothesis proposed by J.V. Neel in 1962 was an elegant way to explain the increased prevalence of obesity (Neel, 1962). Briefly, it was proposed that in times of starvation, individuals that survived to reproduce were those able to store energy and therefore who had the capacity to become obese (Neel, 1962). It is also possible that the intrauterine environment determines propensity to adult obesity (Barker, 1997). Periods of malnutrition during pregnancy are likely to lead to foetal adaption. Correlates were found between birth weight and disease, for example, low birth weight was associated with an increased risk of coronary heart disease and diabetes (Barker, 1997). Support for this theory also comes from studies of children born during times of famine. A study looking at young men born during the Dutch famine (1944-45), demonstrated that low birth weight correlated with a significantly increased risk of obesity in later life (Ravelli et al., 1976).
A genetic basis for obesity has been identified by studying identical twins reared apart (Stunkard et al., 1990). A small number of obese individuals have monogenic syndromes such as leptin or leptin receptor defects (Farooqi and O'Rahilly, 2005). However, the more common form of obesity is polygenic. This is where several genes interact with an “at risk” environment. Linkage studies have identified over 600 genes associated with obesity in this way (Rankinen et al., 2006). These genes only have a small individual effect on weight but their additive effects account for large, 40-70%, variation in BMI in the overall population (Maes et al., 1997). An example of such a gene is the fat mass and obesity associated gene (FTO). This gene was initially identified as part of the Wellcome Trust Case Control Consortium and single-nucleotide polymorphisms (SNPs) in this gene were found to be strongly associated with BMI (Frayling et al., 2007). Therefore, in the vast majority of individuals, a combination of increased consumption of highly calorific foods, reduced energy expenditure (due to increased mechanisation and transport) and an inherent genetic susceptibility ultimately leads to obesity.

1.4 Consequences of obesity

Adipose tissue, particularly visceral adipose tissue, is metabolically active. Adipocytes release inflammatory cytokines and obesity can be regarded as a chronic inflammatory process (Rocha and Libby, 2009). It is well established that inflammation contributes to atherosclerosis and this partly explains the increased risk of ischaemic heart disease and stroke in obese individuals. In addition, obesity increases the risk of type 2 diabetes and insulin resistance. This is disturbing as the complications of diabetes, in particular ischaemic heart disease, that were previously confined to the older generations are now being seen in younger age groups (Lobstein and Jackson-Leach, 2006). Obesity is an independent risk factor for both hypertension and dyslipidaemia (Brown et al., 2000). The combination of central obesity, hypertension, increased blood glucose and dyslipidaemia in an
individual is known as the metabolic syndrome; a clustering of risk factors for ischaemic heart disease and type 2 diabetes (Alberti et al., 2006).

Obesity leads to impaired respiratory function and sleep apnoea, due to the mechanical forces involved (Resta et al., 2001). Osteoarthritis, particularly of the knees and hips, is another example of mechanical problems caused by obesity (Lementowski and Zelicof, 2008). Obesity also increases the risk of certain cancers: colon cancer (Giovannucci, 1995), pancreatic cancer (Patel et al., 2005), breast cancer (Protani et al., 2010) and ovarian cancer (Olsen et al., 2007). The pathophysiology of obesity leading to increased cancer risk is incompletely understood but may be due to increased mitogenic signalling via the Insulin Like Growth Factor-1 receptor (IGF-1 receptor) (Giovannucci, 1995).

1.5 Treatment of obesity

The consequences of obesity are social, psychological and medical. Obesity clinics currently employ a multidisciplinary approach, involving dieticians, psychologists, endocrinologists and surgeons. It is estimated that 10% weight loss significantly improves glycaemic control, lipids, blood pressure and lifespan (Goldstein, 1992). The rising prevalence of obesity and the burden that this places on society has led to a great demand to find a safe, effective and affordable treatment.

1.5.1 Diets

Various diets have been recommended for weight reduction. These can be categorised as low calorie diets, low carbohydrate diets, low fat diets or a combination of these factors. Low calorie diets tend to be high in carbohydrate and low in fat with reduced energy content. The National Cholesterol Education Program (NCEP) (Yu-Poth et al., 1999) diet is an example of this, as are commercial diets
such as Weight Watchers (Freedman et al., 2001). Low calorie diets are considered safe but have limited effectiveness, 8% weight loss over a 3-12 month period dropping to 4% over 4 years (National Heart, Lung and Blood Institute, 1998). Low carbohydrate diets, such as the Atkins diet, have severely restricted carbohydrate content, but are not calorie restricted and fat and protein content is not limited. The body’s glycogen stores are depleted and gluconeogenesis induced with lean tissue and adipose tissue being catabolised. This diet is not recommended for lifelong treatment and is contraindicated in patients with diabetes or with liver or renal impairment (Atkins, 1972).

Low fat diets, such as the Ornish diet have also been recommended (Ornish et al., 1998). In combination with other strategies, such as exercise and stress management, these can be moderately effective with more than 5.8 kg weight loss maintained after 5 years (Ornish et al., 1998). In a two year head to head trial comparing a low fat diet, a low carbohydrate diet and a Mediterranean diet, the low carbohydrate diet achieved the greatest weight loss (Shai et al., 2008). For all three diets, two phases of weight loss were seen; an initial phase where maximum weight reduction occurred, followed by partial rebound and a plateau phase, the weight maintenance phase (Figure 1.3) (Shai et al., 2008).
Figure 1.3: Comparison of low fat, Mediterranean and low carbohydrate diets. Graph showing mean weight changes over 2 years, with vertical bars indicating standard error. Low fat diet (red diamonds), Mediterranean diet (yellow squares) and low carbohydrate diet (blue triangles) (Shai et al., 2008).

1.5.2 Medical treatment

Centrally acting agents have been used to treat obesity by increasing satiety (post meal satisfaction that suppresses further food intake). Serotonin (5-HT) in the form of fenfluramine was widely used in the 1980’s to treat obesity (Stunkard et al., 1980). However, by 1997 all forms of fenfluramine were withdrawn due to side effects: cardiac valvulopathy (Connelly et al., 1997) (Rothman and Baumann, 2009) and pulmonary hypertension (Abenhaim et al., 1996). 5-HT acts at the 5-HT receptor of which 13 subtypes have been identified (Hoyer et al., 2002). Activation of the 5-HT \textsubscript{2C} receptor is responsible for the anorectic effects (Xu et al., 2008) whereas, activation of the 5-HT \textsubscript{2B} subtype leads to valvular heart disease (Fitzgerald et al., 2000). Sibutramine (Reductil, Abbott Laboratories), a serotonin and noradrenaline reuptake inhibitor, was considered to be a more selective agent and
have fewer side effects. However, this proved not to be the case and it was later withdrawn due to side effects of tachycardia and hypertension (European Medicines Agency, 2010). Lorcaserin is a specific 5-HT$_{2C}$ receptor agonist and is currently in Phase III clinical trials (Smith et al., 2009).

The endocannabinoid system has been another target of obesity therapy. Endocannabinoids are involved in the anticipation and wanting of food and blockade of this system may reduce the desire and enjoyment of eating (Di, V et al., 2001). Rimonabant (Acomplia, Sanofi-Aventis), a cannabinoid 1 receptor antagonist, was withdrawn from treatment due to an increased suicide risk, anxiety and depression (European Medicines Agency, 2008).

Orlistat, a pancreatic and intestinal lipase inhibitor, is currently the only available medical treatment for obesity. It is taken before meals and prevents digestion and absorption of fats, resulting in increased fat excretion. This has limited effectiveness and multiple gastrointestinal side effects such as diarrhoea, flatulence, abdominal pain and dyspepsia. At the end of four years treatment, the XENDOS study showed that individuals taking orlistat only achieved 2.8 kg greater weight loss than those taking placebo (Torgerson et al., 2004).
1.5.3 Bariatric surgery

Bariatric surgery treats obesity through modifications of the anatomy of the gastrointestinal tract. The National Institute for Clinical Excellence (NICE) has issued guidance as to who would benefit from these procedures (Figure 1.4) (NICE, 2006).

<table>
<thead>
<tr>
<th>BMI 35-40 kg/m²</th>
<th>BMI 41-50 kg/m²</th>
<th>BMI &gt; 50 kg/m²</th>
</tr>
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<tbody>
<tr>
<td>• Plus comorbidities e.g. type 2 diabetes, hypertension</td>
<td>• Plus comorbidities</td>
<td>• Recommended as a first line option (instead of lifestyle interventions or drug treatment) for adults in whom surgical intervention is considered appropriate</td>
</tr>
<tr>
<td>• Tried and failed non-surgical measures to maintain weight loss for at least 6 months</td>
<td>• Tried and failed non-surgical measures to achieve weight loss for at least 6 months</td>
<td></td>
</tr>
<tr>
<td>• Will receive intensive management in a specialist obesity service</td>
<td>• Will receive intensive management in a specialist obesity service</td>
<td></td>
</tr>
<tr>
<td>• Person is fit for anaesthesia and surgery</td>
<td>• Person is fit for anaesthesia and surgery</td>
<td></td>
</tr>
<tr>
<td>• Commits to long term follow up</td>
<td>• Commits to long term follow up</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.4: NICE guidelines for bariatric surgery (NICE, 2006)

Bariatric surgery can be classified as restrictive or bypass procedures or as a combination of these (Figure 1.5). Restrictive operations include gastric banding (an adjustable, inflatable band wrapped around the stomach to create a small pouch) and vertical sleeve gastrectomy (restriction of the gastric body creates a smaller, tube like stomach). Roux-en-Y gastric bypass is a combination of restriction and bypass procedures. Here the stomach is stapled to produce a small pouch, the small intestine is divided at the midjejunum, the distal portion is anastomosed to the gastric pouch and the proximal portion of the jejunum anastomosed to the distal portion of the stomach. Biliopancreatic diversion with a duodenal switch is a mainly bypass procedure; a portion of the stomach is resected and the remaining part connected to a distal part of the small intestine, bypassing much of the duodenum and jejunum (DeMaria, 2007).
Figure 1.5: Diagram illustrating the various procedures involved in bariatric surgery. Operations for the treatment of obesity include (A) gastric banding, (B) vertical (sleeve) gastrectomy, (C) Roux-en-Y gastric bypass, (D) biliopancreatic diversion with a duodenal switch. Diagram from (DeMaria, 2007)
Gastric banding is the commonest type of restrictive surgery performed. This results in weight loss of about 20% at 12 and 36 months (Maggard et al., 2005). Of the bypass procedures performed, Roux-en-Y gastric bypass is considered the gold standard. This results in a 25% weight loss at 12 and 36 months (Maggard et al., 2005). In general, bypass procedures lead to significantly more weight loss compared to restrictive procedures (Brolin, 2002; Maggard et al., 2005). In addition, patients undergoing gastric bypass have improved glycaemic parameters compared to those undergoing gastric banding (Le Roux et al., 2006a).

Bariatric surgery is associated with significant morbidity and mortality. These procedures should only be performed in centres of excellence by experienced surgeons. The complications of surgery include: dumping syndrome (20%), anastomotic leak (1-2%), incisional hernia (7%), infections (6%), thromboembolism (1-3%) and pneumonia (4%) (Bueter et al., 2009).

This success of gastric bypass surgery in inducing weight loss is due to a number of factors. Firstly, gastric restriction limits the amount of food that can be comfortably swallowed. Secondly, bypass of part of the small intestine mildly reduces absorption of nutrients. Finally, accelerated delivery of nutrients to the distal gastrointestinal tract, to the ileum and colon, is thought to stimulate the release of gut hormones that suppress appetite (Mun et al., 2001; Strader et al., 2005). Gut hormones are peptides that signal information to the brain about nutrient delivery to the gut. They serve as integrators between brain and gut function. This increased release of gut hormones may be the cause of the greater weight loss seen in bypass procedures rather than in restrictive procedures (Le Roux et al., 2006a).
1.6 The hypothalamus and appetite circuits

The concept that the brain regulates appetite is well established. In the 19th century it was observed that patients with pituitary tumours were obese (Bramwell, 1888). In the 1940’s, experimental lesioning of rat hypothalami resulted in obese rodents (Hetherington and Ranson, 1942). Subsequent studies demonstrated that specific destruction of the lateral hypothalamus in rats reduced feeding and this was designated a feeding centre (Anand and Brobeck, 1951). In contrast, lesioning of the ventromedial nucleus (VMN) led to hyperphagia and obesity (Hetherington and Ranson, 1939). A dual centre model of feeding was proposed with a feeding centre in the lateral hypothalamus and a satiety centre in the VMN (Stellar, 1954). It is now accepted that appetite is regulated by a complex network of neurones and circuits via hormones and neuropeptides rather than specific centres located in discrete hypothalamic areas (Woods et al., 1998).

The hypothalamus regulates appetite and metabolism by detecting peripheral signals, e.g. from the gut, adipose tissue and nutrients within the blood. The blood brain barrier (BBB) separates cerebrospinal fluid from blood and prevents circulating, gut derived peptide hormones from entering the central nervous system (CNS) (Reese and Karnovsky, 1967). Peptide hormones must therefore cross at areas where the BBB is incomplete or via specific transport mechanisms. The hypothalamus lies adjacent to three circumventricular organs (CVO) which are areas lacking a BBB: the median eminence, subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT) (Johnson and Gross, 1993). The SFO and OVLT contain neuronal cell bodies and are known as sensory CVOs (Johnson and Gross, 1993). These CVOs are uniquely placed to monitor the blood and transmit this information to the brain by axonal projections. Therefore, it is likely that peptide hormones communicate with the hypothalamus via these CVOs.
1.6.1 The Arcuate nucleus (ARC)

The arcuate nucleus (ARC) is a key area involved in appetite regulation. The ARC is found at the base of the hypothalamus above the median eminence, and may detect peripheral signals due to its proximity to this CVO (Broadwell and Brightman, 1976; Shaver et al., 1992). Insulin (Marks et al., 1990), leptin (Mercer et al., 1996), growth hormone (Kamegai et al., 1996), glucocorticoid (Hisano et al., 1988) and sex hormone (Sar et al., 1990) receptors are all present within the ARC making it a likely centre for integration of metabolic signals. The ARC also receives neuronal information from the paraventricular nucleus (PVN) and nucleus of the tractus solitarius (NTS) and there is reciprocal signalling between these nuclei (Ricardo and Koh, 1978).

Within the ARC are two distinct neuronal populations. The first co-expresses orexigenic (appetite stimulating) neuropeptides (Broberger et al., 1998b; Hahn et al., 1998): agouti related peptide (AgRP) (Ollmann et al., 1997) and neuropeptide Y (NPY) (Chronwall, 1985). AgRP expression is unique to the ARC whereas NPY expression is more widespread (Broberger et al., 1998b). Therefore, AgRP is used as a marker to map the neuronal distribution of NPY neurones originating from the ARC. The second neuronal population within the ARC expresses anorectic (appetite suppressing) peptides, pro-opiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART) (Elias et al., 1998). The orexigenic NPY/AgRP neurones synapse onto the anorectic POMC neurones and exert an inhibitory tone over these neurones (Csiffary et al., 1990). This inhibitory tone is mediated through the inhibitory amino acid neurotransmitter γ amino butyric acid (GABA) (Horvath et al., 1997).

From the ARC, signals are relayed to various downstream effector neurones. As well as the PVN (Bai et al., 1985) and the NTS (Ricardo and Koh, 1978), the ARC relays information to the lateral hypothalamic area (LHA) (Broberger et al., 1998a), dorsomedial nucleus (DMN) (Bai et al., 1985) (Chronwall, 1985) and the VMN (Chronwall, 1985).
1.6.2 The Paraventricular Nucleus (PVN)

The PVN is located at the border of the third ventricle and receives afferent innervation from the ARC, brainstem, cortex and limbic areas (Swanson and Sawchenko, 1980). Both NPY and POMC neurones originating from the ARC project to the PVN (Cowley et al., 1999). The PVN is sensitive to both orexigenic and anorectic signals including: NPY (Lambert et al., 1995), ghrelin (Lawrence et al., 2002), orexin-A (Edwards et al., 1999), cholecystokinin (CCK) (Hamamura et al., 1991), leptin (Elmquist et al., 1997) and GLP-1 (Van Dijk et al., 1996). The PVN is divided into a medial parvocellular division and a lateral magnocellular division. The medial division expresses: thyrotrophin releasing hormone (TRH) (Fliers et al., 1994), corticotrophin releasing hormone (Merchenthaler et al., 1984) and somatostatin (Alonso et al., 1992). The lateral division expresses vasopressin and oxytocin which project to the posterior pituitary (Hatton et al., 1976). NPY neurones originating in the ARC project to TRH neurones in the PVN (Broberger et al., 1999). POMC neurones originating in the ARC potentiate GABA signalling within the PVN (Cowley et al., 1999). The PVN is important for energy balance as it integrates signals from various appetite centres, regulates the thyroid and adrenal neuroendocrine axes, as well as modulating sympathetic activity.

1.6.3 The Dorsomedial Nucleus (DMN)

Experimental lesioning of the DMN leads to hyperphagia and obesity and the DMN may also be considered a satiety centre (Bernardis and Bellinger, 1987). NPY neurones originating in the ARC also terminate in the DMN (Bai et al., 1985) and neurones from the DMN communicate with the PVN (Swanson and Sawchenko, 1980). Therefore, the DMN may serve as an additional point of integration between the ARC and PVN. The DMN may also play a role coordinating circadian rhythm with feeding and energy expenditure (Gooley et al., 2006).
1.6.4 The Ventromedial Nucleus (VMN)

VMN destruction by lesioning (Hetherington AW and Ranson SW, 1939), toxins (Shimizu et al., 1987) or neuronal transport blockade with colchicines (Choi and Dallman, 1999) increase food intake. This led to the hypothesis that the VMN acts as a satiety centre. However, the VMN is sensitive to both orexigenic and anorectic neurotransmitters. Injection into the VMN of NPY (Stanley et al., 1985), GABA (Grandison and Guidotti, 1977), galanin (Kyrkouli et al., 1990) and β-endorphin (Grandison and Guidotti, 1977) increase food intake whilst leptin injection reduces food intake (Jacob et al., 1997). Neurones originating from the ARC containing NPY (Chronwall, 1985), β-endorphin (Finley et al., 1981) and CART (Kristensen et al., 1998) project to the VMN. These may act on neurones within the VMN expressing brain derived neurotrophic factor (BDNF) (Xu et al., 2003). BDNF is regulated by nutritional status, leptin and melanocortin signalling and appears to have an inhibitory role on appetite as reduced BDNF receptor expression or signalling increases food intake and leads to weight gain (Rios et al., 2001; Xu et al., 2003). Efferent neurones from the VMN connect with the amygdala and brainstem (Saper et al., 1976). The amygdala is important in learning and behaviour and has a role in monitoring caloric intake (King et al., 1998) and “overriding” satiety signals (Petrovich et al., 2002). The reward pathways involving the limbic system, cortex, hippocampus and amygdala play an important role in the hedonistic aspects of appetite regulation, and it is likely that these systems are responsible for regulating the homeostatic weight regulation system, leading to over-eating and obesity (Berthoud, 2004).

1.6.5 The Lateral Hypothalamic Area (LHA)

The LHA was previously designated as a feeding centre. NPY and POMC neurones from the ARC both directly synapse onto neurones within the LHA (Broberger et al., 1998a) and these neurones are
sensitive to the orexigenic effects of NPY (Stanley et al., 1993). The LHA contains neurones releasing orexigenic peptides, orexin A and B (Sakurai et al., 1998) and melanin concentrating hormone (MCH) (Bittencourt et al., 1992). In addition to their orexigenic properties, the orexins appear to increase arousal and may initiate food seeking behaviour in starvation (Chemelli et al., 1999). These neurones project widely within the hypothalamus and also connect to the hippocampus, amygdala, basal ganglia and thalamus, areas involved in memory, motivation, behaviour and learning (Peyron et al., 1998).

1.7 The brainstem, vagal nerve and appetite circuit

The brainstem, consisting of the midbrain, pons and medulla oblongata, has also been implicated in appetite regulation (Grill and Norgren, 1978). The dorsal vagal complex, comprising the NTS, the area postrema (a sensory CVO) and the dorsal motor nucleus of the vagus, is located within the medulla oblongata. The presence of nutrients within the gastrointestinal tract is partly relayed to the NTS via the vagus nerve (Berthoud, 2008) and partly via the blood (due to the proximity of the NTS to the area postrema) (Johnson and Gross, 1993). Vagal nerve afferents are stimulated by mechanical distension, chemical stimulation and local production of gut hormones (Schwartz, 2000). Taste receptors within the oral cavity (tongue and palate) are sensitive to sweet, bitter, sour and salty substances and transmit this information via the vagus nerve to the NTS (Scott and Verhagen, 2000). Within the stomach, the presence of food is sensed by receptors on vagal afferent sensitive to stretch and tension (Berthoud et al., 2001). In the upper small intestine, vagal afferent nerves present in the lamina propria have cholecystokinin (CCK) receptors and respond to CCK released from entero-endocrine I cells (Moriarty et al., 1997). Within the liver and portal vein, vagal nerve afferents detect glucose (Berthoud et al., 1992). In the lower gastrointestinal tract it is likely that the anorectic effect of the gut hormone peptide YY (PYY) is partly mediated by the vagus as vagotomy abolishes the anorectic effects of this hormone (Koda et al., 2005). In addition, receptors for the
anorectic gut hormone glucagon like peptide-1 (GLP-1), which is also secreted from the lower intestine, are found both within the nodose ganglion of the vagus nerve (Nakagawa et al., 2004) and also within the area postrema (Yamamoto et al., 2003). All of these signals from the various vagal nerve afferents are integrated with the NTS.

The NTS has a high density of NPY receptor binding sites (Dumont et al., 1998) and contains NPY neurones which project to the PVN (Sawchenko et al., 1985). There is extensive reciprocal connection between the hypothalamus and brainstem, in particular between the NTS and the ARC (Ricardo and Koh, 1978).

Therefore, peptide hormones have two main mechanisms of communicating with central appetite circuits. The first is through CVOs to hypothalamic nuclei or to the NTS, and the second is via stimulation of vagal nerve afferents and the subsequent transfer of information between the NTS and ARC (Figure 1.6).
Figure 1.6: Schematic diagram of the pathways involved in the central regulation of appetite

PYY$_{3-36}$, GLP-1 and oxyntomodulin (OXM) released from entero-endocrine L cells stimulate anorectic pathways in the hypothalamus and brainstem and may also act through the vagus nerve. Pancreatic polypeptide (PP) signals directly to neurones in the brainstem. Ghrelin released from the stomach during fasting may signal directly to the hypothalamus or act through the vagus nerve to stimulate food intake. NPY/AgRP and POMC neurones within the ARC signal to the PVN and other hypothalamic nuclei to increase or decrease appetite, respectively. Green arrows indicate orexigenic signals and red arrows indicate anorectic signals. Diagram from Murphy, K.G. (2006) Nature 14; 444(7121):854-9
1.8 Neuropeptides and neurotransmitters involved in appetite regulation

1.8.1 Neuropeptide Y (NPY)

NPY, a 36 amino acid polypeptide (Tatemoto, 1982b), is the most abundant peptide in the CNS, and the most orexigenic neuropeptide in the hypothalamus (Allen et al., 1983). Acute intracerebroventricular (ICV) NPY administration in rats increases appetite (Clark et al., 1984) and repeated administration leads to weight gain (Stanley et al., 1986). In the hypothalamus, NPY neurones are found in the ARC with projections throughout the hypothalamus (Morris, 1989). Hypothalamic NPY correlates with food intake; protein levels, measured by radioimmunoassay, increase with fasting and decrease with food intake (Sahu et al., 1988). In addition to its effects as an orexigenic neuropeptide, NPY also reduces energy expenditure when injected ICV in rats (Billington et al., 1991). Hormones involved in the control of energy balance, such as insulin and leptin, have a negative feedback effect on hypothalamic NPY expression (Stephens et al., 1995). NPY neurones within the ARC express Ob-R mRNA (the leptin receptor transcript) (Mercer et al., 1996) and CNS leptin administration reduces NPY expression in the hypothalamus (Wang et al., 1997). NPY acts through the NPY family of receptors which will be described in more detail in chapter 2.

1.8.2 The melanocortin system

POMC, a 267 amino acid peptide (Nakanishi et al., 1979; Roberts et al., 1979), is synthesised in the pituitary and ARC (Gee et al., 1983) and has also been localised in the NTS (Gee et al., 1983). Post-translational modification of POMC by prohormone convertases 1 and 2 (PC1) and (PC2) (Benjannet et al., 1991) is tissue specific (Figure 1.7) (Bicknell, 2008).
Figure 1.7: Schematic diagram of major end products of POMC processing. In the anterior pituitary, POMC cleavage products include: adrenocorticotrophin (ACTH), β lipotrophin (β-LPH) and the 16 kDa N terminal fragment. In the hypothalamus and intermediate lobe of the pituitary, POMC is processed further into: α-melanocyte stimulating hormone (α-MSH) and corticotrophin like intermediate lobe peptide (CLIP); β-LPH is processed into β-endorphin and γ-LPH and the N terminal fragment is processed into γ3-MSH. Fragments are generated by enzymatic cleavage by prohormone convertase (PC) 1 and 2 (Benjannet et al., 1991; Nakanishi et al., 1979; Gee et al., 1983; Tatro, 1996)

The biological effects of melanocortins (MC) are mediated through melanocortin receptors (MC-R) (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992) of which 5 subtypes have been identified (MC1 to 5-R) (Gantz et al., 1993a; Gantz et al., 1993b; Gantz et al., 1994). Of these MC3-R (Roselli-Rehfuss et al., 1993) and MC4-R (Mountjoy et al., 1994) are highly expressed within the CNS. MC3-R is localised to the hypothalamus and limbic system (Roselli-Rehfuss et al., 1993). MC4-R is more widely distributed in the hypothalamus, thalamus, cortex and brainstem (Mountjoy et al., 1994; Mountjoy and Wild, 1998). There is evidence that both MC3-R and MC4-R play an important role in appetite. MC3-R mRNA was found in both AgRP and POMC neurones within the ARC (Bagnol et al., 1999). MC3-R may act as an autoinhibitory receptor on these neurones. MC3-R knockout mice have
increased body fat mass but are not hyperphagic; the increased adiposity is therefore likely to be due to reduced energy expenditure (Butler et al., 2000). MC4-R knockout mice have hyperphagia and obesity (Huszar et al., 1997). MC4-R mutations in humans lead to obesity and appear to be the commonest single gene cause of human obesity as well as being implicated in polygenic obesity (Yeo et al., 2000).

MC-R agonists inhibit food intake and MC-R antagonists increase food intake (Giraudo et al., 1998). α-MSH the endogenous MC4-R ligand appears to have a tonic inhibitory effect on feeding (Rossi et al., 1998). α- MSH injection ICV suppresses food intake (Rossi et al., 1998). In addition, SHU919, a synthetic α-MSH antagonist increases food intake (Fan et al., 1997; Giraudo et al., 1998).

Agouti, a 131 amino acid peptide secreted from hair follicle cells that regulates synthesis of pigments, eumelanin and melanin (Bultman et al., 1992), is a high affinity MC-R antagonist (Lu et al., 1994). Agouti mice A\textsuperscript{Y} have bright yellow fur and are a rodent model of obesity due to dominant alleles at the agouti locus (A) leading to agouti peptide over expression (Dickie, 1969). Antagonism at MC1-R leads to the bright yellow fur colour whereas MC4-R antagonism leads to obesity (Lu et al., 1994). Agouti related transcript (AgRP), a 132 amino acid peptide, is another endogenous melanocortin antagonist (Ollmann et al., 1997) co-expressed by many NPY neurones in the ARC (Hahn et al., 1998; Broberger et al., 1998b). It is structurally homologous to agouti and it is also a high affinity MC3-R and MC4-R antagonist (Ollmann et al., 1997). ICV injection of AgRP leads to hyperphagia in rats (Rossi et al., 1998). NPY/AgRP neurones project widely from the ARC and are important in regulating appetite (Hahn et al., 1998). During fasting POMC expression is low (Mizuno et al., 1998) and AgRP increased (Mizuno and Mobbs, 1999).
1.8.3 Cocaine and amphetamine regulated transcript (CART)

CART expressing neurones are found throughout the CNS and CART is expressed abundantly in the hypothalamus, co-localising with POMC within the ARC (Elias et al., 1998) and with MCH in the LHA (Broberger, 1999). Initially CART was thought to be an endogenous anorectic peptide (Kristensen et al., 1998). CART mRNA expression decreases in fasted rats (Kristensen et al., 1998), ICV administration of CART inhibits feeding in rats (Kristensen et al., 1998) and CART antiserum increases feeding in rats (Kristensen et al., 1998). There has, however, been some conflicting evidence suggesting that CART may have a role as an orexigenic peptide (Abbott et al., 2001). In contrast to ICV injection, CART injection into the PVN, VMN, DMN, ARC and LHA all increased food intake in rats (Abbott et al., 2001). The CART receptor and downstream signalling pathways have not been fully elucidated.

1.9 Peripheral adiposity hormones involved in appetite regulation

1.9.1 Insulin

The concept that a marker of adipose tissue energy stores is sent to the hypothalamus to regulate food intake and metabolism was first suggested over fifty years ago (Kennedy, 1953). Insulin levels correlate with body adipose tissue mass and it is considered an adipostat hormone (Woods et al., 1979). In the periphery, insulin acts as an anabolic hormone, promoting energy storage, glucose uptake, protein synthesis and lipogenesis. In contrast, central insulin administration reduces food intake and body weight (Woods et al., 1979). In addition to the impracticality of using insulin as an anti-obesity treatment due to its blood glucose lowering effects, patients with diabetes on exogenous insulin often gain weight, possibly demonstrating central insulin resistance.
1.9.2 Leptin

Leptin (Halaas et al., 1995), the product of the obese (ob) gene (Zhang et al., 1994), is an anorectic, adipocyte derived hormone, secreted in proportion to total body fat content which was discovered as a result of parabiosis experiments between ob/ob and db/db mice (Coleman, 1973). In both ob/ob and db/db mice (the absence of leptin or leptin receptor deficiency respectively) animals are hyperphagic and obese (Coleman, 1973). These mice live in a state of perceived starvation where the hypothalamus does not recognise adipose tissue stores (i.e. the leptin signal is not sent or not recognised). These animals also have abnormal reproduction, hypothermia and stunted linear growth (Friedman, 1997). In man, obesity due to leptin signalling defects is relatively rare (Farooqi and O’Rahilly, 2006).

Leptin exerts its effects via the central appetite circuit. Within the ARC, NPY and AgRP neurones (Mercer et al., 1996) and CART and POMC neurones (Elias et al., 1998) express leptin receptors. Leptin inhibits NPY/AgRP neurones (Mizuno and Mobbs, 1999) and stimulates POMC/CART neurones (Elias et al., 1998; Cowley et al., 2001; Kristensen et al., 1998). In animal studies, leptin administration reduces food intake (Halaas et al., 1995). However, elevated leptin levels, seen in common human obesity, do not suppress appetite (Considine et al., 1996). This has led to two possible hypotheses: firstly, elevated leptin levels may not suppress appetite due to leptin resistance or receptor desensitisation, secondly the role of leptin may be to signal the presence of adequate energy stores. Inadequate leptin levels may prevent energy requiring processes, such as reproduction, to occur whereas adequate leptin or increased leptin may have little or no effect.
1.10 Peripheral gut hormones involved in appetite regulation

There is a vast array of hormones secreted from various entero-endocrine cells located throughout the gastrointestinal tract (Figure 1.8). These hormones communicate with appetite circuits in the CNS, described above. They are able to traverse the BBB via CVOs and specific transport pathways. They also signal via vagal nerve afferent terminals located in the gut to the NTS. These gut hormones signal information about nutrient delivery to the gut and metabolic status to the CNS. A number of gut hormones have anorectic effects including: CCK, GLP-1, OXM, PP, PYY and islet amyloid peptide (IAP). Ghrelin is the only known example of an orexigenic gut hormone.
Figure 1.8: Schematic diagram of the gastrointestinal tract illustrating location of release of specific gut hormones. Ghrelin and gastrin are secreted from the stomach; insulin, glucagon, pancreatic polypeptide and amylin from the pancreas; CCK, secretin, glucose dependent insulinotropic hormone (GIP) from the duodenum; and GLP-1, glucagon-like-peptide 2 (GLP-2), OXM and PYY from the large intestine. Diagram modified from Murphy, K.G. (2006) Nature 14; 444(7121):854-9
1.10.1 Cholecystokinin (CCK)

CCK was the first gut hormone discovered to reduce food intake (Gibbs et al., 1973). It is widely distributed in the gut secreted mainly by entero-endocrine I cells in the duodenum and jejunum (Polak et al., 1975). It is released rapidly post-prandially in response to fat (Schaffalitzky de Muckadell et al., 1986; Lewis and Williams, 1990) and protein (Lewis and Williams, 1990). Its actions include: inhibition of food intake, delayed gastric emptying, stimulation of pancreatic enzyme secretion and stimulation of gall bladder contraction (Dufresne et al., 2006). These effects are mediated via binding to CCK receptors on the vagus nerve (Ritter and Ladenheim, 1985). CCK administration inhibits food intake in animals (West et al., 1984) and in man (Kissileff et al., 1981). However, the reduction in meal size is offset by an increase in feeding frequency (West et al., 1984). At high dose, nausea and taste aversion have been detected limiting the use of CCK as an anti-obesity treatment.

1.10.2 Glucagon-like peptide-1 (GLP-1)

Preproglucagon is a 180 amino acid peptide expressed by: pancreatic α cells (Mojsov et al., 1986), entero-endocrine L cells (Orskov et al., 1987) and in the NTS (Tager et al., 1980). Within L cells and in the NTS, preproglucagon undergoes post-translational modification by PC1/3 to produce bioactive GLP-1 (Figure 1.9) (Mojsov et al., 1986). There are two bioactive forms of GLP-1; GLP-1_{7-36amide} and GLP-1_{7-37}, and the majority of circulating GLP-1 is GLP-1_{7-36amide} (Orskov et al., 1994). GLP-1 is rapidly inactivated by cleavage at the position 2 alanine to GLP-1_{9-37} or GLP-1_{9-36} by dipeptidyl peptidase IV (DPP IV) and the GLP-1 fragments are then cleared by the kidney (Kleefer et al., 1995). DPP IV degradation of GLP-1 occurs as GLP-1 encounters DPP IV impregnated blood vessels draining the intestinal mucosa (Hansen et al., 1999).
Figure 1.9: Differential posttranslational processing of proglucagon in the pancreas, gut and brain.

In the gastrointestinal tract glicentin-related polypeptide (GRPP), OXM, GLP-1, intervening peptide 2 (IP-2) and GLP-2 are formed. Whereas in the pancreas GRPP, glucagon, IP-1 and major proglucagon fragment (MPGF) are formed. Diagram from Nature Reviews Endocrinology (Field et al., 2010a).
GLP-1 is most well recognised for its incretin effects. Incretins are gut derived hormones responsible for the increased secretion of insulin when glucose is given orally compared to intravenously (Figure 1.10) (Elrick, 1964; La Barre and Still, 2009).

**Figure 1.10: Graphical illustration of the incretin effect** (a) Glucose levels over time for healthy subjects given a glucose load orally (pink line) and intravenously (black line). (b) Insulin levels in response to the oral and intravenous glucose loads. The difference between the two responses is known as the incretin effect, the increased secretion of insulin with an oral compared to intravenous glucose load. Diagram adapted from (Holst et al., 2008).
GLP-1 also fulfils several criteria in order to be considered a satiety signal. Circulating levels of GLP-1 rise following food intake and are low in the fasted state (Orskov et al., 1996). GLP-1 secretion after a meal can be divided into two phases; an early phase that occurs between 30-60 minutes and a second overlapping phase between 1 and 2 hours (Elliott et al., 1993; Herrmann et al., 1995). In rodent (Meeran et al., 1999; Turton et al., 1996) and human studies (Flint et al., 1998), GLP-1 administration leads to a reduction in food intake.

GLP-1 mediates its effects via the GLP-1 receptor, a G protein coupled receptor which, when activated, increases cyclic adenosine monophosphate (cAMP) (Thorens, 1992). GLP-1 receptors are expressed in the pancreas, stomach, gut, kidney, lung and heart (Bullock et al., 1996). Within the CNS, GLP-1 R are expressed in the ARC, PVN, NTS, area postrema and supraoptic nucleus (SON) (Goke et al., 1995). Within the ARC, GLP-1 receptors are expressed on POMC neurones (Sandoval et al., 2008). The anorectic effects of GLP-1 may also be mediated via vagal nerve signalling to the NTS (Abbott et al., 2005).

For many years, the short half-life of GLP-1 limited its therapeutic effectiveness as a potential anti-obesity or hypoglycaemic agent. In 1991, a GLP-1 receptor agonist peptide, exendin 4, was discovered in the venom of *Heloderma suspectum*, the Gila monster lizard (Eng et al., 1992). This peptide has 53% sequence homology to GLP-1 and is resistant to cleavage by DPP IV (Holst et al., 2008). Use of exendin 4 for the treatment of people with type 2 diabetes mellitus has the additional benefit of causing a reduction in weight by approximately 3 kg after 30 weeks of treatment (De Block and Van Gaal, 2009). Exendin and other GLP-1 analogues such as liraglutide are now used routinely for the treatment of type 2 diabetes (Agerso et al., 2002).
1.10.3 Oxyntomodulin (OXM)

OXM was originally isolated from porcine jejunono-ileal cells (Bataille et al., 1982). It is a 37 amino acid peptide containing the entire sequence of glucagon and a carboxy terminal extension and is also a product of the pre-proglucagon gene (Figure 1.9) (Bataille et al., 1982). OXM binds to the GLP-1 receptor but with reduced affinity compared to GLP-1 or exendin 4 (Fehmann et al., 1994; Schepp et al., 1996). It is released into the circulation following the ingestion of food (Le et al., 1992), inhibits both gastric acid and pancreatic secretion (Anini et al., 2000) and delays gastric emptying (Schjoldager et al., 1988). OXM levels also increase following gastric bypass surgery and with tropical sprue (Besterman et al., 1979). OXM is co-secreted postprandially along with GLP-1 and PYY from the entero-endocrine L cells and shares the anorectic effects of these co-secreted hormones. In 2001, Dakin et al were the first to demonstrate the effects of OXM as a satiety signal in rats (Dakin et al., 2001). These results have since been confirmed using peripherally administered OXM in human volunteers (Cohen et al., 2003). In a four week study, subjects administered OXM lost weight due to a combined reduction in energy intake as well as an increase in energy expenditure (Wynne et al., 2006).

1.10.4 Pancreatic polypeptide (PP)

NPY, PYY, and PP all share a common tertiary structure characterised by a hairpin structure known as the PP fold (Blundell et al., 1981). PP, a 36 amino acid peptide, was the first member of the family to be identified (Langslow et al., 1973; Kimmel et al., 1975; Kimmel et al., 1968). It is secreted principally from PP cells in the pancreas with a small amount released from the distal gut (Adrian et al., 1976; Larsson et al., 1975). PP is released post-prandially via vagal cholinergic dependent mechanisms (Adrian et al., 1977; Schwartz et al., 1978). High levels of PP binding sites, now known
to be Y4 receptor, are found in the area postrema, NTS, dorsal motor nucleus of the vagus and the interpeduncular nucleus (Whitcomb et al., 1990). PP is considered an anorectic hormone as administration reduces food intake in animals (Malaisse-Lagae et al., 1977; Asakawa et al., 2003) and humans (Batterham et al., 2003b).

1.10.5 Peptide YY (PYY)

PYY is a 36 amino acid peptide originally isolated from porcine intestine (Tatemoto, 1982a). It is a product of the PYY gene located on chromosome 17q21.1 (Hort et al., 1995). Its name originates from the fact that it has a tyrosine at both the carboxy and amino termini (Tatemoto, 1982a). PYY is part of the PP fold family, amino acids 1-8 form a type II proline helix and residues 15-32 form an amphipathic α helix (Blundell et al., 1981; Glover et al., 1984). PYY is synthesised as a pre-propeptide consisting of a hydrophobic signal peptide, followed by the 36 amino acid peptide and a carboxyterminal flanking peptide (Cerda-Reverter and Larhammar, 2000). Post translational processing of the peptide involves cleavage of the precursor and amidation of the carboxyterminal tyrosine (Tatemoto et al., 1988). Carboxyterminal amidation is a feature shared by many biologically active peptides as it protects against carboxypeptidase attack and loss of the amide group may lead to a non-functional peptide (Kimmel et al., 1975; Blundell et al., 1981). The hydrophobic signal sequence allows the peptide to enter the endoplasmic reticulum and to be packaged into secretory vesicles (Von, 1983).

PYY is released from entero-endocrine L cells (Bottcher et al., 1984; Bottcher et al., 1986; Lundberg et al., 1982) and co-exists with glucagon in the pancreatic α-cells (Morley et al., 1985). PYY concentrations increase distally throughout the gastrointestinal tract with the highest concentrations found in the rectum (Adrian et al., 1985). Two molecular forms of PYY exist, PYY 1-36
and PYY$_{3-36}$ (Grandt et al., 1994). PYY$_{3-36}$ is formed by proteolytic cleavage by DPP IV (Mentlein et al., 1993).

Previously it was shown that ICV administration of PYY in rodents increased food intake (Morley et al., 1985). However, PYY is released into the circulation post-prandially and is reduced by fasting, therefore is more likely to be an anorectic hormone (Ghatei et al., 1983). It has now been demonstrated that PYY$_{3-36}$ is responsible for the anorectic effects within the CNS. When PYY$_{1-36}$ is administered it has no effect on appetite in rats lacking DPP IV (Unniappan et al., 2006).

In animal studies, PYY$_{3-36}$ injected directly into the ARC reduces food intake consistent with an anorectic role for this hormone (Batterham et al., 2002). PYY$_{3-36}$ exerts its effects through the NPY family of receptors and binds preferentially to the Y2 receptor found on NPY neurones in the ARC (Keire et al., 2000). The Y2 receptor is an autoinhibitory presynaptic receptor, and may therefore reduce NPY signalling within the ARC leading to reduced appetite. The anorectic effects of PYY$_{3-36}$ are not seen in Y2 receptor null mice (Batterham et al., 2002). PYY$_{3-36}$ may also exert its effects through the vagus nerve signalling to the NTS (Abbott et al., 2005). PYY receptor binding and the physiological degradation of PYY is described in detail in chapter 2.

In human studies, PYY$_{3-36}$ has been shown to reduce food intake in both lean and obese subjects (Batterham et al., 2002; Le Roux et al., 2006b). Some reports suggest that total PYY levels are lower in obese subjects and the PYY$_{3-36}$ response to nutrient ingestion is reduced in obesity (Le Roux et al., 2006b). Concerns regarding possible resistance to PYY$_{3-36}$ in obesity have been unfounded, intravenous infusion of PYY$_{3-36}$ reduced energy intake by 36% in obese subjects demonstrating a potential therapeutic role for this hormone as an obesity treatment (Batterham et al., 2003a).
1.10.6 Ghrelin

Ghrelin is the only known orexigenic gut hormone (Yoshihara et al., 2002). It is a 28 amino acid peptide, octanoylated at the serine 3 residue, principally secreted from the X/A- like endocrine cells in the stomach oxyntic glands (Date et al., 2000). Lower levels of ghrelin secreting cells are found in the small intestine and even fewer in the colon (Date et al., 2000). Ghrelin is an endogenous ligand at the growth hormone secretagogue receptor 1a (GHS-R\textsubscript{1a}) (Kojima et al., 1999), and was originally characterised due to its ability to stimulate growth hormone release (Howard et al., 1996). GHS-R has been identified in hypothalamic neurones (Kojima et al., 1999; Guan et al., 1997) and peripheral ghrelin administration has also been found to activate neurones within the brainstem (Bailey et al., 2000). The majority of circulating ghrelin is not octanoylated, however, the octanoyl group is necessary for activation of the GHS-1a receptor (Bednarek et al., 2000; Hosoda et al., 2000).

Circulating ghrelin levels rise with fasting and fall after food intake, suggesting a role in meal initiation (Cummings et al., 2001). The downstream effects of ghrelin are via activation of NPY and AgRP neurones in the ARC (Chen et al., 2004). Ghrelin administration has been found to be a potent stimulus of feeding in rodents (Tschop et al., 2001) and in man (Wren et al., 2001). Ghrelin levels are highest in cachectic subjects, reduced in lean subjects and the lowest in obese subjects (Druce et al., 2005). This may be an adaptive response, an attempt to stimulate or suppress appetite according to the energy imbalance. Obese subjects are, however, more sensitive to the effects of ghrelin. Ghrelin infusions increased energy intake by 20% in lean individuals compared to 70% in obese individuals (Druce et al., 2005). Therefore, antagonising ghrelin may prove a useful obesity treatment. Conversely, ghrelin treatment may be a useful appetite stimulant, for example in patients with cachexia due to end stage renal failure or cancer.
1.14 Summary

The management of the body’s energy balance is a highly complex process, involving the integration of signals from the gut, adipose tissue, and other parts of the brain (notably reward pathways) at the level of the hypothalamus. In turn, the hypothalamus is able to orchestrate food-seeking behaviour, basal metabolism and elective energy expenditure to maintain and defend the body’s weight against starvation, with a bias towards gaining weight. This has led, in the modern world, to the overall increase in obesity with time. Our increasing understanding of these complex circuits should lead to the design of new drugs which may, in time, prove to be effective treatments for obesity.
1.15 Hypotheses

The anorectic gut hormones, PYY and GLP-1, regulate appetite following nutrient stimulation and release from entero-endocrine L cells in the distal gut. Investigation of the release of these hormones and their metabolism should lead to an increased understanding of the pathophysiology of obesity. This in turn may lead to the development of novel agents for use in obesity.

I hypothesise that

A novel PYY analogue, PYYα, which has been specially designed to have a stable tertiary structure, should lead to a prolonged plasma appearance and therefore have an increased biological effect in reducing food intake.

PYY and GLP-1 are released from L cells due to direct nutrient stimulation and it is possible to determine the main stimulants of hormonal release using a primary L cell model.

In man it is possible to stimulate L cells to release GLP-1 and PYY by delivering nutrients directly to the distal colon where the majority of L cells reside.
1.16 Aims of thesis

The aim of this thesis is to study the physiology of gut hormones involved in the regulation of appetite. This thesis is divided into three sections.

First of all, I will study the degradation of the anorectic gut hormone, PYY, using a specially designed PYY analogue, in *in vitro* studies focussing on proteolytic cleavage and receptor binding, and *in vivo*, in healthy human volunteers.

Secondly, I will investigate the stimulants of release of anorectic gut hormones, GLP-1 and PYY, from the L cell, using a primary L cell model derived from both mouse and human colon.

Finally, I will attempt to stimulate the L cell *in vivo*, in healthy human volunteers, using an orally delivery nutrient supplement, propionate inulin ester, in order to increase the release of GLP-1 and PYY, which should in turn reduce appetite.
Chapter 2: Investigation into the physiological degradation of PYY\textsubscript{3-36}, using PYY analogues

2.1 Introduction

The importance of PYY\textsubscript{3-36} in the physiological control of appetite and energy homeostasis is well established (Batterham and Bloom, 2003). Exogenously administered PYY\textsubscript{3-36} could be a potential anti-obesity treatment. The benefits of PYY\textsubscript{3-36} over other synthetic pharmaceutical agents are that as an endogenous gut hormone it would be unlikely to have major side effects and it would provide a more physiological regulation of appetite, in addition, obese individuals do not display PYY\textsubscript{3-36} resistance (Batterham et al., 2003a).

The effects of endogenously released PYY\textsubscript{3-36} are transient as it is broken down rapidly in the circulation by endogenous peptidases; the half-life in rats is about 9 minutes (Lluis et al., 1989). This is physiologically important, as prolonged effects from endogenously released PYY\textsubscript{3-36} would chronically reduce appetite and lead to weight loss and malnutrition. PYY\textsubscript{3-36} administered peripherally is also cleared rapidly (Sloth et al., 2007). In order to overcome degradation one could either administer specific peptidase inhibitors or design stable PYY analogues resistant to proteolytic degradation. In the treatment of type 2 diabetes, DPP IV inhibitors have been used to extend the half-life of GLP-1 (Drucker and Nauck, 2006). In addition, peptidase resistant GLP-1 analogues, for example Liraglutide, have also been designed and are an effective and practical treatment for diabetes (Agerso et al., 2002). The advantage of stable analogues over peptidase inhibitors is that they exert more specific effects. Peptidase inhibitors may lead to multiple non-specific side effects due to the wide distribution of the peptidases and the multiple substrates they regulate. Knowledge about the proteolytic processing of PYY by various peptidases is important to enable the rational design of stable analogues.
PYY analogues were designed by Professor Stephen Bloom, Imperial College London, to increase resistance to proteolytic cleavage and prolong the half-life of this hormone. One such analogue, PYYα, is the subject of this chapter.

2.1.1 PYY degradation

Circulating peptide hormones are cleared by proteolytic degradation and renal clearance avoiding prolonged, unregulated hormonal signalling (Mentlein, 2009). The human genome encodes over 300 distinct peptidases that perform this task (Turner, 2003). The unique proline rich structures of NPY and PYY have enabled identification of specific peptidases and therefore potential degradation sites (Mentlein, 1988). NPY and PYY share the same amino terminal dipeptide, Tyr-Pro (Blundell et al., 1981; Glover et al., 1984). Proline in the penultimate position protects peptides from most nonspecific peptidases (Walter et al., 1980). However, a number of peptidases exist that specifically cleave proline bonds (Mentlein, 1988). PYY\textsubscript{1-36} is converted to PYY\textsubscript{3-36} by the serine protease, DPP IV (Mentlein et al., 1993), and most circulating PYY is in this amino terminally truncated form which is biologically active (Grandt et al., 1994). Other proline preferring proteases that have been shown to degrade PYY have also been identified, these include: metalloendopeptidases, such as neprilysin (NEP) and meprin, and aminopeptidases (Medeiros and Turner, 1994a). Aminopeptidase P is known to remove the amino terminal tyrosine from PYY but this occurs at low rates compared to DPP IV (Mentlein et al., 1993).
2.1.2 Dipeptidyl peptidase IV (DPP IV)

DPP IV is a cell surface peptidase found on blood vessels (Lojda, 1979), pancreas (Heymann et al., 1986), intestinal and kidney brush borders (Kettmann et al., 1992) and on a subset of T lymphocytes (Mentlein et al., 1984). It is also found within the CNS in the CSF, the CVOs and in leptomeningeal cells (Mitro and Lojda, 1988). Soluble DPP IV, formed by shedding of the membrane anchored forms, can also be found in the plasma (Mentlein, 2009). DPP IV cleaves the amino terminal Pro-Tyr from PYY1-36 with high activity (Mentlein et al., 1993). Addition of specific DPP IV inhibitors, diprotin A and Lys-pyrrolidide, to serum abolished the formation of PYY fragments demonstrating the physiological importance of this enzyme (Mentlein et al., 1993).

2.1.3 Meprins

Meprins are metalloproteinases of the astacin and metzincin superfamily (Villa et al., 2003). They are found in the brush border membranes of kidney proximal tubule cells (Craig et al., 1987) and intestinal epithelial cells (Villa et al., 2003). As meprins are capable of shedding themselves from the cell membrane, soluble forms are also found (Bertenshaw et al., 2003). These secreted forms self associate into complexes thus enabling them to travel through extracellular spaces in a non destructive form (Bertenshaw et al., 2003). They become activated on contact with trypsin-like proteases such as plasmin (Rosmann et al., 2002). The meprin α and β subunits have marked differences in their substrate sequence specificity (Bertenshaw et al., 2001). Substrates for meprins are greater than six amino acids long such as NPY and PYY, bombesin, bradykinin and angiotensin (Villa et al., 2003). Meprins are inhibited by zinc chelating agents such as EDTA and also the hydroxamate containing actinonin and Z-Pro-Leu-Gly-(NHOH) (Villa et al., 2003).
2.1.4 Neprilysin (NEP)

NEP was originally identified as a major antigen of rabbit renal membranes (Kerr and Kenny, 1974). It belongs to the neprilysin (M13) family of zinc-metallopeptidases which cleave the amino terminal bond of hydrophobic residues (Turner et al., 2001). NEP has several cleavage sites for NPY and PYY but the primary site is Asn 29-Leu 30 which is an inactivating cleavage (Medeiros and Turner, 1994b; Medeiros and Turner, 1994a). The highest concentrations of NEP are found in the kidney brush borders (KBB) (Turner, 2003). The KBB are commonly used to study NEP degradation of peptides (Turner and Tanzawa, 1997). A characteristic feature of NEP is its sensitivity to inhibition by the *Streptomyces* metabolite phosphoramidon (Turner and Tanzawa, 1997). In human KBB, degradation of PYY is significantly inhibited (>75%) by phosphoramidon indicating the importance of NEP in degrading PYY (Medeiros and Turner, 1994a).

2.1.5 Design of Stable PYY analogues

A number of PYY analogues were created and tested for bio-efficacy in small rodent studies (carried out by the PYY analogue group, Imperial College London). The most successful analogue, PYYα was taken forward and characterised. PYYα has an altered amino acid sequence designed to stabilise the α-helix. The α-helix is a common motif involved in the folding and stability of peptides. It is thought that the α-helix directs the peptide towards the membrane and holds the carboxy terminus in the correct conformation for receptor binding (Pedersen et al., 2010). The importance of the α-helical region of PYY in Y2 receptor binding has been demonstrated previously by investigation of analogues with a disrupted α-helix (Lerch et al., 2005). These studies demonstrated that disruption of the α-helix led to a large decrease in affinity at all receptor subtypes (Lerch et al., 2005). PYYα has a sequence based on the structure of the neurotoxin α-latrotoxin from the black widow spider,
*Latrodectus tredecimguttatus*, incorporated into its structure. This sequence, also present in the clinically used GLP-1 receptor agonist, exendin-4, is thought to stabilise the tertiary structure of peptides and so reduce degradation (Holz and Habener, 1998).

In order to demonstrate that changes to the PYY amino acid structure did not affect binding at the NPY receptors and affect biological activity, PYYα was investigated *in vitro* to study binding to the NPY receptors.

### 2.1.6 NPY receptors

NPY and PYY are the endogenous ligands for four known NPY receptors in humans: Y1, Y2, Y4 and Y5 (Michel et al., 1998). The NPY receptors belong to the rhodopsin-like superfamily of G protein coupled receptors (Lindner et al., 2008). Y1 (Kanatani et al., 2000) and Y5 (Gerald et al., 1996) appear to mediate the orexigenic actions of NPY, whereas Y2 (Batterham et al., 2002) and Y4 (Yahya et al., 2006) mediate anorectic effects. Y1 and Y5 are co-expressed in several hypothalamic areas including the PVN (Broberger et al., 1999). However, Y2, an autoinhibitory presynaptic receptor, is the predominant NPY receptor in the brain (Berglund et al., 2003). Y3 was identified pharmacologically as a receptor that is bound by NPY with more than ten-fold affinity compared to PYY; however, there is little evidence to support its existence and its gene has not yet been cloned or characterised (Michel et al., 1998). The gene for y6 is located on human chromosome 5q31 (Gregor et al., 1996). It has been given a lower case designation because it encodes a truncated, non functional receptor in most mammals, including humans (Burkhoff et al., 1998; Gregor et al., 1996). In addition to the five cloned receptors, three homologous receptor subtypes have been cloned in fish making it the largest receptor family amongst all peptide receptors that belong to the G protein coupled receptor
superfamily (Cerda-Reverter and Larhammar, 2000). The receptors couple to the G protein Gi and ligand binding leads to inhibition of adenylate cyclase (Aakerlund et al., 1990). Y1, Y2, Y4, and Y5 can also couple to phospholipase C leading to an increase in intracellular calcium (Aakerlund et al., 1990; Berglund et al., 2003).
Figure 2.1: Schematic diagram of the tertiary structure of human PYY$_{1:36}$ and PYY$_{3:36}$ binding to Y1 receptor and Y2 receptor (Nygaard et al., 2006).
2.1.7 Y1 receptor

The Y1 receptor gene is found on human chromosome 4q (31.3-32), located in a cluster with Y2 and Y5 (Michel et al., 1998). Y1 was the first member of the NPY receptor family to be cloned (Eva et al., 1990). It appears in most species and tissues, expressed in the hypothalamus (Eva et al., 1990), adipose tissue and vascular smooth muscle (Wahlestedt et al., 1990). Y1 is a major postsynaptic receptor (Wahlestedt et al., 1986). Y1 is activated by holopeptides (peptides with an intact amino and carboxy terminus) and the receptor interacts with both the amino and carboxy terminus of PYY_136^{36} (Michel et al., 1998). Analogues of NPY/PYY containing a proline at position 34 (Pro 34) are selective for both Y1 and Y5 (Fuhlendorff et al., 1990; Wieland et al., 1995). Pro 34 stabilises the secondary structure and increases the binding affinity at these receptors (Fuhlendorff et al., 1990). The potency order for Y1 is NPY ≥ PYY_136^{36} ≥ Pro 34 > carboxy terminal fragment > PP (Umeda et al., 2009). BIBP3226 ((R)-N²-(diphenylacetyl)-N-[(4-hydroxyphenyl) methyl]-argininamide) is a Y1 antagonist with low affinity (nanomolar) (Entzeroth et al., 1995). The SK-N-MC cell line is a human neuroblastoma cell line which only expresses Y1 receptors. In this cell line Y1 receptor activation leads to inhibition of adenylate cyclase and mobilisation of intracellular calcium (Aakerlund et al., 1990). In the periphery Y1 receptors are found in blood vessels where they mediate vasoconstriction (Wahlestedt et al., 1990). Y1 is also expressed in human adipose tissue and NPY promotes adipocyte proliferation via Y1 (Mashiko et al., 2009).

2.1.8 Y2 receptor

The Y2 receptor gene is found on human chromosome 4q31 (Ammar et al., 1996). Y2 is mainly located presynaptically where it exerts an autoinhibitory effect, preventing further release of the NPY neurotransmitter (Wahlestedt et al., 1990). Y2 receptor is the predominant NPY receptor in the
brain, expressed at the nodose ganglion, amygdala, caudate, corpus callosum, hippocampus and subthalamic nucleus (Rose et al., 1995). Y2 is activated both by holopeptides and carboxy terminal fragments. PYY carboxy terminal fragments bind preferentially to Y2 compared to Y1 (Entzeroth et al., 1995). In general the shorter the remaining carboxy terminus, the lower the affinity (Wieland et al., 1995). The rank order of potencies at this receptor is \( \text{PYY}_{1-36} \geq \text{NPY} > \text{NPY}_{3-36} > \text{NPY}_{13-36} > \text{NPY}_{18-36} > \text{NPY}_{22-36} \) (Wahlestedt et al., 1990). BIIE-0246 is a specific Y2 antagonist (Doods et al., 1999).

2.1.9 Y4 receptor

The gene encoding Y4 receptor is located on human chromosome 10q11.2 (Berglund et al., 2003). It is expressed in the gastrointestinal tract, heart, area postrema, hypothalamus and hippocampus (Lindner et al., 2008). PP is the most potent endogenous agonist at this receptor; however, NPY and \( \text{PYY}_{1-36} \) are still able to activate the receptor with low affinity (Gehlert et al., 1996).

2.1.10 Y5 receptor

The gene encoding Y5 receptor is located on human chromosome 4q (4q31-32) (Gerald et al., 1996). It is adjacent to the gene encoding the Y1 receptor but in an opposite orientation (Hu et al., 1996; Gerald et al., 1996). The rank order of potencies at this receptor is \( \text{NPY} \geq \text{PYY}_{1-36} = \text{Pro34} \approx \text{NPY}_{2-36} \approx \text{PYY}_{1-36} \geq \text{NPY}_{13-36} \) (Hu et al., 1996; Gerald et al., 1996). The Y5 receptor is predominantly expressed in the CNS with the mRNA found in the ARC, PVN, midline thalamic nuclei and lateral hypothalamus (Statnick et al., 1998; Hu et al., 1996; Gerald et al., 1996). Y5 receptor mRNA expression has also been found in the testis, spleen and pancreas with lower levels found in the heart, placenta and
Many of the NPY neurones within the ARC display Y5 immunoreactivity and are GABAergic (Campbell et al., 2001). Therefore, the effects of NPY on neuronal activity may be via modulation of GABA release (Campbell et al., 2001).

YS receptor KO mice paradoxically have higher body weights and increased body fat compared to wild type mice. Receptor knockout effects may be masked or reversed by compensatory mechanisms. To clarify this, the changes in gene expression of various neuropeptides in these Y5 KO mice have been investigated. This demonstrated that NPY/AgRP levels increase and POMC and CART levels decrease (Higuchi et al., 2008). This would lead to hyperphagia and negate the effect of the Y5 receptor knockout. In 2006, Erondu carried out the first clinical trial of a Y5 receptor antagonist, MK 0557 (Erondu et al., 2006). This showed some efficacy in terms of weight loss at 12 weeks and 52 weeks (Erondu et al., 2006). However, the degree of weight loss at 52 weeks was significantly less than that observed for other weight loss drugs (Erondu et al., 2006). Although Y1 and Y5 both play a role in feeding, the relative contribution of each receptor has not yet been fully established. Dominance of the Y1 component may explain the lack of weight loss seen with this drug. Also multiple redundant pathways defend against alterations in body weight; compensation by other orexigenic systems may have overridden the effects of Y5 antagonism.
2.2 Aims and hypothesis

The aim of this work was to characterise and study the PYY analogue, PYYα, in humans. PYY analogues were developed as part of a research portfolio investigating breakdown in order to better understand PYY physiology.

2.2.1 Hypothesis

1. Alteration to identified peptidase recognition sites and insertion of a stabilising peptide sequence will render the PYY analogue resistant to degradation
2. Altering these sites will lead to prolonged appearance of PYY analogues in plasma
3. The prolonged half-life of the PYY analogue will reduce appetite as a biomarker of the effect of PYY

2.2.2 Aims

1. To study the degradation of PYY analogue, PYYα, following incubation with known peptidases.
2. To determine whether changes in analogue structure affect affinity to NPY receptors, measured by receptor binding assays.
3. To study the pharmacokinetics of PYY analogue, PYYα, after a single subcutaneous injection in man.
4. To explore the effects on appetite in man.

The analogues were tested in two animal models prior to use in human studies. This was to demonstrate prolonged pharmacological effect and effectiveness in appetite and weight reduction.
2.3 Materials and Methods

2.3.1 PYY analogue - PYYα synthesis

PYY analogues were produced by Bachem Ltd. (Merseyside, UK) using automated Fluorenly Methoxy-Carbonyl solid phase peptide synthesis (Fmoc) technology. Amino acids were sequentially added from the carboxy to the amino terminus and the completed peptide cleaved from the solid support. Peptides were purified by high resolution HPLC and purity confirmed by matrix assisted laser desorption ionised time of flight (MALDI-ToF) mass spectroscopy.

2.3.2 Safety testing of PYYα

Representative vials of PYYα were sent for microbiological analysis (Microbiology Department, Hammersmith Hospital) and endotoxin analysis (Limulus Amoebocyte Lysate test, Associates of Cape Cod, Liverpool, UK). Further vials were sent for amino acid analysis to confirm that the amino acid content was consistent with the specified sequence (Alta Bioscience, Birmingham, UK). This information was used to calculate the peptide content of the vials. Toxicity testing was carried out in small rodents. A dose greater than 10 times the maximum dose intended for humans was administered intraperitoneally to 20 mice and saline was administered to a control group. Half of the animals in each group were sacrificed by CO₂ asphyxiation after 48 hours, the other half after 14 days. The internal organs were dissected out, fixed and sent for histopathological examination. The bioactivity of PYYα was assessed in an acute feeding study in fasted mice over 24 hours.
2.3.3 Proteolytic degradation of peptides

To determine if PYYα is resistant to enzymatic cleavage, it was incubated with either NEP or KBB preparations. The resulting peptide fragments were separated by HPLC and detected by spectroscopy and MALDI-ToF mass spectroscopy.

2.3.4 KBB degradation assay

KBB had been previously prepared by Dr. Melisande Addison using a standard protocol. Digestion buffer (12 mM HEPES pH 7.4, 300 mM L mannitol) containing 2 nmol peptide and 1.25 mg/ml or 2.5 mg/ml KBB were incubated at 37°C in a volume of 140 µl. The reaction was terminated at 30 minutes by the addition of 10 µl 10% trifluoroacetic acid (TFA). Inhibitor studies were also carried out with 100 nmol phosphoramidon (Sigma- Aldrich, Poole, Dorset, UK) in the presence and absence of the peptide. The reactions were fractionated using reverse phase HPLC. Samples were centrifuged at 12500 x g for 5 minutes at room temperature prior to HPLC analysis (Jasco HPLC system (solvent delivery system PU-2080 plus, autosampler AS-2057 plus, degasser DG2080-53, dynamic mixer 2080-32, UV detector uv-2075 using a Phenomenex Gemini C18, 5 µm particles, 250 mm x 4.6 mm column). The samples were applied to a C18 column and eluted over an acetonitrile gradient as indicated in individual experiments (v/v) over 40 minutes. The eluted peptides were collected and detected sequentially at a wavelength of 214 nm. Percentage degradation of peptides was calculated by comparing area under peaks of reactions with and without enzyme. To validate the method the same volume of sample with and without enzyme has to be injected onto the column for analysis. Absorbance is measured at 214 nm as this is the spectroscopic wavelength where the peptide bond absorbs, minimising any change in absorbance by quantity of any single amino acid.
2.3.5 Neprilysin (NEP) protease assay

The NEP assay was carried out as the KBB protocol above but in a different buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl). 2 nmol of PYY<sub>3-36</sub> was incubated with 200, 400, 800 ng of recombinant human NEP (R&D Systems, Abingdon, UK). As a positive control, 2 nmol human gonadotrophin releasing hormone (hGnRH) (Bachem, UK) was used. 2 nmol of PYYα was added to 200 ng NEP. The incubation time for these reactions was 120 minutes and the reaction terminated with 10µl 10% TFA. The incubated samples were run and analysed as above.

2.3.6 Matrix-assisted laser desorption/ ionisation- Time of flight analysis (MALDI-ToF) of KBB hydrolysates

MALDI-ToF mass spectroscopy of digests was performed by Advanced Biotechnology Centre (Imperial College London). Digests were performed as above except 5 nmol peptide was digested in a reaction volume of 340 µL for 30 minutes.

2.3.7 Receptor binding studies- production of hY1 and hY5 receptor over-expressing cell lines

The human Y1 receptor and human Y5 receptor genes were obtained from the University of Missouri-Rolla cDNA resource centre (www.cdna.org). These were incorporated into expression plasmids (pcDNA3.1+) which also contained a Neo resistance gene, to be used for positive selection with Geneticin (G418).
Figure 2.2: Schematic diagram of the hY1 receptor insert and map of the restriction enzyme cleavage sites.

Figure 2.3: Schematic diagram of the hY5 receptor insert and map of the restriction enzyme cleavage sites.

2.3.8 Preparation of competent bacteria

100 µl of lysogeny broth (LB: 1% bacto-tryptone, 1% NaCl, 0.5% yeast extract) supplemented with 5 µg/ml tetracycline (LBtet) was inoculated with a colony of Escherichia Coli strain XL1B (Stratagene Ltd), and incubated overnight in a shaking incubator (37°C). The bacteria were recovered by
centrifugation (800 x g, 15 minutes). The pellet was resuspended in 40 ml ice cold TFB I (100 mM RbCl / 50 mM MnCl₂ / 30 mM K acetate / 10 mM CaCl₂ / 15% glycerol, adjusted to pH 5.8) and incubated on ice for 10 minutes. This was then centrifuged; the pellet resuspended in 4ml TFB II (0.2 M MOPS-HCl pH 6.5 / 10 mM RbCl / 75 mM CaCl₂ / 10% glycerol) and incubated on ice for 15 minutes. These were aliquoted into 50 µl aliquots, frozen in a dry ice/ ethanol bath and stored at -70°C.

2.3.9 Transformation of competent bacteria

Bacteria were heat shocked to take up the plasmid. The plasmid attaches to the bacterial wall during incubation on ice and passes through the wall on heating. An aliquot of frozen bacteria was thawed on ice, 1 µl (20 ng/µl) hY1R or hY5R plasmid was added and this mixture incubated for 15-20 minutes on ice. This was heated in a water bath at 42°C for 40 seconds and rested on ice for a further 2 minutes. 200 µl of pre-warmed LB broth was added and this was incubated at 37°C for 30 minutes. Transformed bacteria were spread evenly over Agar plates supplemented with ampicillin (100 µg/ml) inverted and incubated overnight at 37°C.

2.3.10 Amplification of plasmid (mini-preparations)

Small scale preparations of plasmid were carried out to confirm the presence of the hY1 or hY5 receptor insert. To isolate the plasmids from the bacteria, the cell wall is disrupted with alkaline SDS, and contaminating proteins, genomic DNA and RNA removed by precipitation of the bacterial debris
with potassium acetate. As genomic DNA is anchored to the cell wall, precipitation removes both the broken cell walls and the genomic DNA. RNA is removed by treatment with RNase A.

2 ml LBamp was inoculated with a single bacterial colony and incubated overnight in a shaking incubator (37°C). 1.5 ml of culture was centrifuged (12300 x g, 3 minutes), the supernatant discarded and the pellet resuspended in 100 µl of GTE (50 mM glucose, 25 mM Tris- HCl pH 8.0, 10 mM EDTA, 0.1 mg/ml RNase A). 200 µl of 0.2 M NaOH/1% SDS was added, this was mixed and the sample incubated on ice for 5 minutes to lyse the cells. 150 µl of 5 M potassium acetate was added to neutralise the NaOH, this was mixed and incubated for a further 5 minutes on ice. This was centrifuged (12300 x g) for 5 minutes at 37°C. 350 µl of the supernatant was transferred to a clean tube, an equal volume of phenol/ chloroform added and the sample mixed. The phases were separated by centrifugation (12300 x g, 3 minutes) and the aqueous phase (top layer) transferred to a fresh tube. DNA was precipitated by the addition of 0.6 volumes (i.e. 200 µl) of propan-2-ol and incubated at room temperature for 10 minutes. This was centrifuged (12300 x g for 7 minutes) and the supernatant discarded. The pellet containing DNA was dissolved in 100 µl glass distilled water (GDW). 10 µl of 2 M Na acetate pH 5.2 and 250 µl of ice cold 100% ethanol was added and the sample mixed and incubated at -20°C for 1 hour. DNA was recovered by centrifugation at 12300 x g, the supernatant was discarded and the pellet was dissolved in 10 µl GDW.

2.3.11 restriction endonuclease digestion of plasmid DNA

To select bacterial clones with the correct plasmids inserted, DNA was digested with restriction endonuclease enzymes. For hY5 receptor restriction enzymes BamHI and XhoI were used. For hY1, BamHI and Pmel were used. Each reaction was carried out in a total volume of 10 µl containing 8 µl
of a mastermix made to the ratio of (1 µl of the appropriate 10x restriction buffer, 1 µl RNase, 0.4 µl each of each restriction enzyme, 1 µl of 10x bovine serum albumin (BSA) and 5 µl water) plus 2 µl of the extracted DNA from the mini-preparation.

2.3.12 Electrophoresis of DNA fragments

The resulting fragments were analysed by gel electrophoresis on an agarose gel. This was run alongside a 1 kb DNA ladder (Invitrogen) to determine if the insert was present. A 1% (w/v) agarose gel was prepared by dissolving 1.5 g agarose in 150 ml GDW. This was microwaved for 3 minutes, the gel cooled to 45°C and 3 ml 50x TAE (0.04% final concentration) and 7.5 µl ethidium bromide added before the gel was poured.

To load the samples, 3 µl loading buffer was added to 10 µl of the restriction enzyme digest. For the ladder, 1µl ladder was added to 9 µl GDW and 3 µl loading buffer. The samples were loaded onto the agarose gel and this was electrophoresed at 120-130 mV. DNA was visualised under UV light (300 nm). Clones containing the correct size inserts where selected for large scale amplification. The hY1 insert size is approximately 1155 base pairs and the hY5 insert is 1422 base pairs.

2.3.13 Amplification of the plasmid (maxi-preparations)

Once the clone had been selected this was amplified further. This is a scaling up of the previous method followed by an additional step, a caesium chloride gradient to further purify the plasmid.
500 ml LBamp was inoculated with a small quantity (1.5 ml) of a starter culture of the plasmid transformed bacteria and this was incubated overnight in the shaking incubator (37°C). The bacteria were recovered by centrifugation (3000 x g, 8 minutes, 4°C). The pellet was resuspended in 25 ml GTE supplemented with 2 mg/ml lysozyme, this was incubated at room temperature for 5 minutes.

50 ml of 0.2 M NaOH/ 1% SDS was added, the sample mixed until clear and this was then incubated on ice for 5 minutes. 38 ml of 5 M potassium acetate was added to neutralise the NaOH, the sample mixed and left for a further 10 minutes. This was then centrifuged (9000 x g, 15 minutes, 4°C). The supernatant was filtered through nylon gauze into a clean tube, 0.6 volumes of propan-2-ol added and incubated on ice for 15 minutes. DNA was recovered by centrifugation (15 minutes, 9000 x g, 4°C). The supernatant was discarded and the pellet was dissolved in 10 ml of GDW. 100 µl of 100x TE (final concentration 10 mM Tris-HCl pH 8.0, EDTA 1mM) and 100 µl RNase A was added and the reaction incubated at 37°C for 30 minutes. Addition of an equal volume of phenol/ chloroform terminates the reaction. The phases were separated by centrifugation (10000 x g, 20 minutes, 4°C). The aqueous phase (top layer) was transferred to a clean tube and 0.1 volumes of 2 M sodium acetate pH 5.2, 1 volume propan-2-ol added and the reaction incubated at -20°C for 1 hour. This was then centrifuged (20 minutes, 24000 x g, 4°C), the supernatant discarded and the pellet allowed to air dry. This was finally dissolved in 8.25 ml TES (10 mM Tris-HCl pH 8.0, EDTA 1 mM, NaCl 150 mM) and 8.4 g of CsCl₂ added to the solution.

2.3.14 Caesium chloride gradient purification

Ethidium bromide intercalates into the DNA helix causing it to partially unwind. Binding occurs to a higher degree in linear DNA or nicked plasmid DNA compared to closed circular, supercoiled plasmid
DNA. This allows these different forms of DNA, with different densities, to separate into bands in the caesium gradient generated by ultra-centrifugation.

150 µl ethidium bromide (10 mg/ml stock solution) was added to the TES, DNA, CsCl₂ solution and this was mixed. The sample was transferred into a 2 Sorvall poly-alumina centrifuge tube using a syringe and blunt needle. This was overlayed with mineral oil, sealed and ultra-centrifuged (185500 x g, 16 hours, 20°C). After centrifugation the DNA bands were visualised in a dark room under UV light and the band containing the closed circular DNA loop removed using a 20 gauge needle and 2 ml syringe (this step was performed by Dr. James Gardiner). Ethidium bromide was removed by repeated extraction with an equal volume of caesium chloride saturated propan-2-ol until both phases were colourless. DNA was precipitated by the addition of 2 volumes of GDW and 6 volumes of room temperature absolute ethanol. DNA was recovered by centrifugation (24000 x g, 15 minutes, room temperature), the supernatant discarded and the pellet dissolved in 0.4 ml GDW. The DNA was ethanol precipitated, dissolved in 1 ml GDW and quantified.

2.3.15 Quantification of DNA

DNA was diluted 1:100 and 1 ml placed into a quartz cuvette. Absorbance was read at 260 nM for DNA and 280 nm for contaminants (Shimadzu UV160 spectrophotometer, Kyoto, Japan). DNA concentration was calculated using the formula

\[
\text{DNA concentration (mg/ml)} = 50 \times (A_{260} \times \text{dilution factor}).
\]
2.3.16 Polyethylenimine (PEI) mediated gene transfer

Plasmids containing the DNA constructs for the human Y1 and Y5 receptor were transfected into
human embryonic kidney (HEK) 293 (not T) cells. PEI is a cationic polymer, every 3rd atom is an amine
nitrogen. In the presence of 5% glucose, PEI and DNA interact to form complexes which are able to
move across the cell membrane by pinocytosis.

2.3.17 Maintenance of HEK hY1 and hY5 receptor transfected cells

HEK 293 (not T) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% foetal
calf serum (FCS) and 1% antibiotics (ABx – penicillin and streptomycin); this is referred to as DMEM
complete. Medium was changed every 2-3 days and cells passaged when 70% confluent using
trypsin. Medium was aspirated from the flask and the cells washed briefly in Versene (recipe in
appendix). The Versene was aspirated and replaced with 1 ml 2.5% trypsin 10x (Gibco) and 9 ml of
Versene. This was incubated until cells were seen to detach and the cell/Versene/trypsin mix was
added to a centrifuge tube containing 10 ml of DMEM complete. This was centrifuged (100 x g, 5
minutes, room temperature), the supernatant discarded and the pellet resuspended in fresh
medium and transferred to a new flask at a dilution of 1:10.

2.3.18 PEI transfection of cells

HEK 293 (not T) cells were plated out on 6 well plates at a density of 20,000 cells/well using a cell
cytometer. Transfections were carried out when the cells were 50% confluent. PEI/DNA complexes
were prepared. A 0.1 M PEI (25 kDa Aldrich) solution was adjusted to pH 7 with HCl and sterile
filtered using a 0.2 µm filter. The amount of PEI required was calculated according to the ratio of PEI amine nitrogen equivalents to DNA phosphate (where 1 µl of 0.1 M PEI is equivalent to 100 nmol amine nitrogen and 1 µg DNA is equivalent to 3 nmol DNA phosphate). The ratio of phosphate to amine nitrogen to promote stable transfection is 1 phosphate to 9 nitrogens. Therefore, for 2.2 µg of DNA (containing 6.6 nmol phosphate), 59.4 nmol of amine nitrogen are required (0.594 µl of the 0.1 M PEI). This DNA/PEI mixture was prepared in a 0.3 M NaCl solution to give a final concentration of 150 mM NaCl. PEI solution was slowly added to the DNA and NaCl solution, vortexed for 30 seconds and then left to stand at room temperature for 10 minutes prior to use.

On a 6 well plate, 4 wells were transfected with the plasmid and 2 wells were used as a positive and negative control. The DNA/PEI mix was slowly added to the cells, incubated for 3 hours at 37°C, 5% CO₂ and the medium was then removed and replaced with fresh DMEM complete. 48 hours later the medium was changed to DMEM containing 2% Geneticin (G418). One of the controls was left in DMEM complete the other treated with Geneticin. As the pcDNA3.1+ expression plasmids contain a Neo resistance gene only the successfully transformed cells should survive. In the control treated with Geneticin all of these cells died. The control plus DMEM with no Geneticin showed survival of all cells. When all the controls treated with Geneticin had died, this was an indication that the only remaining cells had been successfully transformed and stably incorporated the pcDNA3.1+ plasmid DNA. The cells were then passaged from each well into a separate T75 flask and grown to confluence.

2.3.19 Maintenance of SK-N-MC cell lines

SK-N-MC is a human neuroblastoma cell line expressing human Y1 receptors. These cells were cultured for use in the Y1 receptor binding assay. These cells were maintained in a 1:1 mixture of
DMEM: F12 supplemented with 2 mM L-glutamine, 10% FCS and 1% Abx (Penicillin and Streptomycin).

2.3.20 Receptor purification- membrane preparation

HEK 293 (not T) transfected cells with hY1 and hY5 receptor and SK-N-MC cells were cultured in T175 flasks. Approximately forty to sixty T175 flasks were grown for each cell line. Culture medium was removed and the cells detached using ice cold, sterile filtered 0.02 M PBS and a rubber policeman. The PBS containing the detached cells was then centrifuged (200 x g, 5 minutes, room temperature), the supernatants discarded and the pellet resuspended in 1 ml PBS. This was then slowly dropped into 50 ml of 1 mM HEPES buffer and mixed for 5 minutes. This was centrifuged (1000 x g, 20 minutes, 4°C) and the pellets homogenised in 50 mM HEPES buffer using an Ultra Turrax homogeniser (IKA Labortechnik, Staufen, Germany) and then centrifuged (1000 x g, 20 minutes, 4°C). This time the supernatants were kept and ultra-centrifuged (100000 x g, 1 hour, 4°C) (Sorvall OTD 55B ultra-centrifuge, Du Pont). Supernatants were discarded and the pellets resuspended in 50 mM HEPES buffer using a hand held homogeniser (Jencons). Pellets were aliquoted and stored at -70°C.

2.3.21 Receptor binding assays (RBA)

For receptor binding assays investigating binding to the Y1 and Y5 receptor I used membrane that I had prepared myself from specially transfected HEK 293 cell lines and the SK-N-MC cell line. For studies looking at Y2 receptor binding membrane was provided by Dr. James Minnion and Ms. Joyce
Cell membranes were incubated for 90 minutes at room temperature in siliconised polypropylene tubes (Sigma-Aldrich, Poole, UK) with $^{125}$I–PYY$_{1-36}$ in RBA buffer in a final assay volume of 0.5 ml. Varying amounts of cold peptide was added to displace the label and determine binding of various peptides. The receptor $^{125}$I-PYY$_{1-36}$ complex was separated from free $^{125}$I-PYY$_{1-36}$ by centrifugation (15600 x g, 3 minutes, 4°C) (Sigma Laboratory Centrifuges 3, K18). The supernatant was discarded, the pellet resuspended in 500 ml RBA buffer and then re-centrifuged. The supernatant was again discarded and the bound label measured using a gamma counter (NE 1600, NE Technology Ltd, Reading UK). Specific binding was calculated as the difference between binding of $^{125}$I-PYY$_{1-36}$ in the presence and absence of unlabelled peptide. For competition binding curves membranes were incubated in the presence of increasing concentrations of unlabelled peptide and results expressed as specific binding. All curves were performed with points in triplicate. IC$_{50}$ values were calculated using Prism 4 (GraphPad Software Inc., San Diego, USA).

2.3.22 Physiological investigation in man of PYYα

Healthy overweight subjects, men and women aged between 18 to 60 years, with body mass indices between 19 to 35 kg/m$^2$, were recruited by advertisement. Inclusion and exclusion criteria are listed in the table below (Table 2.1). All subjects were screened. They had a medical examination, history, blood tests and ECG. Those with eating disorders were excluded using SCOFF (Morgan et al., 1999) and those with high levels of restraint excluded with a Dutch eating behaviour questionnaire (DEBQ) (Caccialanza et al., 2004). A three day home food diary was used to screen for unusual food eating.
patterns. During the screening visit a sample of the buffet meal was provided and palatability assessed with a Likert scale.

The study was approved by the Hammersmith and Queen Charlotte’s Research Ethics Committee (08/H0707/140). All subjects were given a verbal explanation of the study, written information and time to decide whether to enter the study before they were consented. Study participants were warned of these potential side effects of nausea and vomiting. The study was planned and performed in accordance with ICH Good Clinical Practice and the Declaration of Helsinki.
### Inclusion criteria

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<th>Inclusion criteria</th>
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<tr>
<td>Healthy volunteers</td>
<td>History of alcoholism/substance abuse within the last 5 years</td>
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<tr>
<td>Aged between 18 and 60 years.</td>
<td>History of major haematological, renal, gastrointestinal, hepatic, respiratory, cardiovascular or psychiatric disease. Any other illness or use of over the counter medications which would interfere with the study or cause harm to the volunteer.</td>
</tr>
<tr>
<td>Male or female</td>
<td>Medical or psychological conditions or social circumstances which would interfere with the ability to participate reliably in the trial.</td>
</tr>
<tr>
<td>BMI between 19 and 35 kg/m²</td>
<td>Lack of access at home to a telephone.</td>
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<tr>
<td>Weight stable for at least 3 months</td>
<td>Women who are pregnant, breast feeding or unable to maintain adequate contraception for the duration of the study and one month afterwards.</td>
</tr>
<tr>
<td>In good health determined by medical history, clinical examination and baseline clinical investigations</td>
<td>History of hypersensitivity to any of the components of the infusions.</td>
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<tr>
<td>Treatment with an investigational drug with the two preceding months.</td>
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<tr>
<td>Volunteers who have donated, or intend to donate, blood within three months before or following study completion.</td>
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**Table 2.1:** Inclusion and exclusion criteria for the Investigation of the physiological breakdown of peptide YY
2.3.23 Protocol for the investigation of the physiological breakdown of peptide YY

Eight healthy male and female volunteers were recruited. Study visits were at least three days apart. During the 24 hour period prior to each study visit, participants were requested to refrain from strenuous exercise and alcohol consumption. From 10 pm the night before the study they were asked to drink only water. On the morning of each study visit, participants attended the Clinical Investigations Unit (Hammersmith Hospital). Female volunteers had a urine β-hCG test to exclude pregnancy. An intravenous cannula was inserted into the arm for blood sampling. A subcutaneous injection (saline or PYYα) was administered into the anterior abdominal wall. The subject was blinded as to whether they were receiving a saline or a peptide injection. Blood samples (8 ml) were taken at 0, 15, 30, 45, 60, 90, 120, 150, 180 and 210 minutes relative to the injection. At 150 minutes after the injection a buffet meal was served (food provided to excess) and the amount of food eaten was weighed and energy calculated. Visual analogue scales (VAS) were used to record hunger, satiety, nausea and food palatability. Participants were supervised at all times by two medically qualified study investigators of specialist registrar or consultant grade.

Individuals received a saline injection on their first visit and also on another occasion, randomly replacing one of the visits. This was to acclimatise them to the study environment and provide two sets of control data. In the non saline studies, they received a subcutaneous injection of the study analogue, PYYα. This was given at 10, 20, 40, 80 and 100 nmol. Safety data (vital signs and adverse effects) were reviewed following each study visit. In accordance with the Duff report (Duff, 2006) the first administration of each dose of analogue was only administered to a single volunteer (a sentinel). The development of antibodies was monitored throughout the study. A blood sample taken at the beginning of each visit was tested in a highly sensitive radioactive PYY binding system that detects the development of extremely low titre antibodies. An independent monitoring
committee reviewed the safety data (vital signs, adverse effects and immunogenicity). Participants were free to withdraw from the study at any time without providing a reason.

2.3.24 Plasma hormone analysis

PYYα like immunoreactivity was measured with a specific and sensitive radioimmunoassay. The antiserum (R2463) was produced in rabbits against synthetic porcine PYY analogue coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:17,500. This antibody cross-reacts fully with PYYα, less than 0.25% with PYY and has no cross-reactivity with pancreatic polypeptide, neuropeptide Y, or any other known gastrointestinal hormone. The $^{125}$I-PYYα was prepared by the iodogen method and purified by high pressure liquid chromatography. The specific activity of the $^{125}$I-PYYα label was 51 Bq/fmol. The assay was performed in total volume of 0.7 ml of 0.06 M phosphate buffer pH 7.2 containing 0.3% bovine serum albumin. The assay was incubated for three days at 4°C before separation of free and bound antibody label by charcoal absorption. The detection limit of the assay was 12 pmol/l and the intra and inter-assay coefficient of variation were 7.9% and 12.1% respectively.

2.3.25 Statistical analysis

Statistical advice for the study power calculation was obtained from Dr. Joseph Eliaahoo, Statistical Consultant at Imperial College London. As this was a pilot study a formal power calculation could not be performed. Experience within the department suggested that in order to allow for individual variation a minimum of 6 subjects was required per group where food intake was the measured
endpoint. At least 6 subjects are needed to detect a difference of 143.5 kcal intakes if the standard deviation is 100 kcal on a 1000 kcal meal. This gives 80% power to detect a difference in means with a significance level of 0.05. Combined data are represented as mean ± SEM.
2.4 Results

2.4.1 Peptide degradation studies

In order to compare the degradation of the PYY analogue, PYYα, with the native peptide, PYY$_{3-36}$, both peptides were incubated separately with NEP and KBB. A representative graph for each peptide incubation study is displayed below. In addition, PYY$_{3-36}$ was incubated with KBB and sent for analysis by MALDI-TOF mass spectroscopy. This enabled identification of the fragments formed to delineate vulnerable amino acid sequences which are targets for the peptidases.

Figure 2.4: Representative reverse-phase HPLC absorption profile following in vitro digestion of 2 nmol PYY$_{3-36}$ with 1.25 mg/ml KBB or 1.25 mg/ml KBB & 100 nmol phosphoramidon for 30 min at 37°C. The left y axis shows absorbance, the right y axis shows % acetonitrile and the x axis shows the time over which the peptides were eluted. The black dotted line demonstrates the acetonitrile gradient. Within the graph, the black line represents the PYY$_{3-36}$ alone; the blue line is with the addition of KBB and the red line with the addition of KBB and phosphoramidon.
Figure 2.5: Representative reverse-phase HPLC absorption profile following in vitro digestion of 2 nmol PYY<sub>α</sub> with 1.25 mg/ml KBB for 30 min at 37°C. The left y axis shows absorbance, the right y axis shows % acetonitrile and the x axis shows the time over which the peptides were eluted. The green dotted line demonstrates the acetonitrile gradient. Within the graph, the yellow line represents the PYY<sub>α</sub> alone; the blue line is with the addition of 2.5mg/ml KBB and the red line with the addition of 12.5mg KBB.
Figure 2.6 Reverse-phase HPLC absorption profile following in vitro digestion of PYY3-36 with 200ng, 400ng and 800ng NEP over 120 min at 37°C. The left y axis shows absorbance, the right y axis shows % acetonitrile and the x axis shows the time over which the peptides were eluted. The black dotted line demonstrates the acetonitrile gradient. Within the graph, the black line represents the PYY3-36 alone; the blue line is with the addition of 200 ng NEP, the red line with the addition of 400 ng NEP and the yellow line with the addition of 800 ng NEP.
Figure 2.7: Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol GnRH with 200ng NEP for 60 min at 37°C PYY\textsubscript{3-36}. The left axis shows absorbance, the right axis shows % acetonitrile and the x axis shows the time over which the peptides were eluted. The black dotted line demonstrates the acetonitrile gradient. Within the graph, the black line represents the GnRH alone; the red line with the addition of 200 ng NEP.
Figure 2.8: Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2 nmol PYYα with 200ng NEP for 120 min at 37°C. The left y axis shows absorbance, the right y axis shows % acetonitrile and the x axis shows the time over which the peptides were eluted. The green dotted line demonstrates the acetonitrile gradient. Within the graph, the yellow line represents the PYYα alone and the black line represents the addition of 200ng of NEP.
Figure 2.9: MALDI-ToF Degradation studies – Incubation with KBB and analysis by MALDI-ToF. The x axis represents the mass to charge ratio and the y axis represents signal intensity. PYY$_{3-36}$ was incubated with KBB and the resulting fragments were separated by HPLC and measured by MALDI-ToF mass spectroscopy. The peaks in the graph above represent the fragments formed and the numbers are the various molecular weights of the fragments formed. The second peak (Mw = 2859.01) may represent PYY$_{15-36}$, the third peak Mw = 3043.42 may represent PYY$_{13-36}$, and the forth peak Mw = 3229.27 may represent either PYY$_{11-36}$ or PYY$_{4-31}$. 
2.4.2 Summary of degradation data findings

The preliminary experiments using PYY\textsubscript{3-36} and KBB demonstrated, as expected, that PYY\textsubscript{3-36} is degraded by peptidases found within the KBB (53% degradation). NEP is a key enzyme found within the KBB and the addition of phosphoramidom to the KBB is known to inhibit NEP. Figure 2.4 illustrates the degradation of PYY\textsubscript{3-36} by KBB and the partial protection afforded by the addition of phosphoramidom (24% degradation with the addition of phosphoramidom). This suggests that other peptidases, not within the NEP family, are also responsible for the proteolytic degradation of PYY\textsubscript{3-36}. The incubation of PYY\textsubscript{α} with KBB demonstrates that it is more resistant to degradation (24% degradation). From these studies it is not clear if the increased α-helix stability protects the peptide from degradation by NEP or from other peptidases.

To clarify this, PYY\textsubscript{3-36} and PYY\textsubscript{α} were incubated separately with NEP (Figures 2.6 and 2.8). These studies demonstrated that NEP did not significantly degrade PYY\textsubscript{3-36} \textit{in vitro} (<5%). This finding was surprising as it has been previously documented that PYY\textsubscript{3-36} is a known substrate for NEP (Turner et al., 2001). Even increasing the amount of NEP added to the PYY\textsubscript{3-36} up to 800ng had no effect on peptide degradation. To confirm that the NEP was active, a control assay was run using GnRH, a known substrate for NEP (Figure 2.7). This demonstrated that the peptidase was active. Therefore, under these assay conditions \textit{in vitro} PYY\textsubscript{3-36} is not a substrate for NEP. As the degradation of PYY\textsubscript{3-36} by the KBB was reduced by the addition of phosphoramidom, this suggests that the peptidases responsible for PYY\textsubscript{3-36} degradation are still of the neprilysin family.

To help further investigate which peptidases within the KBB were responsible for PYY\textsubscript{3-36} proteolytic breakdown, the degradation products of the KBB and PYY\textsubscript{3-36} incubation were sent for MALDI-ToF mass spectroscopy analysis. This provides further information on the size of the fragments formed by degradation. The fragments formed were of Mw 3229, 3043 and 2859 and these may represent PYY\textsubscript{11-36}, PYY\textsubscript{13-36} and PYY\textsubscript{15-36} respectively. The production of PYY\textsubscript{11-36} may occur with meprin β (Bertenshaw, 2001). It is not currently known which enzymes could cleave the peptide to produce the PYY\textsubscript{13-36} or PYY\textsubscript{15-36} fragments.
2.4.3 Receptor binding assays

Following transformation of E.coli with plasmids containing hY1 and hY5 receptor, restriction enzyme digests were carried out to confirm the presence of the hY1 and hY5 receptor inserts.

![Restriction digest images](image)

**Figure 2.10: Restriction digests for hY1 and hY5:** The left hand agarose gel confirmed the presence of a correctly sized insert of 1338bp within the hY5 expression plasmid. The right hand agarose gel confirmed the presence of a correctly sized insert of 1155bp within the hY1 expression plasmid.

HEK 293 (not T) cell lines were successfully transfected with the hY1 and hY5 receptors and selected for stable expression of these receptors utilising Geneticin. A number of different stable cell lines were produced. RNA was extracted from the clones and rt-PCR reactions carried out using specific primers for hY1 and hY5 receptor.
Figure 2.11: rt-PCR for hY1 and hY5 receptor. The gel on the left represents successful transfection of 3 different hY1 receptor cell lines: lines 11, 12 and 13. The (–) column is a negative control which is the rt-PCR reaction run without any reverse transcriptase and the (+) is the same reaction run with reverse transcriptase. The presence of a band in the negative column would have represented DNA contamination of the sample. The absence of a band represents a non-contaminated sample. For the three clones above (11,12 and 13) all had a band seen between 1018 and 506 and this represents the size of the hY1 and hY5 receptor insert 779bp which was amplified on rt-PCR. The gel on the right is for the hY5 receptor cell lines, line B and C. A band is seen below the 506.5 band on the ladder which represents the size of the hY5 receptor sequence amplified, 307bp.

For each successfully transformed clone (as demonstrated by a positive band on rt-PCR) a membrane preparation was carried out to extract the receptors. This was used to carry out small receptor binding assays to demonstrate the successful expression of the hY1 and hY5 receptor. For two clones, hY1 receptor (cell line 11) and hY5 receptor (cell line B), functional receptor was demonstrated, as judged by the high affinity binding of NPY.
Figure 2.12: Receptor binding curve for hY5 receptor (Cell line B)

Figure 2.13: RBA curve for hY1 receptor (Cell line 11)
For both receptor binding assays the amount of non-specific binding was extremely high (40%). In order to reduce this, a number of assays were carried out varying the assay conditions (time of incubation, various buffers and different amounts of BSA). This had no effect on the non-specific binding. This led me to investigate the use of a hY1 receptor expressing cell line, SK-N-MC. To characterise this cell line, receptor binding assays were carried out with fragments of PYY. This was done to confirm that the rank order of potencies was in line with those expected for a hY1 receptor. The receptor binding affinities demonstrated that this cell line expressed a Y receptor with the expected order of binding affinities for Y1 receptor (Figure 2.14). This led me to use this cell line to investigate the binding affinities of PYYα (Figure 2.15).

![Graph](image)

**Figure 2.14:** Receptor binding assay - SK-N-MC used as a model for hY1 receptor demonstrates the order of binding affinities expected for a hY1 receptor: $\text{PYY}_{1-36} \geq \text{NPY} \geq \text{PYY}_{2-36} \geq \text{PYY}_{3-36} \geq \text{Y2A}$. The Y2 agonist was N-acetyl Leu28, Leu31 NPY$_{24-36}$.
Figure 2.15: Receptor binding assays for the hY1 receptor (obtained from SK-N-MC membranes). Cell membrane preparations were incubated with $^{125}$I-PYY$_{1-36}$ and varying amounts of unlabelled PYY$_{\alpha}$ (black line), PYY$_{1-36}$ (lilac line) and PYY$_{3-36}$ (purple line).
Figure 2.16: Receptor binding assays for the hY2 receptor (obtained from a hY2 receptor HEK293 over-expressing cell line). Cell membrane preparations were incubated with $^{125}$I-PYY$\text{1-36}$ and varying amounts of unlabelled PYY$\alpha$ (blue line) and PYY$\text{3-36}$ (grey line).
2.4.4 Summary of results for receptor binding assays

The successful expression of hY1 and hY5 receptor in HEK 293 (not T) cell lines was demonstrated, however, the receptor binding assays were not reliable for these receptors as the amount of non-specific binding for both was extremely high (40%). For this reason, I used an hY1 receptor expressing cell line SK-N-MC (Figure 2.15). This demonstrated that the PYYα bound to this receptor with comparable affinity to the naturally occurring ligand, PYY1-36. The receptor binding assay for hY2 receptor demonstrated a 2.5 fold reduced affinity of PYYα for this receptor compared to its naturally occurring ligand. This was a surprising finding as the α-helix has previously been shown to increase affinity at the Y2 receptor (Lerch et al., 2005).

Work within the Department of Investigative Medicine, Imperial College London, has shown a dose dependent effect of PYYα with significant reductions in appetite and body weight. This effect was demonstrable in two species, mice and rats, and lasted for up to 48 hours. In addition, these analogues were found to be safe as demonstrated by formal toxicity testing. In view of these findings these analogues were administered in a clinical study, designed to look at the biological effects of the analogues as well as the pharmacokinetic properties of the analogues.
2.4.5 Investigation of the physiological breakdown of peptide YY in man

Eight healthy volunteers were recruited for this clinical study. For each dose of PYYα (10, 20, 40, 80, 100 nmol) four subjects were tested. Administration of PYYα led to no change in blood pressure or heart rate and had no adverse effects.

2.4.6 Effect of PYYα on energy intake in healthy volunteers

![Bar chart representing total energy intake (kJ) consumed at a buffet meal, 150 minutes following subcutaneous administration of PYYα. The x axis represents the treatment given and the y axis represents the total energy intake. Each bar represents a total of four different volunteers who were administered the peptide. All eight volunteers were administered saline on their first visit (saline 1). 4 out of 8 received saline in a subsequent visit. The error bars represent SEM.](chart.png)
Doses of PYYα up to a maximum dose of 100 nmol had no effect on food intake as measured by buffet meal. This finding was surprising and was in contrast to the anorexic effects of PYYα in rodents.

2.4.7 Effect of PYYα on subjective assessment of appetite as measured by visual analogue scales

All subjects were requested to complete visual analogue scales during their study visit. These were completed at 0, 15, 30, 45, 60, 90, 120, 150, 180 and 210 minutes following the administration of saline or PYYα. In addition, following the meal subjects were requested to score the tastiness of the meal. There was no difference seen between the groups in terms of the subjective ratings of appetite at the doses of PYYα given. VAS are notoriously unreliable and with such a small n number it may be expected that a difference is not seen, especially as there was no change in the objective measurement of food intake.
Figure 2.18: Subjective measures of appetite during the feeding study following administration of PYYα in healthy volunteers.
Figure 2.18 (continued)

All subjects received a meal at 150 minutes. Subjects recorded their subjective assessment in response to a questionnaire asking (A) “How hungry do you feel now?” (B)“How full do you feel now?” (C)“How pleasant would it be to eat right now?” (D)“How much can you eat right now?” (E)“How full do you feel right now?” (F) How tasty was the meal?” The blue lines represent the saline visit, the lilac line the 10 nmol, the mauve line the 20 nmol, the purple line the 40 nmol, the emerald line the 80 nmol and the light green line the 100 nmol dose of PYYα administered subcutaneously at 0 minutes. There was no significant difference in scores following any of the doses of PYYα.
2.4.8 Effect on subcutaneous PYYα administration on plasma levels

Blood samples were taken for PYYα throughout the feeding study at 0, 15, 30, 45, 60, 90, 120, 150, 180 and 210 minutes following the administration of saline or PYYα. These were assayed by radioimmunoassay. This demonstrated an early rise in the plasma levels of PYYα at 30 minutes followed by a gradual reduction in levels over 210 minutes. In contrast to data from the rodent studies, PYYα in humans did not have a prolonged half-life.

The overall effects of PYYα in humans were quite the opposite of those previously demonstrated in mice and rat studies, carried out by the PYY analogue group, Imperial College London. Despite having a strengthened α-helix, PYYα have a shorter plasma appearance, as measured by the RIA and also by food intake, which was a surrogate marker of biological effect.
Figure 2.19: Plasma levels of PYYα measured by RIA following subcutaneous injection of PYYα. For all doses a meal was given at 150 minutes.

For all the graphs, the y axis represents the PYYα concentration (pmol/l) in plasma and the x axis represents time in minutes. Graph (a) PYYα 10 nmol, (b) 20 nmol, (c) 40 nmol, (d) 80 nmol and (e) 100 nmol. Each dose was administered to 4 volunteers, n = 4. The error bars represent SEM.
2.5 Discussion

Dysregulation of PYY$_{3-36}$ metabolism is a potential cause of altered appetite regulation and obesity. Therefore, knowledge of PYY$_{3-36}$ breakdown by endogenous peptidases is important and PYY analogues are a useful mechanism to study this in vivo. PYY$_\alpha$ was previously shown to have an increased effect, compared to native PYY$_{3-36}$, in reducing food intake in mice and rats and had a prolonged half-life compared to the native peptide. For this reason, this peptide analogue was chosen to be investigated in depth in vitro and in human studies.

2.5.1 Pharmacological profile

Peptide hormones are often administered parenterally to avoid the harsh gastric environment. The ideal pharmacological profile of a PYY analogue would be as follows: firstly, following subcutaneous administration it would have a slow and constant release, secondly there would be prolonged plasma levels which fall within the therapeutic window. In animal studies, PYY$_\alpha$ has a favourable pharmacological profile with levels detectable up to 48 hours after administration. In the clinical study, food intake was measured as a biomarker for peptide activity. For doses up to 100 nmol of PYY$_\alpha$, no reduction in food intake was demonstrated. This surprising finding could be explained in two ways:

1. The construction of PYY$_\alpha$ has led to alteration of receptor binding affinities and therefore its bioactivity.

2. The degradation of PYY$_\alpha$ is different between rodent species and man, and it is degraded more rapidly in humans, leading to loss of bioactivity.
2.5.2 Y receptor binding

Previously it has been demonstrated that disruption of the α-helix led to reduced affinity at all NPY receptors (Lerch et al., 2005). From this it could be extrapolated that by strengthening the α-helix this would lead to increased affinity at all the receptors. I have found that, in receptor binding assays, PYYα had an affinity approximately 2.5 fold lower than PYY$_{3-36}$ at the Y2 receptor. I have also found that PYYα had a comparable affinity to native PYY$_{1-36}$ at the Y1 receptor. This finding demonstrates that the α-helix plays a differential role within the peptide in the binding to these two receptors. This finding could explain the lack of effect of PYYα on food intake. The Y1 receptor is implicated in orexigenic appetite pathways, and it is possible that PYYα is counterbalancing any anorexigenic effects with off-target orexigenic effects.

2.5.3 Peptidase resistance

In animal studies, it was previously demonstrated that PYYα had anorectic effects lasting up to 48 hours, far longer than those seen with PYY$_{3-36}$. It was hypothesised that these prolonged effects were due to reduced proteolytic clearance of the peptide by the proline preferring peptidases. Therefore, I carried out assays looking at the degradation of PYYα incubated with NEP and KBB. Previously NEP was found to be a key peptidase important in the in vitro degradation of PYY$_{3-36}$. However, I was unable to replicate this finding in my degradation study. PYY$_{3-36}$ and PYYα were both resistant to proteolytic cleavage by NEP. KBB is a rich source of proteolytic enzymes (including NEP) and the addition of KBB to PYY$_{3-36}$ and PYYα led to degradation of these peptides. PYYα was slightly more resistant to KBB degradation compared to PYY$_{3-36}$ (24% degradation of PYYα compared to 53% degradation of PYY$_{3-36}$). This demonstrates that strengthening the α-helix offers some protection to the peptide against proteolytic degradation. Phosphoramidon is known to inhibit NEP and other
members of its peptidase family for example, neprilysin-2, Kell blood group protein and endothelin-converting enzyme. In my study, the addition of phosphoramidon only partially inhibited the degradation of PYY\textsubscript{3-36} indicating the presence of other peptidases, not within the neprilysin family, important in degradation. Other candidate peptidases for PYY\textsubscript{3-36} cleavage include the meprins and aminopeptidases. It would be of interest, for further work, to compare the degradation of PYY\textsubscript{3-36} and PYY\textalpha{} \textit{in vitro} with these peptidases, and utilise KBB with specific inhibitors of these peptidases in order to understand if they are responsible for the degradation seen with KBB.

2.5.4 Conclusions

The PYY\textalpha{} analogue was a useful tool to investigate the physiological degradation of PYY. The increased degradation of PYY\textsubscript{3-36} compared to PYY\textalpha{} demonstrates that α-helix stability is important in terms of tertiary structure. When the α-helix is strengthened it led to reduced proteolytic degradation by peptidases within the KBB. In animal studies it was previously shown that PYY\textalpha{} led to a prolonged anorectic effect. In this study, I have shown that in man, PYY\textalpha{} had no effect on appetite. I found that stabilising the α-helix by the insertion of an α-latrotoxin insert led to enhanced affinity at the Y1 receptor and reduced affinity at the Y2 receptor. This could be expected to lead to counterbalanced effects on appetite as the Y1 receptor is involved in the orexigenic appetite circuits and the Y2 receptor is involved in the anorectic appetite circuit. However, the receptor binding assays do not predict whether the ligand has agonist or antagonistic effects. It would be useful, in future studies, to investigate the effects of PYY and PYY\textalpha{} on second messenger activity. This could be achieved by measuring changes in intracellular cAMP or calcium levels in cellular models.
Chapter 3: Investigation into the physiological stimuli leading to the release of GLP-1 and PYY from the entero-endocrine L cell

3.1 Introduction

There is a drive towards creating analogues of the anorectic gut hormones, GLP-1, PYY and OXM, which could be used as potential anti-obesity agents (Hussain and Bloom, 2011). The successful study of GLP-1 led to the creation of GLP-1 analogues which in turn yielded effective treatments for type 2 diabetes (Drucker and Nauck, 2006). These treatments have the additional advantage of causing weight loss due to the anorectic properties of GLP-1 (Pratley et al., 2011). The weight loss seen with these GLP-1 treatments, though significant, is only of the order of approximately 3.5 kg, achieved after 52 weeks of treatment (Pratley et al., 2011). In order to achieve more substantial weight loss it may be necessary to consider a poly-pharmaceutical approach. In many medical conditions, this type of approach is used. For example, patients with hypertension often take a number of different medications to lower the blood pressure. This approach is also being considered within the field of obesity treatment. Currently co-administration of OXM and PYY is being investigated (Field et al., 2010b).

Exogenous administration of gut hormones often results in supraphysiological circulating levels. This can cause adverse effects, such as nausea and vomiting, and has been demonstrated with GLP-1 (Williams et al., 2009) and PYY (Zambanini et al., 1999). Drug delivery of peptide hormones is technically difficult and often necessitates a needle based delivery system which patients may find unacceptable. In addition, with the injection of peptide hormones there is also a risk of precipitating immune reactions and antibody formation. There is great demand for a needle free, safe, effective peptide hormone delivery system.
PYY, GLP-1 and OXM are co-released from the entero-endocrine L cells in the distal gut. Therefore a means of stimulating L cells to secrete these hormones could be a potentially effective treatment for obesity. An ideal treatment would be an orally delivered secretagogue that stimulates the release of anorectic hormones from L cells. In order to find such a secretagogue, we can investigate existing models of L cell hyper-secretion, for example following gut bariatric surgery and with malabsorption. Following bariatric gut surgery, the rise in endogenous gut hormones is thought to be a consequence of the increased nutrient delivery to the distal gut (Le Roux et al., 2006a; Taqi et al., 2010). An identical pattern is seen in malabsorptive syndromes, such as tropical sprue, where failure to absorb nutrients proximally leads to an increased nutrient load to the distal gut (Besterman et al., 1979). These iatrogenic and pathological models suggest that it is specific nutrients being delivered to the distal gut that are responsible for L cell stimulation and gut hormone release.

In order to be able to manipulate L cells and increase the production and release of gut hormones, it is necessary to study the physiology of the L cell and elucidate factors that lead to PYY and GLP-1 release. In this chapter, I will attempt to investigate L cell physiology in a primary cell culture model. This will enable a greater understanding of the various potential physiological and pharmacological stimulants for gut hormone release.

3.1.1 Distribution of L cells within colonic crypts

L cells are distributed throughout the gastrointestinal tract and increase in number distally with the highest numbers found in the colon and rectum (Eissele et al., 1992). The epithelial surface of the distal gastrointestinal tract is constantly renewed by colonic crypts generating vast quantities of cells (Malaterre et al., 2007). At the base of each crypt, stem cells give rise to a dividing cell population that differentiates into three lineages: columnar cells, goblet cells and entero-endocrine cells.
Malaterre et al., 2007). It has been estimated that the entero-endocrine cells constitute approximately 1-5% of the total number of cells within each crypt (Halm and Halm, 1999). Within the L cells PYY and GLP-1 have been co-localised within granules and these are thought to be secreted from the cells (Bottcher et al., 1984).

3.1.2 GLP-1 and PYY release

GLP-1 and PYY are released post-prandially. The release of GLP-1 is biphasic with an initial rise at 15 to 30 minutes after a meal followed by a second peak at 90 to 120 minutes (Elliott et al., 1993; Herrmann et al., 1995). PYY release occurs at 30 minutes following a meal, and can remain elevated for up to 2 hours with ingestion of protein and fats (Adrian et al., 1985b). Despite the fact that the overall effect of food intake is to stimulate PYY and GLP-1 release, the mechanism of activation of the different populations of L cells located in various parts of the gastrointestinal tract is not fully understood.

3.1.3 Neural stimulation

The majority of L cells are located distally in the gut and this is the main source of both GLP-1 and PYY (Adrian et al., 1985b). After a meal the rapid rise in GLP-1 is unlikely to be due to direct L cell stimulation and is more likely to be the result of a proximal distal loop. Nutrients in the proximal gut may stimulate distal release of gut hormones by either a neural or circulating signal. In support of this, L cells are located in close proximity to the enteric nervous system (Anini and Brubaker, 2003b) and the gut microvasculature (Hansen et al., 1999) (Figure 3.1). The enteric nervous system has been
described as the “second brain” as it is extensive and contains every known neurotransmitter that exists within the CNS. The vagus nerve has been implicated in the proximal to distal loop and vagotomy reduces the release of PYY (Fu-Cheng et al., 1997) and GLP-1 from the distal gut (Rocca and Brubaker, 1999). In addition, in NCI-H716 cells it has been demonstrated that carbachol, a cholinergic agonist, leads to increased GLP-1 secretion (Reimer et al., 2001).

Figure 3.1: Structural representation of the intestinal wall. This illustrates the contact of the cells with the gut lumen and with the gut microvasculature. Within each crypt there are approximately 500 cells and the entero-endocrine cells constitute approximately 1-5% of the total cell number. Figure from (Chin et al., 2008).
3.1.4 Hormonal stimulation

Several hormones including leptin (Anini and Brubaker, 2003a), GIP (Roberge and Brubaker, 1993), and gastrin releasing peptide (GRP) (Roberge et al., 1996) have been shown to stimulate GLP-1 secretion. Somatostatin is a potent inhibitory gut hormone which is secreted from intestinal D cells and reduces GLP-1 and PYY release from L cells (Hansen et al., 2000).

3.1.5 Intestinal L cells and nutrient sensing

L cells within the small intestine are ideally situated to detect changes in gut luminal contents and may sense nutrients directly. The L cells are polarised with an apical surface facing into the gut lumen and a basolateral surface adjacent to blood vessels and nerves (Eissele et al., 1992). It is likely that the L cells are able to sense gut luminal contents directly by the interaction of the nutrients with receptors located on the L cell apical surface. The vast majority of L cells are located distally in the colon and rectum. These colonic L cells are less likely to experience changes in sugar and amino acids levels directly. They are more likely to be subjected to changes in short chain fatty acid (SCFA) concentrations. SCFA are produced in the colon as a result of the fermentation of non digestible carbohydrates (NDC) (Cummings, 1981). Therefore, the second phase of GLP-1 and PYY secretion may be due to the direct contact between these nutrients within the gut and the L cells.

3.1.6 Glucose sensing

L cells are sensitive to glucose and, in GLUTag cells, GLP-1 release is triggered by 0.5 – 25 mM glucose (Reimann and Gribble, 2002). To date, three separate mechanisms enabling L cells to
recognise sugars have been identified. In pancreatic β cells, glucose stimulated insulin release is a consequence of: glucose entering the cell via the GLUT 2 transporter, phosphorylation of glucose by glucokinase, an increase in the intracellular ATP/ADP ratio and finally closure of the $K_{ATP}$ channel leading to increased intracellular calcium and finally insulin release (Rorsman, 1997). In comparison with β cells, L cells also contain $K_{ATP}$ channels and glucokinase (Gribble et al., 2003). Secondly, the demonstration that GLUTag cells and mouse L cells are also sensitive to non-metabolisable sugars revealed the presence of the sodium glucose co-transporter (SGLT) within the cells, these transporters are used by the intestinal brush border to take up sugars (Gribble et al., 2003). Finally, components of the taste receptor pathway, Tas 1 receptor (Jang et al., 2007) and α-gustducin (Sutherland et al., 2007), have been characterised within L cells. The physiological implications of glucose sensing by the L cells are unclear. The apical L cell surface is unlikely to come in contact with glucose as most will have been previously absorbed in the gastrointestinal tract. In contrast, the basolateral L cell surface would be in constant contact with glucose within the circulation. It may be that glucose sensing by the L cell is only of importance under pathological conditions, such as malabsorption, when the capacity of the duodenum to absorb sugars is impaired.

3.1.7 Amino acids

Protein hydrolysates stimulate GLP-1 release in vivo (Cordier-Bussat et al., 1998) and various amino acids, in particular glutamine (0.01 mM to 10 mM), have been found to have direct stimulatory effects on both Glutag cells (Reimann et al., 2004) and primary isolated L cells (Tolhurst et al., 2011). $Na^+$ dependent amino acid transporters and members of the class C G protein coupled receptor family are thought to mediate this response to glutamine (Tolhurst et al., 2011).
3.1.8 Lipids and fatty acid stimulation

Lipids and fatty acids have been shown to be potent stimulants of PYY release (Pironi et al., 1993; Adrian et al., 1985). As glucose does not reach the distal gut in high concentrations, fatty acids are more likely to be physiological stimulators of GLP-1 release (Schirra et al., 1996). However, under conditions such as malnutrition or bariatric surgery, glucose and fatty acids may be equally important in triggering release of anorectic gut hormones from L cells. The recognition of fatty acids by L cells may be mediated via G protein coupled receptors located on the L cell surface that have affinity to both medium and long chain fatty acids. GPR119, present on both the apical and basolateral surfaces of the L cell, is known to bind the endogenous ligand oleoylethanolamide (OEA) and it has been demonstrated that OEA administration in vivo and in vitro can increase GLP-1 and PYY secretion (Cox et al., 2010). In vivo studies using a small molecule GPR119 agonist have also demonstrated increased GLP-1 secretion (Chu et al., 2008).

3.1.9 Short chain fatty acids (SCFA)

Discovery of the SCFA receptors, GPR41 and GPR43, on the apical surface of L cells, has led to interest in SCFA as a potential secretagogue (Karaki et al., 2006). SCFA and other small molecule pharmaceutical agonists of receptors located on the L cell surface may be therefore beneficial as potential triggers of the release of GLP-1, PYY and OXM. SCFA are described extensively in chapter 4.
3.1.10 L cell second messenger systems leading to release of gut hormones

Following nutrient recognition at the cell surface by receptors coupled to Gs G protein and activation of adenylate cyclase, elevated cAMP is thought to be the main determinant of GLP-1 release. Increased cAMP signals through protein kinase A (PKA) pathways and exchange proteins directly activated by cAMP, for example EPAC (Drucker et al., 1994). Increased cAMP leading to gut hormone release has been demonstrated in a number of systems including: perfused intestine, cultured primary cells and immortalised cell lines (Simpson et al., 2007). The role of cAMP has been further elucidated and elevated cAMP is associated with an increase in the transcription of the preproglucagon gene (Drucker et al., 1994) as well as a direct stimulation of hormone release (Reimann and Gribble, 2002). Forskolin, an adenylate cyclase activator, and isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor have previously been used to increase cAMP and activate the PKA dependent pathway in L cell cultures. This is an accepted positive control which has been used to demonstrate that GLP-1 secretion increases in response to stimulation of the cAMP dependent protein kinase A (PKA) pathway (Brubaker et al., 1998).

Gut hormone release has also been associated with an activation of Gq coupled G proteins and an increase in intracellular calcium. The fatty acid receptors GPR40 and GPR120 are both associated with Gq and are implicated in nutrient sensing (Brown et al., 2005). In addition, Gi has also been implicated in the L cell second messenger pathways. The main mode of action of Gi is to inhibit cAMP production by adenylate cyclase and currently its role in second messenger signalling within the L cell is not fully understood.
Figure 3.2: A schematic diagram of an entero-endocrine L cell. (Tolhurst et al., 2009). Ingested nutrients travel through the gastrointestinal tract and may be detected by G protein coupled receptors located on the L cell apical surface. In addition, glucose may be detected by sodium and glucose co-transporters (SGLT1) as well as by GLUT2 on the basolateral L cell surface. Activation of these receptors and transporters leads to an increase in intracellular cAMP and intracellular calcium which in turn leads to exocytosis of the secretory granules containing PYY and GLP-1. In addition, L cells are stimulated by neural signals from the enteric nervous system and circulating hormones.
3.1.11 Immortalised L cell lines used to study L cell physiology

Various models have been used to study L cells including: isolated primary mouse cells (Reimann et al., 2008), immortalised cell lines GLUTag cells (Drucker et al., 1994), secretin tumour cell line (STC-1) (Rindi et al., 1990), NCI-H716 (Reimer et al., 2001) and isolated intestinal loops (Plaisancie et al., 1994). The NCI-H716 cell line represents a human L cell model derived from a human caecal adenocarcinoma (Park et al., 1987). It was initially described as a cell line with the potential to differentiate into endocrine cells and its ability to secrete GLP-1 has been established (Reimer et al., 2001). NCI cells do not, however, secrete PYY (work carried out within this lab). The GLUTag cell line is derived from a colonic tumour from a transgenic mouse expressing SV40 large T antigen under the control of a proglucagon promoter (Drucker et al., 1994). This cell line has been extensively used to study the mechanism of GLP-1 release (Gribble et al., 2003). This cell line is not known to release PYY or OXM and therefore it can not be used to study the mechanism of release of these hormones. STC-1 cells are also a mouse derived cell line and secrete a wide range of hormones including secretin, CCK, GIP and GLP-1, this cell line therefore is not comparable with the L cell in vivo (Abello et al., 1994; Rindi et al., 1990).

Many of the existing L cell models described above serve as poor physiological models due to a marked difference in gut hormone secretion profile compared to human L cells in vivo. In addition, immortalised cells lack paracrine stimulation from neighbouring cells as well as neuronal input from the diffuse enteric nervous system. In an attempt to rectify some of these issues I intend to establish a primary cell culture of cells from mouse and human colon. This would include a variety of cell types including enterocytes, epithelial cells, immune cells, fibroblasts and adipocytes. This would give a more realistic picture of the in vivo situation. As GLP-1 and PYY are only released from the L cells, measurement of these hormones would specifically indicate the presence of L cells within the cultures.
3.2 Aims and hypothesis

3.2.1 Aims

To establish an L cell model that can be used to determine the mechanism of release of gut anorectic hormones, GLP-1 and PYY.

1. To set up a primary mouse L cell model
2. To replicate published work on this model with glucose and glutamine to determine if this model is viable and physiological
3. To stimulate the L cells with SCFA and measure GLP-1 and PYY release
4. To set up a primary human L cell culture model for future studies and screening of various physiological and pharmacological agents

3.2.2 Hypothesis

I hypothesise that SCFA, which are present at high concentrations in the distal gut, at the site where the L cells are the most abundant, will stimulate release of PYY and GLP-1 from the primary L cells in both mouse and human models.
3.3 Methods

3.3.1 Mouse L cell isolation

A method of isolating primary L cells was obtained from Dr. Fiona Gribble, Cambridge University. This method has been published and is an accepted method of isolating L cells. Further attempts by Dr. Gribble to separate out the individual L cells by fluorescence activated cell sorting (FACS) and establish a pure L cell culture have been unsuccessful due to the requirement of L cells for paracrine stimulation. Therefore, currently the only method of culturing L cells is within a mixed colonic culture.

All animal procedures were approved by the Local Ethical Committee and conformed to Home Office regulations. 6-8 week C57 Black male mice were sacrificed and the colon, from the caecum to the rectum, collected into ice cold L-15 Leibowitz medium (PAA, UK). The intestine was cleaned thoroughly in Leibowitz medium and then chopped into 1 mm² squares. The tissue was then transferred into a laminar flow cabinet and all further work carried out with aseptic technique. The sections of colon were digested with 0.4 mg/ml collagenase XI (Sigma, C9407) in Dulbecco’s Modified Eagle Medium (DMEM) at 37°C. Resulting cell suspensions were filtered through 200 µM nylon mesh, centrifuged (5 minutes, 300 x g) and the pellets resuspended in DMEM (supplemented with 10% FCS and 1% Abx and referred to as DMEM complete). The digestion process was repeated up to four times and the resulting cell suspension plated out onto 24 well, 1% Matrigel (BD Matrigel) coated plates.
3.3.2 Human L cell isolation

Ethical approval for this study was obtained from the Hammersmith & Queen Charlotte’s REC (Registration number 2000/ 5795). This study was performed in collaboration with Dr. Julian Walters, Dr. Jonathan Nolan and Dr. Ian Johnston, Imperial College London, who kindly obtained the biopsies for use in these studies. Subjects undergoing diagnostic colonoscopy for idiopathic diarrhoea were approached to obtain additional samples for research purposes. Eight biopsies measuring 2 mm$^2$ were obtained from each volunteer. These colonic biopsies were collected into ice cold L-15 Leibowitz medium without glutamine (PAA). The samples were washed thoroughly and then digested as described above (section 3.3.1). As these crypts were larger in diameter, the filtration stage through the nylon mesh was omitted.

3.3.3 Secretion experiments

Hormone secretion experiments were carried out 24 hours after plating the cells. The cells were washed three times with 500 µl of a physiological secretion buffer (4.5 mM KCl, 138 mM NaCl, 4.2 mM NaHCO$_3$, 1.2 mM NaH$_2$PO$_4$, 2.6 mM CaCl$_2$, 1.2 mM MgCl$_2$ and 10 mM HEPES, this was adjusted to pH 7.4 with NaOH). The cells were then incubated in 300 µl of the physiological buffer containing the test reagents for 4 hours at 37°C at 5% CO$_2$. Forskolin and IBMX were prepared as 1000x stocks in DMSO and the final concentration adjusted to 0.1-0.2% DMSO. Na acetate, Na propionate, Na butyrate, glucose and glutamine were obtained from Sigma. Solutions were prepared on the day of the secretion experiment and the pH checked to ensure a neutral pH. The GPR43 agonist, phenylacetamide 1, was kindly donated to us by Amgen. The molecular structure has previously been published (Lee et al., 2008).
Following incubation, the cell supernatants were collected into eppendorfs and centrifuged (3 minutes, 100 x g) to remove any dead, floating cells. The supernatants were separated from the pellets and stored in clean eppendorfs at -20°C until analysis. The cells remaining in the plates were treated with a 500 µl per well of a cell lysis buffer (40 ml GDW, 0.25 g Na deoxycholic acid, 0.5 ml Igepal, 1.5 ml 5 M NaCl and 2.5 ml of 1 M Tris HCl, pH 7.4), frozen at -80°C, then scraped and stored at -20°C until analysis.

To calculate percentage release the following formula was used

\[
\text{% hormone released} = \frac{100 \times \text{hormone measured in the supernatant}}{\text{Hormone in the supernatant plus hormone in the lysed cells}}
\]

### 3.3.4 GLP-1 and PYY assay

**Plasma GLP-1 RIA**

GLP-1- like immunoreactivity (IR) was measured by a specific and sensitive RIA, previously established (Kreymann et al., 1987). The antibody was produced in rabbits against GLP-1 coupled to bovine serum albumin (BSA). The antibody cross reacted 100% with all amidated forms of GLP-1 but did not cross react with glycine extended forms [GLP-1\textsubscript{1-37} and GLP-1\textsubscript{7-37}] or any other known pancreatic or gastrointestinal peptide. \textsuperscript{125}I-GLP-1 was prepared by the iodogen method (Wood et al., 1981) and purified by HPLC. The specific activity of the \textsuperscript{125}I GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.35 ml of 0.06 M sodium barbitone buffer (pH 8) containing 0.3 % BSA. The assay was incubated for three days at 4ºC before separation of the free from antibody bound label by charcoal absorption. The limit of detection was 7.5 pmol/l with an intra-assay variation of 5.4%.
Plasma PYY RIA

PYY-like IR was measured using a specific and sensitive RIA (Adrian et al., 1985). The assay measured the hormone fragment, PYY_{3-36}, and the full length hormone, PYY_{1-36}, both of which are biologically active. The antiserum (Y21) was produced in rabbits against synthetic porcine PYY coupled to BSA by glutaraldehyde and used at a final dilution of 1:50,000. This antibody cross-reacts fully with the biologically active circulating forms of PYY, but not with PP or other known gastrointestinal hormones. The $^{125}$I-PYY was prepared by the iodogen method and purified by high pressure liquid chromatography. The specific activity of the $^{125}$I PYY label was 54 Bq/fmol. The assay was performed in total volume of 0.35 ml of 0.06 M phosphate buffer PH 7.2 containing 0.3% BSA. The assay was incubated for three days at 4ºC before separation of the free from antibody bound label by sheep anti-rabbit antibody. The detection limit of the assay was 2.5 pmol/l, with an intra-assay coefficient of variation of 5.8 %.

3.3.5 Statistical analysis

One way ANOVA was used to compare differences between the treatment groups. Error bars represent standard error of the mean. Results were considered significant if the P value was < 0.05. Analysis was carried out using Graph Pad Prism software, version 4.
3.4 Results

Initial experiments demonstrated that the digestion protocol was effective in isolating individual colonic crypts from mouse intestine. The photograph below (Figure 3.3) illustrates the abundance of crypts isolated using this method. The crypts had a clearly defined morphological appearance immediately following digestion but soon lost this appearance within 24 hours as the migration of cells out of the crypt became apparent (Figure 3.4). The mouse crypts had a different morphological appearance compared to the human crypts (Figure 3.5) which were longer (250 µm) and had a broader appearance.

Figure 3.3: Photomicrograph of mouse colonic crypts obtained immediately following colon digestion. (10x magnification) Multiple elongated crypts can be seen in cross section. One crypt is approximately 100µm in length.
Figure 3.4: Photomicrograph of mouse colonic crypt cultures 24 hours after plating. (10x magnification). Numerous cells can be seen migrating out of the crypts.

Figure 3.5: Photomicrograph of colonic crypts isolated from human colonic biopsies. (10x magnification). These crypts have a slightly different morphological appearance compared to mouse crypts. They have a broader opening and are larger in size (1 crypt is approximately 250 µm in length).
3.4.1 Stimulation experiments to increase cAMP

Initial experiments were carried out using the protocol established by Dr. Gribble. To validate that this was an effective method of isolating mouse L cells, a series of stimulation experiments were conducted using a combined stimulus of 0.01 mM forskolin and IBMX. This demonstrated a reliable increase in GLP-1, \( P < 0.001 \) (student’s t test).

**Figure 3.6: Stimulation experiment.** The x axis represents the treatment group; the y axis represents the GLP-1 released. The graph on the left demonstrates the amount of GLP-1 released from the cells into the cell supernatant (GLP-1 measured by RIA in pmol/l), the graph on the right represents the % of GLP-1 released from the cells as a whole. \( N = 4 \), number of wells per experiment = 12.
3.4.2 Time course experiment

In order to optimise the L cell cultures further, the cells were plated out and the amount of PYY released measured at various different time points (15, 30, 60, 120, 180 and 240 minutes). The assay to measure GLP-1 and PYY was an in house RIA developed primarily for a human plasma matrix. The small amounts of PYY and GLP-1 released from the cells were initially difficult to detect, consequently I had to modify the RIA and reduce the total volume by half (total volume 0.35 ml) whilst still maintaining the same amount of sample (100 µl) added to each tube. The time course experiment demonstrates an increase in release of PYY over time, from 60 minutes until 240 minutes. The error bars increase over this time period possibly demonstrating an increase in cell death and cell rupture leading to variable amounts of PYY being released. In spite of this, the 4 hour time point was used for all following experiments in order to be able to detect the PYY adequately with the RIA.

![Figure 3.7: Time course experiment. PYY released into the supernatant was measured at various time points. The x axis represents the time (minutes), the y axis represents the amount of PYY released into the supernatant (pmol/L). The error bars represent SEM.](image-url)
For all graphs in the following section, these are representative graphs. Each mouse colonic crypt experiment was carried out a minimum of 3 times, n = 3, with number of wells per experiment ranging from 4-6. Where there are no error bars this indicates that for several of the samples the GLP-1 or PYY was either undetectable in the sample or was too high and consequently off the standard curve. Due to differences between experiments in terms of quality and quantity of cells isolated (due to difficulties of primary cell culture), it is not possible to combine the results from separate experiments. For the human colonic crypt cultures, this is a newly established technique and these experiments have only been carried out once.
3.4.3 Secretion experiments with glucose

Figure 3.8: Mouse primary colonic crypts incubated with glucose, PYY measured. Graph (a) represents PYY measured in cell supernatants, graph (b) represents % of PYY released from the cells. Error bars indicate mean + SEM.

Figure 3.9: Mouse primary colonic crypts incubated with glucose, GLP-1 measured. Graph (a) represents GLP-1 measured in cell supernatants, graph (b) represents % of GLP-1 released from the cells. Error bars indicate mean + SEM.
The secretion experiments with glucose and the primary mouse colonic crypts demonstrated a non-significant increase in both PYY and GLP-1. However, a trend of increasing PYY and GLP-1 was apparent with the increasing concentrations of glucose (from 1 mM to 100 mM). For all experiments a positive control (0.01 mM IBMX and forskolin) and a negative control (physiological buffer alone) were carried out which demonstrated that the cells were viable. These experiments unusually demonstrate that high, non-physiological concentrations of glucose are required to stimulate the L cells, and that even at these high levels the response seen is unreliable. This is in contrast to published data and may demonstrate destruction of the glucose sensing apparatus during the L cell digestion process.
3.4.4 Secretion experiments with glutamine

Figure 3.10: Mouse primary colonic crypts incubated with glutamine, PYY measured. Graph (a) represents PYY measured in cell supernatants, graph (b) represents % of PYY released from the cells. Error bars indicate mean + SEM. 100+ represents 100mM glutamine with IBMX and forskolin.

Figure 3.11: Mouse primary colonic crypts incubated with glutamine, GLP-1 measured. Graph (a) represents GLP-1 measured in cell supernatants, graph (b) represents % of GLP-1 released from the cells. Error bars indicate mean + SEM.
The secretion experiments with glutamine and the primary mouse colonic crypts demonstrated a non significant increase in both PYY and GLP-1. Again a trend of increasing PYY and GLP-1 was apparent with the increasing concentrations of glutamine (from 1 mM to 100 mM). For all experiments a positive control (0.01 mM IBMX and forskolin) and a negative control (physiological buffer alone) were carried out. Again these experiments are not in line with published data carried out using the same protocol of isolating mouse L cells.

The only variation between the protocol I used to establish the primary cell cultures and detect gut hormones and the published protocol is in the method of measuring GLP-1. The RIA I used measured the total GLP-1 level, whereas the experiments carried out by Dr. Gribble, measured active GLP-1 using an ELISA.
3.4.5 Secretion experiments with Na propionate

Figure 3.12: Mouse primary colonic crypts incubated with Na propionate, PYY measured. Graph (a) represents PYY measured in cell supernatants, graph (b) represents % of PYY released from the cells. Error bars indicate mean + SEM.

Figure 3.13: Mouse primary colonic crypts incubated with Na propionate, GLP-1 measured. Graph (a) represents GLP-1 measured in cell supernatants, graph (b) represents % of GLP-1 released from the cells. Error bars indicate mean + SEM.
In contrast to the secretion experiments incubating the L cells with glucose and glutamine, I found that the SCFA propionate had a significant and dose dependent effect on PYY release. The effect on GLP-1 was less marked and was not significant. This was a surprising finding given that the PYY and GLP-1 are supposedly co-released from the L cell.

To further examine the effect of SCFA on the release of gut hormones, the effects of acetate and butyrate were also investigated. I focused on the effect of SCFA on PYY release as this proved a robust and reliable parameter to measure whereas the inter-assay variation of the GLP-1 assay was becoming increasingly problematic and the non specific binding of the label increased, indicating that the GLP-1 label was unstable.
Figure 3.14: Mouse primary colonic crypts incubated with (a) Na acetate (b) Na propionate and (c) Na butyrate, PYY measured. The x axis represents the test substance, the y axis represents the amount of PYY released into the supernatant.
The SCFA, propionate and butyrate led to a significant increase in the release of PYY from the mouse colonic L cells. The response to acetate was not statistically significant but there was a trend towards significance ($P = 0.192$). The response of the mouse L cells to SCFA has previously not been demonstrated and is a novel finding.

Mouse colonic crypt cells were used as an L cell model. However, it is known that there is marked species variation between L cells; for example, rat L cells are not known to be glucose responsive which is in contrast to mice L cells (Brubaker et al., 1998). To determine if the effects of SCFA seen in the mice L cells were a species specific effect I next established cultures of primary human L cells.
3.4.7 Human colonic crypt cultures

Figure 3.15: Human primary colonic crypts incubated with glucose, PYY measured. Graph a represents PYY measured in cell supernatants, graph b represents % of PYY released from the cells. Error bars indicate mean + SEM. This experiment has only been performed once. The number of wells used for each group was 4-5. P = 0.07 for supernatants, P = 0.17 for lysed cells.

Figure 3.16: Human primary colonic crypts incubated with Na propionate, PYY measured. Graph a represents PYY measured in cell supernatants, graph b represents % of PYY released from the cells. Error bars indicate mean + SEM. This experiment has only been performed once. The number of wells used for each group was 4.
The studies carried out using the human colonic biopsies, demonstrated that L cells within these cultures were more responsive to glucose, $P = 0.07$, compared to the mouse L cells and that the effect of the SCFA propionate on PYY release in human L cells was still present and strongly significant, $P < 0.001$. As SCFA are present at high concentration in the colon, up to 800 mM, these findings demonstrate a physiological response of the L cells to the SCFA. SCFA are known to have affinity for GPR43 and GPR41 receptors on the L cell surface (Brown et al., 2003).
3.4.8 Phenylacetamide 1 – GPR43 agonist

In order to investigate the mechanism of action of SCFA in stimulating the release of PYY, I obtained a specific GPR43 allosteric agonist (Amgen), Phenylacetamide 1, for use in secretion experiments in mouse colonic crypt cultures. Incubation of the mouse colonic crypts with the GPR43 agonist did not cause an increase in release of PYY. However, when given in combination with 800 mM propionate there was a trend towards an increase in PYY release.

![Bar chart](image)

Figure 3.17: Mouse primary colonic crypts incubated with phenylacetamide 1 (Amgen), PYY measured. Graph (a) represents PYY measured in cell supernatants, graph (b) represents % of PYY released from the cells. Error bars indicate mean + SEM.
3.5 Discussion

Preliminary experiments with mouse colonic crypt cultures demonstrate that PYY and GLP-1 are detectable within the lysed cells and also in the cell supernatants. This indicates the presence of L cells within the mixed colonic cultures. In addition, there was a strongly significant GLP-1 response to the PKA pathway activators, forskolin and IBMX, this indicated that the cells were viable and that the isolation from the colon did not significantly disrupt the intracellular signalling pathways. In addition, some response was seen to increasing concentrations of both glucose and glutamine. This finding was not statistically significant; however, the small increase in PYY seen was reproducible.

The most significant finding in this study was the response of the cells to the SCFAs, propionate and butyrate. These led to a significant and dose dependent increase in PYY that was reproducible up to a maximal concentration of 800 mM. The usual concentration of SCFA in the gut is between 20-200 mM, however, after a fibre rich meal, SCFA concentrations of this magnitude (800 mM) have been observed. This statistically significant increase in PYY seen with propionate and butyrate is in keeping with a putative role for these molecules as physiological stimulants of gut hormone release. SCFA are thought to interact with the L cell via G protein coupled receptors GPR41 and GPR43, these are discussed in detail in the following chapter. In light of this a GPR43 specific allosteric agonist was obtained from Amgen. This did not lead to a dose dependent increase in PYY release unless given in combination with propionate when it appeared to enhance the effects of propionate. These results may be misleading. The agonist was a highly insoluble molecule requiring DMSO to increase solubility. It may be that this increased concentration of DMSO was toxic to the cells and so increased cell lysis. This would have increased PYY levels in the cell supernatants. In this experiment, the percentage release of PYY was higher than that seen for all other experiments and this is likely to be due to cell death. It would be important to make some assessment of cell death in these secretion experiments for example by using typtan blue. Markers of L cell proliferation would not be
appropriate as L cells do not proliferate; once these cells are differentiated from the stem cells, they migrate out of the crypt and then undergo apoptosis.

For all experiments the GLP-1 measured in response to stimulants was far less reliable and reproducible. The reason for this is not clear. Protease inhibitors were added to the lysis buffer and physiological buffer, however, it is possible that the GLP-1 was being degraded and therefore difficult to detect. It was also apparent that the GLP-1 assay being used was becoming increasingly unreliable indicating degradation of the labelled GLP-1. For this reason I focussed on measuring PYY within these secretion experiments. As published data with similar protocols uses an ELISA to measure active and not total GLP-1 it is also important to attempt to replicate these results. A failure to do this may indicate that the glucose sensing machinery is being damaged by the process of L cell isolation leading to requirement of high, non physiological doses of glucose to stimulate hormonal release.

Primary L cell cultures are an excellent model of L cells as they contain all of the anorectic gut hormones that they would contain in vivo. Therefore, despite the difficulties in culturing these cells, they are superior to the available immortalised L cell lines which all lack PYY. The added benefit of these mixed colonic crypt cultures is that the L cells are in a physiological environment surrounded by cells that they would normally be in contact with in vivo and therefore they are privy to their usual paracrine stimulation.

There are a number of limitations of this primary L cell model and it is important to understand these in order to be able to interpret the results. Firstly, the L cells are highly polar with an apical surface in contact with the luminal gut contents and the basolateral surface in contact with the
microvasculature of the gut (Eissele et al., 1992). In vitro, the L cells polarity is disrupted and both sides of the L cell are in contact with the cell culture medium. This may possibly overexpose or underexpose the apical or basolateral surface of the L cell to the stimulants within the culture medium giving misleading results. It may be possible to culture these mixed colonic crypts in vitro, maintaining their polarity, for example with an Ussing chamber or using a cell culture insert. This would be technically extremely difficult but would be more physiological. Another method of investigating the L cells would be within a whole tissue model, for example, by studying intestinal loops with a preserved blood supply (Plaisancie et al., 1996). It may also be possible in animal models to inject nutrients directly into the colon and take blood samples to measure gut hormones. This approach would be useful to determine the stimulants of L cell secretion. However, this would not inform us further on the mechanism of the L cell nutrient recognition or on how this leads to intracellular signalling and release of gut hormones.

In addition, the removal of the L cells from their colonic epithelial also deprives them of their contact with the enteric nervous system. As described previously, the enteric nervous system is rich in neurotransmitters. L cells are known to have receptors for various neurotransmitters on their surfaces and it is likely that this is a key component of L cell regulation. My studies demonstrated the need for a far higher concentration of nutrients, such as glucose, than would otherwise be available to the L cells in the colonic environment. It is possible that the presence of certain neurotransmitters, for example acetylcholine, is required in order to prime the L cells making them more responsive to lower levels of nutrients. In addition, it is likely that the presence of certain hormones, for example GIP is also required in cellular milieu for the L cells to be maximally responsive.
Despite these limitations this L cell model is a useful method of studying the stimulants required for PYY release. In addition, the human L cell model is a novel system that can be used to study L cell physiology and to screen pharmacological and physiological stimulants of gut hormone release. I would like to further use these primary cultures to study the intracellular signalling following L cell recognition of SCFA. The GPR43 and GPR41 receptors are coupled to Gi and Gq, therefore it may be possible to measure the changes in intracellular cAMP and calcium that occur following incubation with SCFA. It may also be possible to isolate the L cells by FACS based on the unique granularity of the L cells (Buffa et al., 1978). It would not be possible to culture these isolated L cells, however, it would be possible to extract RNA and therefore measure changes in gene expression with different nutrient stimulants, for example it would be useful to investigate expression of preproglucagon mRNA following incubation with SCFA.
4. The investigation of short chain fatty acids in the physiological regulation of appetite in man

4.1 Introduction

4.1.1 Non digestible carbohydrates (NDC)

Non digestible carbohydrates (NDC) or dietary fibre are terms used to describe carbohydrate resistant to mammalian enzyme hydrolysis. NDC pass, relatively intact, into the large intestine where they undergo fermentation by commensal gut microbiota (Cummings and Stephen, 2007). In man, fermented NDC are the main source of short chain fatty acid (SCFA) production (Cummings, 1981). NDC are degraded into SCFA: acetic acid, propionic acid and butyric acid, hydrocarbons with a chain length under six carbon atoms (Cummings, 1981). These are usually present as their respective anions: acetate, propionate and butyrate (Figure 4.1) (Cummings, 1981).

![Propionic acid and Propionate](image)

**Figure 4.1 Diagram of the structure of propionic acid and its related anion, propionate**

4.1.2 Inulin

Inulin is a form of NDC found naturally in onions, garlic, Jerusalem artichokes and chicory (Van et al, 1995). Structurally, inulins are chains of fructose moieties joined together by β(2-1) frucosyl fructose linkages, with or without a starting D – glucose (Figure 5) (Waterhouse and Chatterton, 1993). The
The number of fructose moieties linked together is known as the degree of polymerisation (DP). For inulin this varies between 2 units and 60 units, with an average DP of 10-12 (Roberfroid, 2005). The unique \(\beta(2-1)\) bonds within inulin prevent its breakdown by mammalian enzymes which are specific towards \(\alpha\)-glycosidic bonds (Oku et al., 1984). Bifidobacteria species produce inulinase (\(\beta\)-fructosidase) which specifically cleaves the \(\beta(2-1)\) bonds (Damian et al., 1999). Fermentation of inulin by Bifidobacteria leads to the production of propionate (Niness, 1999).

![Diagram of inulin structure](image)

**Figure 4.2**: Diagram of inulin structure – fructose units linked with beta glycosidic bonds with a terminal glucose unit.

### 4.1.3 Non digestible carbohydrates (NDC) and effects on appetite

Many studies have demonstrated that dietary supplementation with NDC suppress appetite (Keenan et al., 2006; Bosch et al., 2006; Shen et al., 2009; Delzenne et al., 2005). The mechanism by which this occurs remains unclear. One hypothesis is that NDC dilute the energy density of the diet. However, animals eating low energy dense foods, such as cellulose, compensate by increasing food intake, thereby maintaining a constant energy intake (Keenan et al., 2006). Animals fed NDC do not
have increased food intake (Keenan et al., 2006) so it is likely that there is an additional factor inducing satiety, other than pure energy dilution.

The satiating effects of NDC may also be attributable to a bulking effect in the stomach or colon, and the stimulation of stretch receptors and vagal nerve afferents. However, studies looking at the effects of gastric distension alone, using intragastric balloon distension, have failed to show appetite suppression (Oesch et al., 2006).

Finally, the production of SCFA by NDC fermentation in the colon may induce satiety due to a direct effect on entero-endocrine L cells and the release of gut anorectic hormones, PYY and GLP-1. The discovery that SCFA are produced distally in the gut (Cummings et al., 1987) and the abundance of SCFA receptors in this area, particularly on entero-endocrine L cells (Karaki et al., 2006), are likely to be of physiological importance. In line with this hypothesis is the finding that plasma PYY and GLP-1 are increased in: animals fed a NDC supplemented diet (Zhou et al., 2008; Delzenne et al., 2005; Keenan et al., 2006; Zhou et al., 2006), animals administered SCFA (Longo et al., 1991; Tappenden et al., 1996; Cuche et al., 2000) and humans administered SCFA (Freeland and Wolever, 2009).

### 4.1.4 Short chain fatty acids (SCFA)

The process of SCFA production by fermentation of NDC by anaerobic, commensal gut bacteria is complex. NDC are composed of hexoses and pentoses. Within gut bacteria, hexoses are broken down via the Embden-Myerhoff-Parnas glycolytic pathway to pyruvate (Miller and Wolin, 1996). Hexoses and pentoses may also be converted to 6-phosphogluconate and metabolised via the pentose phosphate pathway to produce pyruvate (Cummings, 1981). Acetate is formed through oxidative decarboxylation of pyruvate (Cummings, 1981). Butyrate is formed by the reduction of acetoacetate (Duncan et al., 2004; Miller and Wolin, 1996). Propionate is formed by fixation with
carbon dioxide via the succinate decarboxlase pathway or the acrylate pathway to produce propionate (Prins RA, 1977; Miller and Wolin, 1996; Cummings, 1981).

The normal concentration of SCFA in the colonic lumen is 130 mmol/L (Cummings et al., 1987). Of this the ratio of acetate: propionate and butyrate is 57: 21: 22% (Cummings et al., 1987). The SCFA produced by colonic fermentation are absorbed via passive diffusion (McNeil et al., 1978) or monocarboxylic acid transporters (Ritzhaupt et al., 1998). Absorption is extremely efficient with only 5-10% SCFA excreted in the faeces (Cummings, 1981). SCFA transport across the colonic epithelium is complicated by their metabolism within the epithelial cell. Roughly 50% of propionate is metabolised within the epithelial cell and the remainder transported to the liver (Cummings, 1981). Once absorbed, propionate and butyrate are used for gluconeogenesis (Wolever et al., 1991). Acetate is taken up by the liver and used as a substrate for cholesterol synthesis (Wolever et al., 1991). In contrast, propionate inhibits cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase) (Wolever et al., 1991).

4.1.5 SCFA fluctuation

The concentration of acetate, propionate and butyrate in peripheral blood is 100-150 µM, 4-5 µM and 1-3 µM respectively (Wolever et al., 1997). SCFA produced by bacterial NDC fermentation in the colon enters the peripheral circulation via the splanchnic circulation. This mixes with circulating propionate produced endogenously by the degradation of either branched chain amino acids (leucine, isoleucine and valine) (Brindle et al., 1988) or odd numbered fatty acids (Seufert et al., 1974; Sbai et al., 1994) (Figure 4.3). Under fed conditions, SCFA production from colonic fermentation predominates. However, under conditions of starvation and increased fat oxidation, endogenous production predominates (Wolever et al., 1997). In the peripheral circulation, SCFA levels fluctuate throughout the day; levels tend to decrease after breakfast and increase after lunch.
and dinner (Wolever et al., 1997). The reason for this is unclear. It has been hypothesised that this reduction in circulating SCFA may be due to a reduction in endogenously produced SCFA (occurs following change from fasted to fed state) which does not compensate for increased colonic SCFA availability (Wolever et al., 1997).

**Figure 4.3: Schematic diagram of whole body SCFA turnover**

Peripheral circulating SCFA are derived from either endogenous production or exogenous supply. The main exogenous source is bacterial fermentation of NDC in the distal gut. From the gut, these SCFA are either metabolised by the colon epithelial cells or enter the splanchnic circulation and are removed by the liver. Only a small percentage enters the peripheral circulation.

### 4.1.6 Roles of SCFA

SCFA have a number of important physiological roles. Through metabolism of fermentable fibre, the host is able to gain energy from food not digested in the upper gastrointestinal tract (Bergman, 1990). In ruminants, SCFA contribute to over 70% of dietary energy supplied, and in non ruminants
about 5-10% (Bergman, 1990). As mentioned previously, acetate increases serum cholesterol whereas propionate inhibits the use of acetate as a cholesterol precursor (Daubioul et al., 2002; Wolever et al., 1991). Additionally, SCFA in the colon lumen may reflect the activity of commensal gut microbiota and SCFA receptors possibly monitor the bacteria for host defence (Le et al., 2003). SCFA modulate intestinal mucosal growth and butyrate has antiproliferative effects on neoplastic cell lines (Cherbut et al., 1998).

### 4.1.7 Effect of SCFA on colonic motility

The effects of SCFA on colonic motility have been studied in detail. There are two types of muscle important for colonic motility, longitudinal muscle and circular muscle. Acetate reduces the frequency of giant contractions in longitudinal muscle in vitro (Ono et al., 2004) whereas propionate has the opposite effect (Ono et al., 2004). Propionate also increases contractions in circular muscle (Tazoe et al., 2008). SCFA may exert these effects through the enteric nervous system, via release of 5-HT from mucosal mast cells or the release of PYY (Fukumoto et al., 2003; Tazoe et al., 2008; Ono et al., 2004; Cherbut et al., 1998).

### 4.1.8 Effects of SCFA on adipose tissue

The plasma level of free fatty acids (FFA) is determined by the rate of triglyceride (TG) synthesis and TG breakdown (lipolysis). TG synthesis occurs in both the liver and adipose tissue. However, lipolysis by hormone sensitive lipase (HSL) occurs mainly in the adipose tissue. Phosphorylated HSL hydrolyses TG to glycerol and three free fatty acids. Control of lipolysis is mainly by insulin, one of the most potent anti-lipolytic hormones. Insulin exerts its effects by dephosphorylating HSL. Acetate
and propionate have also been found to reduce lipolysis in a dose dependent manner comparable to insulin (Ge et al., 2008; Hong, 2005). SCFA are able to stimulate leptin production \textit{in vitro} at concentrations similar to circulating levels of SCFA (Xiong et al., 2004). This is supported by an \textit{in vivo} study in mice which demonstrated that peripherally administered SCFA increased circulating leptin (Xiong et al., 2004).

### 4.1.9 Short chain fatty acid (SCFA) receptors

In 1997, the G protein coupled receptors (GPCR) GPR 40 (also known as free fatty acid receptor 1, FFAR-1), GPR41 (FFAR-3), GPR42 and GPR43 (FFAR-2), were discovered as a gene cluster on chromosome 19q13.1 (Sawzdargo et al., 1997). GPR41 and 42 are adjacent and it is possible that GPR42 may have arisen from gene duplication and is likely to be a pseudogene (Sawzdargo et al., 1997). These receptors form part of a subfamily of related G protein coupled receptors, the rhodopsin (class A) family, and share 30-40% sequence identity (Brown et al., 2003).

In 2003, independent groups screened libraries of molecules against GPR41 and GPR43 transfected cell lines, measured intracellular calcium and reporter genes and independently demonstrated that SCFA are endogenous ligands of GPR41 and GPR43 (Le et al., 2003; Brown et al., 2003; Nilsson et al., 2003). The fatty acid chain length confers activity and specificity at the receptor. Acetate and propionate are equipotent at the GPR43 receptor followed by butyrate, valerate (C5) and formate (C1) (Brown et al., 2003; Nilsson et al., 2003; Le et al., 2003). At the GPR41 receptor, the rank order of potencies differs slightly with propionate, valerate and butyrate being equipotent, followed to a lesser extent by acetate (Brown et al., 2003; Nilsson et al., 2003; Le et al., 2003).
GPR43 is expressed abundantly in immune cells, bone marrow and spleen (Brown et al., 2003; Le et al., 2003; Nilsson et al., 2003). It has also been identified in the pancreas (Kebede et al., 2009) entero-endocrine L cells in the distal gut (Karaki et al., 2006) and adipose tissue (Hong, 2005; Ge et al., 2008). GPR41 is also expressed in adipose tissue (Xiong et al., 2004), pancreatic islets (Kebede et al., 2009) and in a small percentage (4%) of L cells (Tazoe et al., 2009; Samuel et al., 2008).

GPR43 is a G protein-coupled receptor associated with the pertussis sensitive G protein, Gi, and also the pertussis insensitive Gq family of G proteins (Brown et al., 2003; Le et al., 2003; Nilsson et al., 2003). Therefore, activation of GPR43 leads to a reduction in cAMP and increased intracellular calcium. GPR43 is thought mainly to exert its effects through Gi as a 70% reduction in signalling is seen in the presence of pertussis (Nilsson et al., 2003). GPR41 is only associated with the pertussis sensitive Gi G protein family (Brown et al., 2003; Le et al., 2003; Nilsson et al., 2003). Therefore, GPR41 activation leads to a reduction in cAMP.

Evidence that the effects of SCFA are mediated via GPR43 and GPR41 comes from studies of adipose tissue, where both receptors are abundant. GPR43 mRNA has been found to be increased during adipocyte differentiation in 3T3 cell lines and in primary mouse adipocyte cell cultures (Hong, 2005). Additional propionate supplementation also increases adipocyte differentiation and expression of GPR43 mRNA in 3T3 cells (Hong, 2005). This effect of propionate on adipocytes appears to be via GPR43, as GPR43 silenced using siRNA led to decreased adipocyte differentiation and downregulated PPARγ2 (Hong, 2005). In vivo, GPR43 expression is increased in adipose tissue in mice fed a high fat diet compared to a normal diet (Hong, 2005). This evidence may suggest that propionate, via GPR43 signalling, has a role in fat deposition. In particular, propionate stimulates lipid accumulation and has antilipolytic activity. The evidence for SCFA signalling via GPR41 in adipocytes is controversial. Hong
et al were unable to detect the presence of GPR41 in mouse or human adipocytes, however, the presence of these receptors in adipose tissue has been found by other groups (Xiong et al., 2004).

4.1.10 Delivery of SCFA to the colon

Investigations into SCFA supplementation in humans are limited for a number of reasons. Firstly, delivery of SCFA to the colon is unreliable due to their short half-life. Secondly, SCFA production in the gut, by fermentation of NDC, depends on a number of factors including substrate source, gut transit time and host commensal gut microbiota (May et al., 1994). NDC also has a number of side effects owing to its osmotic effects on the colon and the production of large amounts of waste products including methane and hydrogen (Bergman, 1990). This causes bloating, flatulence and increased stool frequency, making this an unattractive obesity treatment option. Extreme measures, such as using rectal infusions of SCFA have been employed (Wolever et al., 1991; Freeland and Wolever, 2009). However, these are clearly impractical in a clinical setting.

A novel solution to deliver SCFA to the colon was developed by Dr. Douglas Morrison, Scottish Universities Environmental Research Centre (SUERC). Propionate inulin ester was developed comprising an inulin backbone with esterified propionate attached. The propionate inulin ester is able to resist fermentation in the proximal gut and release propionate in the colon. Studies using radiolabelled butyrate have shown that this is an effective method of SCFA delivery.
4.2 Aims and hypothesis

4.2.1 Hypothesis

I hypothesise that increased propionate delivery to the entero-endocrine L cells in the colon, using a novel propionate inulin ester delivery system, will increase plasma levels of the anorectic gut hormones, GLP-1 and PYY, and thus reduce food intake.

4.2.2 Aims

To investigate the effects of SCFA on appetite and food intake in overweight or obese human volunteers, using a novel propionate delivery system, propionate inulin ester. The following parameters were measured:

1. Subjective measures of appetite with visual analogue scores
2. Energy intake using a buffet meal
3. Plasma GLP-1 and PYY
4. Markers of colonic fermentation with breath hydrogen analysis
4.3 Materials and methods

4.3.1 Pilot study- Investigating the effects of propionate inulin ester on appetite

As propionate inulin ester had not previously been studied in man, it was necessary to carry out a pilot study. Inulin is present in the Western diet at about 10 g/ day (Van et al., 1995) therefore I hypothesised that this dose would not cause any gastrointestinal side effects. The aims of this pilot study were to determine whether propionate inulin ester had any effects on appetite or food intake prior to performing a larger randomised controlled study.

4.3.2 Propionate inulin ester

Propionate inulin ester (Figure 4.4), was a gift from Dr. Morrison (Morrison et al., 2006). The mechanism of propionate inulin ester production is currently pending patent and therefore the exact method of production is not available. However, the reactants for its synthesis are inulin, water, propionic anhydride and sodium hydroxide. As both propionate and inulin are classed as foodstuffs, Medicines and Healthcare Products Regulatory Agency (MHRA) approval was not required to carry out this study.

Figure 4.4: Schematic representation of butyrate inulin ester kindly supplied by Dr. Douglas Morrison as a representation of the structure of propionate ester.
4.3.3 Nutritional supplements: pilot study

The propionate inulin ester was baked into bread rolls (Premier foods, Holgran - Hovis division Lichfield), each containing 10 g of ester (Table 4.1).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight/ bread roll (g)</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakers pride (flour)</td>
<td>57.56</td>
<td>52.33</td>
</tr>
<tr>
<td>Block yeast</td>
<td>1.73</td>
<td>1.57</td>
</tr>
<tr>
<td>Salt</td>
<td>0.92</td>
<td>0.84</td>
</tr>
<tr>
<td>Zippy +</td>
<td>0.58</td>
<td>0.52</td>
</tr>
<tr>
<td>Propionate inulin ester</td>
<td>9.79</td>
<td>8.90</td>
</tr>
<tr>
<td>Water</td>
<td>39.43</td>
<td>35.85</td>
</tr>
<tr>
<td>Total</td>
<td>110.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Table 4.1: Nutritional composition of propionate inulin ester bread rolls used in the pilot study.

Rolls contained approximately 10 g ester per roll (Holgran, Hovis division, Premier Foods)

4.3.4 Nutritional supplements: randomised controlled study

Inulin was obtained from Beneo HP, Raftiline, Batch HPHPD7BPD, Sudzucker AG Mannheim, Ocshenfurt, Germany. For this study, a sour dough bread roll recipe with 2.85 g extra sugar was used. In addition, the amount of ester in each roll was limited to 5 g. This was to improve palatability of the bread roll. The calculated nutritional information is listed in 4.2.
<table>
<thead>
<tr>
<th></th>
<th>Weight/ bread roll (g)</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flour – goldcrest</td>
<td>95.16</td>
<td>86.51</td>
</tr>
<tr>
<td>Raftiline HPX inulin or inulin ester</td>
<td>6.66</td>
<td>6.06</td>
</tr>
<tr>
<td>Sugar</td>
<td>2.85</td>
<td>2.60</td>
</tr>
<tr>
<td>Yeast- block</td>
<td>2.85</td>
<td>2.60</td>
</tr>
<tr>
<td>Salt</td>
<td>1.52</td>
<td>1.38</td>
</tr>
<tr>
<td>Zippy plus</td>
<td>0.95</td>
<td>0.87</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.2: Nutritional composition of propionate inulin ester bread rolls used in the RCT. Rolls contained approximately 5 g ester per roll (Holgran, Hovis division, Premier Foods)

4.3.5 Subjects enrolled in the pilot study

Six healthy, overweight or obese, men and women, aged 21 to 65 years, with BMI between 25 and 35 were recruited by advertisement. Subjects who had gained or lost more that 3 kg in the three months preceding the study, had a history of drug or alcohol abuse, were on any prescription medications or dietary supplements or who had any history of gastrointestinal disease were excluded from the study. All potential volunteers underwent a medical screening visit which involved a clerking and physical examination, height and weight measurement, blood pressure monitoring, an ECG, and blood tests (full blood count, urea and electrolytes, liver function tests, thyroid function tests and glucose). In addition, subjects were required to complete DEBQ and SCOFF questionnaires (Morgan et al., 1999). Volunteers with a high level of restraint (measured by the DEBQ) or with disordered eating patterns (measured by the SCOFF questionnaire) were excluded (Caccialanza et al., 2004; Morgan et al., 1999).
4.3.6 Subjects enrolled in the RCT

Twenty six healthy volunteers, men and women, aged 21 to 65 years, BMI range 25 to 35, were recruited by advertisement. The inclusion and exclusion criteria were the same as described previously for the pilot study. All potential volunteers underwent the same screening procedure as described in detail in the pilot study.

4.3.7 Ethical approval

Ethical approval was obtained from the Hammersmith and Queen Charlotte’s & Chelsea Research Ethics Committee (08/H0707/99) and is in accordance with the Helsinki declaration. This trial was registered at clinicaltrials.gov (reference number NCT00750438). All recruited participants gave written consent on the first study visit and verbal consent on subsequent visits.

4.3.8 Feeding study protocol

Study visits took place at the Sir John McMichael Centre, Clinical Investigations Unit, Hammersmith Hospital. Participants were requested to arrive at 8am, having fasted for 12 hours prior to their visit and to refrain from alcohol and strenuous exercise the day before the study day. Subjects were also asked to use the same means of transport to get to the study centre on each study day. This was to ensure adequately filled glycogen stores and similar macronutrient balance on the test days. On the day prior to each study visit, subjects were asked to maintain the same diet. They were told to eat the same type and quantity of food and were required to complete a 24 hour food diary on this day to confirm compliance. In order to assist them in this task they were allowed to keep their food
diaries to remind them of what to eat prior to the next study visit. The food diaries were assessed with the help of Dietplan software, version 5.

On arrival, a 20 gauge cannula was inserted into the forearm and a baseline sample of venous blood was withdrawn. After 15 minutes a second baseline sample was drawn. Immediately after the second baseline blood sample was drawn the subject was served a standardised breakfast. A standardised lunch was served at 180 minute and a buffet meal at the end of the study, at 360 minutes.

4.3.9 Standardised meals and buffet meals

A standardised breakfast was served, following the baseline blood samples, and the subject was isolated and asked to consume the meal within 10 minutes. The standardised breakfast consisted of Tropicana orange juice, a packet of cereal (from a multi-variety pack) with milk and a bread roll with butter. This had an energy value of between 1190 and 1255kcal (depending on the cereal chosen) and was constant for each participant for each visit. At 180 minutes, the subjects were given a standardised lunch, from a limited choice of ready meals, either 513 kcal for a non vegetarian ready meal (Sainsbury’s Taste the Difference chilli con carne with coriander rice) or 658 kcal for a vegetarian ready meal option (Sainsbury’s macaroni cheese). The variation in caloric intake between subjects represents different meal choices made by the subjects based on their meal preferences. At 360 minutes subjects were offered a buffet meal. This consisted of a large bowl containing either 3 packets of ready meals (for a woman) or 4 packets (for a man) of a non vegetarian ready meal (Sainsbury’s Italian spaghetti bolognese) or vegetarian option (Sainsbury’s tomato and mozzarella pasta bake). This was designed for the food to be available in excess. The subjects were isolated using screens and were asked to eat until they were comfortably full. Unlimited water was available
throughout the day. Energy intake in the *ad libitum* meal was measured by weighing the remaining food on completion of the buffet meal.

**4.3.10 Visual analogue scales**

Measures of appetite were scored using 100 mm visual analogue scores (VAS) with text expressing the extremes of feelings at each end of the line (Flint et al., 2000). VAS were used to measure hunger, satiety, fullness, prospective food consumption and desire to eat. These were completed by the subjects at 30 minute intervals. At hourly intervals, subjects completed a set of VAS measuring potential side effects associated with the ester: stomach discomfort, bloating, nausea, heart burn, belching, flatulence, urge to defecate and diarrhoea.

**4.3.11 Sample processing**

Blood samples were immediately transferred to a lithium heparin tube containing 2000 kallikrein inhibitor units (0.2ml) aprotinin. All samples were stored on ice and were centrifuged at 4000 rpm (1780 RCF) at 4 degrees Celsius for 10 minutes. The plasma was then separated and stored in eppendorfs at minus 20 degrees Celsius for later analysis.

**4.3.12 Breath hydrogen analysis**

Breath hydrogen levels were measured as a marker of colonic fermentation at half hourly intervals (Simren and Stotzer, 2006).
4.3.13 Pilot study protocol

Subjects had a control feeding study on day 1 and an interventional feeding study (with 10 g propionate inulin ester) on day 7. At the end of day 1, the subjects were given five bread rolls to consume at home. Each bread roll contained 10 g of propionate inulin ester. The subjects were asked to take half a roll with breakfast and the other half with their evening meal. They were requested not to start any new diets or change their exercise regime during the trial period. To confirm compliance, subjects were asked to return any uneaten rolls at the end of the study. The day 7 feeding study enabled assessment of the acute effects of propionate inulin ester on appetite and food intake (Figure 4.5).

During the study, ten ml venous blood samples were withdrawn from the cannula at the following time points: -10 and 0, 30, 60, 90, 120 and 180 minutes. During the pilot study, new information was received regarding the gut transit time for inulin and oligofructose, revealing that following oral administration these took four to six hours to reach the colon (personal communication received from Professor Gary Frost). Therefore the original study protocol was amended and the blood sampling times were changed to: -15, 0, 60, 120, 210, 300 and 360 minutes. At the time of the change in protocol, all six volunteers had completed their first visit (control study).
4.3.14 RCT protocol

The study was a randomised, double blind, crossover study comparing both inulin and propionate inulin ester to control. Randomisation was by sealed envelopes by a colleague in the same department but not associated with the study. The timeline for the study is shown below (Figure 4.6). The first study visit for all subjects was an acclimatisation study to familiarise the participant with their surroundings, the researchers and the study protocol. All conditions during the acclimatisation day were similar to the actual study days. The only difference was that only 1ml of blood was taken at each time point on the acclimatisation days instead of 5ml of blood. The feeding study protocol was the same as for the pilot study. The only difference was that the 10 g dietary supplement was divided into two, half given with breakfast and the rest with lunch.
After the first control study day, subjects were randomised to either propionate inulin ester or inulin groups. They were given 12 bread rolls containing either 5 g propionate inulin ester or 5 g inulin and were instructed to take two rolls a day for 6 days, starting the day following the control study and ending the day prior to the next study. After these six days of fibre supplementation, the subjects returned to the study centre and underwent a similar feeding study to the first study. In this second feeding study, the standardised breakfast and lunch were supplemented with 5 g of propionate inulin ester or 5 g inulin. The plan was to compare this second feeding study to the control day 7 days previously. After this second feeding study, subjects had a washout week where they did not take any supplements. Subjects returned to the study centre and had a third feeding study (the second control feeding study day). After this they were given twelve bread rolls to take home, containing the opposite of what they had taken previously, i.e. the inulin group now took ester and vice versa. At the end of the second week of supplementation they returned to the study centre and had a final feeding study, supplemented with either ester or inulin, depending on the group they were in. It was intended that this fourth feeding study day be compared to the third feeding study day. The purpose of the two control feeding studies in addition to the acclimatisation study was to control as far as possible for the adaptive changes in bowel flora that would have taken place during the course of the feeding study.
Figure 4.6: **Timeline for randomised, controlled, crossover study of the effects of propionate inulin ester on food intake.** During the acclimatisation feeding study and the 1st and 2nd control feeding studies the participants were given bread rolls without added propionate inulin ester or inulin on the day of the study. During the 1st and 2nd interventional feeding studies the participants received either 10g of propionate inulin ester or 10g of inulin for the six days prior to the study day as well as 10g on the study day. 5g of this was given in the form of a bread roll with the standardised breakfast and the other 5g given in the form of bread roll with the standardised lunch.
4.3.15 Plasma hormone assays

All samples were assayed in duplicate and within a single assay to eliminate inter assay variation. GLP-1 and PYY were measured using established in house RIAs. These assays were full volume assays performed in total volume 0.7 ml. See methods section 3.3.4.

4.3.16 Statistical analysis for the pilot study

For the pilot study, it was estimated that a study number of six would be sufficient for a preliminary investigation of the ester. Results are expressed as mean +/- SEM. End points were compared using a Wilcoxon signed rank test or a repeated measures ANOVA using Graphpad prism version 4.03. P < 0.05 was taken as significant.

4.3.17 Statistical analysis for the RCT

Using the Russ Lenth power calculator, it was determined that 22 volunteers would be needed, based on a power of 90%, P value 0.05, a difference in means of 20 pmol/L of the gut hormone PYY and a standard deviation of 18 in each group. Results are expressed as mean +/- SEM. End points were compared using a paired Student’s t test or repeated measures two tailed ANOVA using Graphpad prism version 4.03. P < 0.05 was taken as statistically significant.
4.4 Results of pilot study

4.4.1 Subjects studied

Six healthy, overweight or obese volunteers, four men and two women, were recruited and all six completed the study. Mean age was 36.8 ± 5.76 years, range 21-55 years and mean BMI 30.5 ± 1.30 kg/m² (27.2 - 35.2 kg/m²).

4.4.2 Adverse effects

All participants complained that the propionate inulin ester was unpalatable due to a strong, bitter taste. One participant complained of nausea and three complained of bloating during the trial period. All participants claimed to have eaten all of the study rolls as requested, no excess bread rolls were returned to the study investigators.

4.4.3 Energy intake

Energy intake measured by buffet meal during the control feeding study was 631.5±123.5 kcal. After dietary supplementation with 10 g propionate inulin ester per day for five days and the addition of 10 g of propionate inulin ester to the standardised breakfast during the feeding study, the energy intake was 654.5 ± 169.5 kcal. There was no significant difference between food intake on the control study day and the second study day.
Figure 4.7: Pilot study energy intake (kcal) measured by buffet meal during a control and propionate inulin ester feeding study, n = 6. The x axis represents the control and interventional study days and the y axis represents the energy intake (kcal). The red line on the graph represents the mean energy intake during the buffet meal on the control day and the propionate inulin ester day.

4.4.4 Plasma PYY

Due to the change in the blood sampling time points between the control study and the propionate inulin ester study, comparison of data was only possible at 60 minutes and 120 minutes. At 120 minutes, there was a trend towards an increase in plasma PYY in the propionate inulin ester group compared to the control (P = 0.16).
Figure 4.8: Pilot study plasma PYY following either a control feeding study (black circles, black line) or a study supplemented with 10 g of propionate inulin ester (purple squares, purple line), n = 6. X axis represents time in minutes, y axis represents plasma PYY (pmol/l). Timings of meals are indicated by black arrows. Red arrows indicate time points which correspond in both studies and can be used for comparison.
Figure 4.9: Pilot study plasma PYY levels measured (a.) 60 minutes or (b.) 120 minutes following either a control study (blue) or a propionate inulin ester study (purple), n = 6. X axis represents study day, y axis represents plasma PYY (pmol/l).
4.4.5 Plasma GLP-1

Figure 4.10: Pilot study plasma GLP-1 following either a control study (black circles, black line) or a propionate inulin ester study (purple squares, purple line), n = 6. X axis represents time in minutes, y axis represents plasma GLP-1 (pmol/l). Timings of meals are indicated by black arrows. Red squares indicate time points which correspond in both studies.
a. Plasma GLP-1 at 60 minutes

![Graph showing plasma GLP-1 at 60 minutes]

b. Plasma GLP-1 at 120 minutes

![Graph showing plasma GLP-1 at 120 minutes]

**Figure 4.11**: Pilot study plasma GLP-1 at time points (a.) 60 minutes and (b.) 120 minutes following either a control study (blue) or a propionate inulin ester study (purple), n = 6. X axis represents study day, y axis represents plasma GLP-1 (pmol/l).
4.4.6 Effect of propionate inulin ester on breath hydrogen levels

There were higher levels of breath hydrogen in the propionate inulin ester group, indicating increased colonic fermentation ($P < 0.0001$).

![Graph showing breath hydrogen levels](image)

**Figure 4.12: Pilot study breath hydrogen levels** taken during the control feeding study (black circles) and during the propionate inulin ester study (purple squares), $n = 6$. During the feeding studies subjects received a standardised breakfast at 0 minutes and a standardised lunch at 180 minutes (indicated by black arrows).
4.4.7 Effect of propionate inulin ester on appetite – VAS

There was no significant difference between any of the groups comparing VAS scores for the subjective measures of appetite (Figure 14).
Figure 4.13: Pilot study visual analogue scales for fullness, hunger, prospective food intake and desire to eat. X axis represents time in minutes, y axis represents VAS measurement. The black line is the control and the purple line the propionate inulin ester.
4.4.8 Results of randomised controlled trial

Subjects studied

Out of the 26 volunteers, 24 completed the study and 2 volunteers dropped out and were lost to follow up. For this reason, intension to treat analysis was not possible for these two participants. Four participants whose energy intake was more than two standard deviations away from the mean were also excluded. The final number of subjects included in this analysis is 20, 5 men and 15 women.

4.4.9 Demographics

Age and BMI in each group were normally distributed and no significant difference was found between the two groups (table 4.3).

<table>
<thead>
<tr>
<th>Subjects randomised to receive propionate inulin ester followed by inulin</th>
<th>Subjects randomised to receive inulin followed by propionate inulin ester</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age - years</strong> Mean ± SEM</td>
<td>41.64 (± 4.66)</td>
</tr>
<tr>
<td>Range</td>
<td>21-65</td>
</tr>
<tr>
<td><strong>Race or ethnic group</strong></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>6</td>
</tr>
<tr>
<td>Asian</td>
<td>2</td>
</tr>
<tr>
<td>Afrocarribean</td>
<td>2</td>
</tr>
<tr>
<td>Mixed</td>
<td>1</td>
</tr>
<tr>
<td><strong>BMI</strong> Mean ± SEM</td>
<td>29.5 (±2.76)</td>
</tr>
<tr>
<td>Range</td>
<td>26.3 – 35.2</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>Number of subjects in each group</strong></td>
<td>11</td>
</tr>
</tbody>
</table>

Table 4.3: Baseline characteristics of the participants in the randomised, controlled trial, according to study group - propionate inulin ester first followed by a washout week then inulin or vice versa.
4.4.10 Adverse effects

All participants were requested to keep a food diary and a record of any adverse effects. Only three participants complained of adverse effects which included bloating and increased stool frequency. These three participants experienced these side effects with both the propionate inulin ester and with the inulin.

4.4.11 Food diaries

From the food intake the day before each study day, the calorie content was measured using Dietplan 5 software. Two subjects (subjects number 15 and 20) failed to return their food diaries so this analysis was incomplete, n = 18.

Food diary analysis demonstrated wide inter and intra subject variation for the energy intake for the evening meal prior to the study day. There was a trend towards a higher caloric intake on the evenings prior to the interventional feeding studies compared to the control studies. Comparing food intake prior to the feeding studies before and after the propionate inulin ester supplementation, this difference was not significant (P = 0.06). However, it was significant for the feeding studies before and after the inulin study day (P < 0.05).
Table 4.4: Caloric content (kcal) of the evening meal consumed by the subjects the day prior to each study visit, n = 18. PIE = propionate inulin ester. Measured using self assessments (food diaries) and Dietplan 5 software.

<table>
<thead>
<tr>
<th>Energy intake Meal prior to (kcal)</th>
<th>PIE control</th>
<th>PIE</th>
<th>Inulin control</th>
<th>Inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>845.2 ± 94.55</td>
<td>1029 ± 87.76</td>
<td>874.3 ± 79.90</td>
<td>1038 ± 80.71</td>
</tr>
</tbody>
</table>

Figure 4.14: Energy intake during evening meal prior to study day, n = 18. PIE = propionate inulin ester. Subjects had a significantly higher energy intake prior to the feeding study where they received an inulin supplement compared to control (P < 0.05)
4.4.12 Effect of propionate inulin ester and inulin on energy intake

Energy intake, measured by food eaten during a buffet meal, was recorded on each visit. All data were normally distributed. In feeding studies, administration of propionate inulin ester over six days reduced energy intake at a buffet meal by 18.8% compared to control (energy intake 392.2 ± 59.4 kcal [control] to 318.3 ± 57.5 kcal [propionate inulin ester], n = 20, P< 0.05). Inulin alone reduced energy intake by 18.1% (energy intake 330.1 ± 47.5 kcal [control] to 270.4 ± 43.9 kcal [inulin], n = 20, P< 0.05).

![Figure 4.15: Energy intake measured during buffet meal on each study day, n = 20.](image)

Control 1 (dark blue), propionate inulin ester (PIE) (purple), inulin group (green) Control 2 (light blue). X axis represents study day, y axis represents energy intake.
Figure 4.16: Comparing energy intake measured during the two control visits. The dark blue bar represents the control prior to the propionate inulin ester (PIE) study and the light blue represents the control prior to the inulin study.

The difference in means is not significant. (mean 393.2 ± 59.38) vs (330.1 ± 47.54) kcal, $P = 0.192$ (paired t test).
Figure 4.17: Energy intake (kcal) measured by buffet meal during (a.) control and propionate inulin ester feeding study and (b.) control and inulin feeding study, n = 20.
Comparing the change in food intake between control and interventional study days, there was no significant difference between the two groups (propionate inulin ester and inulin) (P value 0.80). However, subjects ate more the evening prior to the inulin visit which may have an impact on food intake and confound these results.

Figure 4.18: Comparing the energy intakes on study days, measured by buffet meal during the propionate inulin ester study day, and the control study day 7 days previously; and the energy intake during the buffet meal during the inulin study day and the control study day 7 days previously, n = 20
4.4.13 Plasma PYY and GLP-1

There was no significant difference between the groups in the plasma PYY or GLP-1. This unexpected result was in contrast to the trend in increased PYY seen in the pilot study and suggests that the propionate is working through an alternate mechanism to that hypothesised.

![Graph](image)

**Figure 4.19:** Plasma levels of PYY (a.) and GLP-1 (b.) n = 20. The standardised breakfast was at 0 minutes and the standardised lunch at 180 minutes. x-axis represents time and y-axis represents plasma levels of (a.) PYY and (b.) GLP-1.
4.4.14 Effect of propionate inulin ester and inulin on breath hydrogen levels

There was no significant difference between the four groups (\( P = 0.28 \)). However, there is a trend towards higher levels of fermentation in the group receiving the inulin intervention (Figure 4.20).

Figure 4.20: Levels of breath hydrogen measured during feeding studies. Propionate inulin ester group (purple triangles), control 1 (blue squares), inulin group (green diamonds), control 2 (blue triangles). Black arrows indicate timing of standardised meals. At 0 minutes subjects received a standardised breakfast and at 180 minutes a standardised lunch, \( n = 20 \).
4.4.15 Effects of propionate inulin ester and inulin on appetite- visual analogue scales

There was no effect of the either the propionate inulin ester or the inulin on the subjective measures of appetite (Figure 4.4.21). VAS are notoriously unreliable as a subjective measure of appetite and are only useful in the context of objective measures of appetite.
4.4.21 Effects of propionate inulin ester and inulin on appetite - visual analogue scores

Propionate inulin ester group (purple triangles), control 1 (blue squares), inulin group (green diamonds), control 2 (blue triangles). Black arrows indicate timing of standardised meals. There was no significant difference between any of the groups by comparing VAS scores.
4.5 Discussion

The pilot study was necessary as this was the first use of the propionate inulin ester in man. Subjects had a control study visit, five days of ester supplementation and a second study day to determine the acute effects of propionate inulin ester on appetite. Acclimatisation is important as it is necessary for the host gut microbiota to adapt to a new diet to enable fermentation of the inulin. The pilot study provided some useful information. The increased levels of hydrogen gas produced with the propionate ester intervention demonstrated increased colonic fermentation, peaking at around 270 minutes indicating that the propionate inulin ester was delivered to the colon and fermented. There was a trend towards an increase in plasma PYY levels at 120 minutes in the intervention group compared to the control study day. This increase in PYY at 120 minutes did not correlate with the rise in breath hydrogen (which occurs much later between 200 and 300 minutes). This suggests that propionate inulin ester may exert some of its effects independently of its fermentation in the colon. This may be due to more proximal absorption of free propionate.

Having investigated the effects of propionate inulin ester on appetite with my pilot study, I designed a randomised, double blind, crossover study looking at the effects of propionate inulin ester on appetite and energy intake and compared this to the effects of inulin alone on appetite. This study design included an extra feeding study at the start of the study to acclimatise the subjects to the study so that they would be less stressed on subsequent visits. As inulin is fermented to propionate in the colon, the aim of this study was to determine if inulin esterified with propionate had any additional effects on appetite and energy intake. Discussions with Dr. Morrison and Hovis, Premier Foods, led to a change in ester production and bread roll recipe and a resolution of the taste issues, prior to the randomised, controlled study.
This study demonstrated that both propionate inulin ester and inulin significantly reduced energy intake. This is not unexpected as inulin is fermented to propionate in the colon and would be expected to have similar effects in increasing satiety and reducing food intake. I had hypothesised that the effects of inulin would be magnified by the additional esterified molecules of propionate but this was not the case. This may be due to the fact that this study was not suitably powered to look for this difference. The power calculation for this study was for 22 subjects; however, after 2 subjects withdrew and 4 were excluded, the final number of subjects studied was only 20.

It may also be due to a reduction in fermentation of the propionate inulin ester. This may be due to a conformational change of the inulin caused by the addition of propionate. Dr. Morrison states that 50% of the possible binding sites on the inulin molecule are esterified, this may lead to a significant change in the shape of the inulin backbone and the propionate molecules may also block accessibility of the hydrolysis sites to the gut bacterial enzymes. This effect of resistance to hydrolysis has been demonstrated in previous similar compounds such as acetyl and methyl inulins (Damian et al., 1999).

I did not see any difference in subjective measures of appetite between the different groups. This is not an unexpected finding as VAS may be unreliable (Flint et al., 2000). I endeavoured to make this as accurate as possible by printing out each VAS on a separate sheet and removing all distractions whilst the participants completed them (e.g. turning off the movies). Flint et al showed that VAS were reliable if used to compare scores within subjects but that a large number of subjects was required. For a crossover study, with power 0.8, 18 subjects are needed to demonstrate an effect of more than 10% (Flint et al., 2000). With my sample size of 20 and with my crossover study design, I
should have been able to demonstrate a difference in the various measures of appetite between the different groups.

Despite requesting that subjects eat a standardised meal on the evening prior to each study day, there was still wide variation between visits. Part of the reason for this was the consumption of the propionate inulin ester or inulin bread roll in the evening prior to the study. Four participants did eat the same meal but took the intervention roll in addition to instead of substituting it for part of their meal. As the study rolls had a significant caloric value of 352kcal, this was a major cause of variation between study visits. A large number of participants also ate completely different meals on the evening prior to their study visits with different caloric values and different composition of macronutrients. In a study by Chandarana et al, it was shown that baseline hunger and plasma GLP-1 and PYY\textsubscript{3-36} concentrations were affected by the previous evening’s meal (Chandarana et al., 2009). This widespread variation is likely to be a confounding factor in my analysis. To overcome this problem in the future, I would alter the study design to include a standardised evening meal, e.g. a pasta ready meal with instructions of when to eat it and what to drink with it, prior to each study day.

In order for a factor to fulfil the criteria to be considered a satiety factor, it is important to determine that the reduction in food intake achieved is not caused by any other physiological states, such as nausea or malaise. Inulin and other forms of resistant starch are associated with side effects such as bloating, abdominal discomfort, increased stool frequency, belching and flatulence. These side effects were experienced in this study with both inulin supplementation and propionate inulin ester. These side effects are not seen in interventions using non fermentable starch, such as cellulose. Rats fed diets with 20% cellulose compared to 20% inulin or normal chow, had no side effects and no
change in colon weight or morphology (unpublished data, personal communication from Ms Veronique Peters, Department of Investigative Medicine). In order to avoid possible reductions in energy intake caused by side effects, a cellulose propionate ester might offer a suitable solution.

In addition, the propionate inulin ester has a slightly bitter taste and for this reason, the amount of ester that could be added to each bread roll was limited to 5 g of ester. Inulin is not associated with adverse taste; therefore, this can be attributed to the bitter taste of unesterified propionate. Dr. Morrison has carried out mass spectroscopy to determine the esterification ratio of the inulin backbone and has revealed that the esterification ratio is 0.5 (i.e. half of the potential esterification sites on the inulin backbone have been joined to propionate). To limit the effects of taste between the two groups, I reduced the amount of ester in each roll, used a bitter tasting sour dough bread recipe and added 2.85 g of sugar to each roll to disguise the flavour. This was successful and none of the participants were able to tell the difference between the rolls. However, many of the participants found the rolls heavy and they did have a higher caloric value compared to standard bread rolls (352 kcal in the inulin or ester rolls compared to 240 kcal in standard brown bread rolls).

The data regarding hydrogen breath gas analysis is interesting. Although there was no significant difference between any of the groups, there is a trend in the inulin group towards increased levels of hydrogen gas, indicating increased colonic fermentation. My data also showed that there is no difference between the propionate inulin ester group and controls. This is at odds with results from my pilot study which showed that there was a significant increase in fermentation in the propionate ester group compared to the controls. There are two possible reasons for this. Firstly, in the pilot study, the intervention was given in a single dose at the start of the day. In the randomised, controlled study, the intervention was given as a divided dose, with 5 g of either propionate inulin
ester or inulin given with the standard breakfast and 5 g at lunch. It may be that the split dose failed to reach the maximum peak fermentation as seen with the single dose. Secondly, the method of ester production changed between the first and second studies. It may be that the extent of esterification has an impact on the degree of fermentation.

In order to determine the efficacy of this method of propionate delivery it is important to measure plasma levels of SCFA. Dr. Morrison has previously used a radiolabelled butyrate tracer to demonstrate that ester is delivered to the colon (Morrison et al., 2006). However, little is known about whether the SCFA are released, absorbed and metabolised. It would be useful to measure absorption of SCFA by measuring plasma levels of propionate and also faecal SCFA levels. This clearly presents some difficulties as SCFA are produced endogenously as well as from fermentation in the colon. In addition, only 50% of the absorbed SCFA escape into the circulation where they are largely removed by the liver. Chronic SCFA supplementation may lead to a slightly higher basal level of SCFA. However, SCFA metabolism is complex and it is unknown at present what effect external SCFA supplementation would have on endogenous SCFA production. A study by Robertson et al demonstrated higher systemic concentrations of acetate and propionate after chronic resistant starch supplementation (Robertson et al., 2005).

The results from my pilot study and from the randomised, double blind, controlled study are encouraging. They demonstrate that both inulin and propionate inulin ester are able to reduce energy intake. This is likely to be due to increased propionate in the colon leading to a reduction in appetite. However, this remains to be proven. If successful, this could have implications for future development of novel stimulators of L cells receptors which increase plasma levels of the anorectic gut hormones. This could have implications for future development of treatments for obesity.
Chapter 5 General discussion

Obesity is a cause of chronic ill health and specific metabolic disorders (World Health Organisation, 2006). The rising prevalence of obesity is placing a significant burden on society. Clinicians only have a limited number of pharmaceutical agents available to treat obesity; this is partly due to the many side effects associated with these treatments. Bariatric surgery is associated with significant morbidity and mortality; however, it is currently the only effective, available treatment of obesity. In trying to understand the pathophysiology of obesity and the mechanism of appetite regulation it may be possible to identify potential targets that could be used to treat obesity.

Hormones released from various regions of the gastrointestinal tract play an important role in regulating appetite (Murphy and Bloom, 2006). These gut hormones provide short and long term signals of nutritional status to central appetite regulating circuits. However, gut hormones have an extremely short half-life and are rapidly degraded by peptidases and through renal clearance (Lluis et al., 1989). Prolonging the circulation of the anorectic gut hormones could in turn stimulate central anorectic signalling pathways within the hypothalamus and brainstem to reduce appetite and weight.

The therapeutic potential of GLP-1 as an anti-obesity treatment was initially limited due to its short half-life. However, investigation of the degradation and metabolism of GLP-1 led to the production of GLP-1 analogues (Agerso et al., 2002) with an increased half-life in the circulation, and to enzyme inhibitors which reduce the degradation of endogenous circulating GLP-1 (Drucker and Nauck, 2006). It is of interest that these enzyme inhibitors, DPP IV inhibitors, do not cause weight loss. DPP IV is widespread and has a number of substrate targets (Mentlein et al., 1993). The non specific inhibition of DPP IV is likely to lead to off target effects. PYY$_{1-36}$ is a known substrate of DPP IV and is cleaved to the active form, PYY$_{3-36}$, which is responsible for the anorectic effects of this peptide (Mentlein et al.,
1993). By non-specifically reducing active PYY_{3-36} and increasing active GLP-1 it is likely that equilibrium is reached in terms of reduced anorectic and orexigenic signalling.

In order to answer my first research aim regarding the degradation of PYY_{3-36}, I used a novel PYY analogue, PYYα. This analogue was specially designed to have a stable tertiary structure by strengthening the α-helix within the peptide. This PYY analogue was found to be effective in reducing appetite in rodents and had an increased half-life in the circulation. In order to determine the specific properties of this analogue that are responsible for these beneficial effects, I carried out degradation studies of PYYα and endogenous PYY_{3-36}. I found that PYY_{3-36} was readily degraded by KBB, whereas PYYα was more resistant to degradation, a property afforded by the increasingly stable α-helix. In addition, my studies demonstrated that the peptidase NEP was not responsible for PYY_{3-36} degradation within the KBB. PYYα and PYY_{3-36} were resistant to degradation by NEP \textit{in vitro}. In order to determine the peptidases responsible for PYY degradation, MALDI-ToF mass spectroscopy analysis was performed on the fragments produced by KBB degradation. This confirmed the presence of a fragment, PYY_{11-36}. It is possible that this fragment is produced by meprin β. To confirm this, it would be useful to study the degradation of PYY_{3-36} with meprin β. In addition, it would be useful to study the KBB degradation of PYY_{3-36} with the addition of specific meprin β inhibitors. This could elucidate which peptidases are involved in PYY_{3-36} degradation.

In addition to studying the degradation of PYY, I studied the receptor binding properties of this peptide. Y1 and Y5 receptors are thought to mediate the orexigenic actions of PYY_{1-36} (Kanatani et al., 2000; Gerald et al., 1996), whereas the anorectic actions of PYY_{3-36} are mediated via Y2 receptors (Batterham et al., 2002). In rodent studies, PYYα displayed a reduction in Y2 receptor binding affinity; however, this did not reduce its anorectic effect. I proposed that a reduction in either Y1 or
YS binding would counter balance this effect. To further investigate this, I created over-expressing hY1 and hY5 receptor cell lines for use in receptor binding assays. Although functional receptors were expressed, this system was insufficiently reliable to determine small differences in the binding affinities of PYY and its fragments. To address this, I used a specific Y1 receptor expressing cell line SK-N-MC. Receptor binding studies using this cell line demonstrated that PYYα had increased affinity at the Y1 receptor. However, this analogue did not produce an increased orexigenic effect. It is also possible that the analogue has reduced affinity at Y5. So far this has not been studied. Specific novel cell lines expressing hY5 receptor are now available, for example BT-549, a breast cancer cell line. Such cell lines could be used to investigate this hypothesis further. The receptor binding affinities of the PYY analogues at the various receptors do not inform us of the agonist or antagonist activity of the ligand at the receptor. It would be interesting to further investigate the second messenger pathways, in particular changes in cAMP and intracellular calcium following PYYα binding at all the Y receptor subtypes.

In contrast to the anorectic action of PYYα in rodent studies, PYYα had no anorectic effects in man. This may be for a number of reasons. Firstly the PYYα may be being degraded more rapidly in man than in rodents. This is extremely likely as the plasma analysis of the PYYα demonstrated an initial peak at 30 minutes and a rapid reduction by 240 minutes. Secondly, PYYα may act differentially at the Y receptors in man compared to in rodents. It would also be useful to study in vitro second messenger signalling of the analogue in rodent compared to human Y receptor systems.

There are two possible mechanisms to increase circulating levels of anorectic gut hormones. The first would be to reduce the degradation and clearance of these hormones. The second would be to
increase production and release of these hormones. Following my initial studies of gut hormone degradation, I subsequently focused on increasing gut hormone secretion.

The anorectic gut hormones, PYY and GLP-1 are released from the entero-endocrine L cell (Bottcher et al., 1984). These cells are located throughout the gastrointestinal tract and are abundant in the colon and rectum (Adrian et al., 1985b). In order to answer my second research aim regarding the physiology of the L cell, I established a reliable method of isolating mouse L cells. Subsequently, I adapted this method to isolate L cells from human colon biopsies. I have used these L cell cultures to investigate various nutrient stimulants and found that the SCFA propionate was a reliable stimulant of PYY release. SCFA is a ligand for the recently discovered receptors, GPR43 and GPR41 on the L cell surface (Brown et al., 2003; Le et al., 2003). I hypothesised that SCFA were stimulating the L cell through the GPR43 receptor. In contrast to SCFA, a GPR43 agonist, Phenylacetamide 1 (Amgen) (Lee et al., 2008) does not produce a dose dependent increase in PYY from L cells in vitro. However, consistent with its role as an allosteric agonist, this GPR43 agonist increased PYY release when administered in combination with propionate. Interpretation of data from these agonist studies was limited due to the effects of DMSO, the solvent used in these studies which is known to have toxic effects on cells. For this reason and due to the great difficulties of investigating an insoluble chemical, further studies with this GPR43 agonist were terminated. An alternate approach to studying the 2nd messenger systems through which SCFA exert their effects would be to use either GPR43 antagonists or to study L cells lacking the GPR43 receptor. GPR43 knockout mice are not yet available but are in development. An interesting future study would be to determine the effects of SCFA on L cells derived from these mice.
SCFA are the products of NDC fermentation (Cummings, 1981). Studies have demonstrated that NDC reduce appetite *in vivo* (Keenan et al., 2006). In order to answer the third research aim, I carried out a double blind, randomised, placebo controlled crossover study in 20 healthy overweight to obese volunteers to investigate the effects of propionate on appetite. Inulin, an NDC, was used as a carrier molecule to transport the propionate to the colon. Inulin is also fermented into propionate (Niness, 1999). Comparing propionate inulin ester to control demonstrated a significant reduction in food intake. However, inulin alone also significantly reduced food intake. There was no difference in food intake between the propionate inulin ester and inulin alone. This reduction in food intake was consistent with my hypothesis. However, plasma levels of PYY and GLP-1 were not increased in participants receiving the inulin or propionate inulin ester. This suggests that the observed reduction in food intake was not attributable to changes in circulating levels of these gut hormones and suggests another possible mechanism of appetite reduction. This finding is in contrast to my data using the *in vitro* L cell model, which clearly demonstrated that SCFA stimulate PYY release in both mice and human L cells. It is also possible that *in vivo*, the SCFA ester was not being delivered effectively to the colon. Further studies are needed to investigate the mechanism of delivery of the SCFA to the colon to ensure that firstly this is reaching the colon, and secondly that the propionate is being released from the inulin carrier molecule in order to exert its effects.

In order to assess the efficiency of propionate delivery to the colon, it is necessary to measure levels of propionate within the blood. Circulating propionate is derived from NDC fermentation and also from the endogenous production of propionate (Wolever et al., 1997). The effect of ingested propionate on endogenous propionate production is currently unknown. To measure plasma propionate and determine the proportion of this derived from the colon is difficult. A method of reliably assessing the delivery of colonic propionate would be to use a stable carbon isotope,
carbon, incorporated into the propionate inulin ester. This could be used to assess delivery and release of the propionate from the inulin backbone and would be an interesting future study.

5.1 Future work

Following the exciting results from the L cell studies, I propose to elucidate further stimulants of gut hormone release and also to uncover the intracellular signalling pathways leading to granular exocytosis. In addition, I would like to investigate L cells within the context of diabetes. Obesity and diabetes are often related and this may be due to a common fault in a signalling pathway. In diabetes, L cell number and function has been found to be reduced and it is currently not known if a similar pattern exists in obesity. If such a pattern does exist, then a method of recovering L cell number and function would be an extremely useful treatment for both obesity and diabetes. It would be interesting therefore to examine human colon biopsies from both diabetic, obese and normal volunteers and examine expression of the cell surface receptors and also determine if the intracellular signalling pathways differ between these models. It would also be useful to determine the factors that lead to recovery of L cell function, as is seen following gastric bypass surgery demonstrable by an extremely rapid improvement in glucose tolerance.

Obesity is a complex, polygenic, multi-factorial biological problem of immense importance for human survival and a healthy lifestyle. Studies within this thesis provide avenues for further research into the regulation of appetite and body weight. In particular, the primary L cell culture model that I have developed could become an important tool for studying the physiological release of gut hormones and also to screen potential pharmacological agents for the treatment of obesity.

World Health Organization Overweight and Obesity Fact Sheet No. 311. 2006. Ref Type: Internet Communication


European Medicines Agency. European Medicines Agency recommends suspension of marketing authorisation for sibutramine. 2010. Weight loss medicine associated with increased risk of cardiovascular events to be removed from all markets in the European Union. Ref Type: Report


Appendices
## Appendix A: Amino acids

<table>
<thead>
<tr>
<th>Acronyms</th>
<th>Full Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala (A)</td>
<td>Alanine</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>Glycine</td>
</tr>
<tr>
<td>His (H)</td>
<td>Histidine</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>Lysine</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>Leucine</td>
</tr>
<tr>
<td>Met (M)</td>
<td>Methionine</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Proline</td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>Arginine</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>Serine</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>Threonine</td>
</tr>
<tr>
<td>Val (V)</td>
<td>Valine</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>
6.2 Appendix B: Solutions used in this thesis

Alkaline SDS (0.2 M NaOH/1% SDS):

Mix 2 ml 10 M NaOH, 5 ml 20% SDS and 93 ml water.

2 M Calcium chloride:

Dissolve 5.88 g CaCl$_2$·2H$_2$O in 20 ml water and sterilise by passing through a 0.22 µm filter. Store in 1.0 ml aliquots at -20°C.

Caesium chloride saturated propan-2-ol:

Mix 100 g CsCl with 100 ml water and 1 L propan-2-ol. Leave to settle before use.

Dextran coated charcoal:

Dissolve 2.40 g charcoal and 0.24 g dextran in 100 ml phosphate buffer (RIA buffer) with gelatine (0.0025% w/v). Mix for 20 minute at 4°C.

0.5 M EDTA pH 8.0:

Dissolve 146.1 g \((\text{HO}_2\text{CCH}_2)_2\text{NCH}_2\text{CH}_2\text{N(CH}_3\text{CO}_2\text{H})_2\) in 800 ml water and adjust to pH 8.0 with 1 M NaOH. Make up to 1 L with water.

10 mg/ml Ethidium bromide:

Dissolve 200 mg ethidium bromide in 20 ml water.
Gel loading buffer:

3.13 ml 80% glycerol, 50 µl 0.5 M EDTA and 6.10 ml water. Add 10 mg orange G.

GTE:

Mix 2.5 ml 1 M Tris-HCl, pH 8.0, 2.0 ml 0.5 M EDTA and 5.0 ml 18% glucose. Make up to 100 ml with water. Sterilise by passing through a 0.2 µm filter.

20 mM HEPES pH 11:

Dissolve 4.76 g HEPES in 900 ml water. Adjust to pH 11 with 0.5 M NaOH and make up to 1 L with water.

LB:

Mix 10.0 g NaCl, 10.0 g tryptone and 5.0 g yeast extract mixed in 450 ml water. Adjust to pH 7.5 with NaOH and make up to 500 ml with water. Sterilised immediately by autoclaving.

LB agar:

Add 7.0 g agar to 500 ml LB then autoclave.

1 M Magnesium chloride:

Dissolve 203.3 g MgCl₂·6H₂O in 1 L water.
0.1 M PEI pH 7.0:

Dissolve 450 mg PEI in 80 ml water. Adjust to pH 7.0 with HCl. Make up to 100 ml with water. Filter solution.

3 M Potassium acetate pH 4.6:

Dissolve 294.4 g CH₃COOK in water, adjusted to pH 4.6 with glacial acetic acid and make up to 1 L with water.

Phosphate buffer (RIA buffer):

Dissolve 48 g of Na₂HPO₄.2H₂O, 4.13 g KH₂PO₄, 18.6 g (HO₂CCH₂)₂NCH₂CH₃N(CH₂CO₂H)₂, 2.5 g NaN₃ in 5 L of water. Confirm pH is 7.6 ± 0.1 and store buffer at 4°C.

Phosphate buffer with gelatine:

Buffer is produced as above with 12.5 g of gelatine dissolved in 5 L warmed water. Cool solution before addition of other ingredients.

0.1 M Phosphate buffered saline (PBS):

Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ in 800 ml of water. Adjust to pH 7.6 with HCl and make up to 1 L with water.

1 M Potassium chloride:

Dissolve 74.5 g KCl in water and make up to 1 L.
0.3 M Sodium chloride:

Dissolve 17.55 g NaCl in 800 ml water. Make up to 1 L with water.

5 M Sodium chloride:

Dissolve 292.2 g NaCl in 800 ml water. Make up to 1 L with water.

10 M Sodium hydroxide:

Dissolve 400.0 g NaOH in 800 ml water. Make up to 1 L with water.

50 x TAE:

Dissolve 242.0 g trizma base in 843 ml water and 57 ml of glacial acetic acid and 100 ml 0.5 M EDTA.

TES:

Mix 25 ml 1 M Tris-HCl pH 8.0, 5 ml 5 M NaCl and 5 ml C_{10}H_{14}O_{6}N_{2}.2H_{2}O. Make up to 500 ml with water.

TFB I:

Dissolve 589 g CH_{3}COOK, 2.42 g RbCl, 1.98 g CaCl_{2}.2H_{2}O and 438 mg MnCl_{2}.4H_{2}O in 100 ml water. Add 37.5 ml 80% glycerol and make up to 200 ml with water. Sterilise by passing through 0.2 μm filter.

TFB II:

Dissolve 418 mg C_{7}H_{15}NO_{4}S, 3.28 g CaCl_{2}.2H_{2}O and 242 mg RbCl in 100 ml water. Add 37.5 ml 80% glycerol and make up to 200 ml with water. Sterilise by passing through 0.2 μm filter.
1 M Tris-HCl, pH 7.5:

Dissolve 121.1 g trizma base in 800 ml water. Adjust to pH 7.5 with HCl and make up to 1 L with water.

2 M Tris-HCl, pH 8.0:

Dissolve 121.1 g trizma base in 450 ml water. Adjust to pH 8.0 with HCl and make up to 500 ml with water.
6.3 Appendix C: Radioimmunoassay Methods

6.3.1 General Principle of a Radioimmunoassay
All radioimmunoassays used were derived and maintained by Professor MA Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) unless otherwise stated. All reagents and materials other than peptides were supplied by Sigma.

The principle of radioimmunoassay is the competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. When unlabelled antigen from standards of samples and a fixed amount of labelled antigen are allowed to react with a constant and limiting amount of antibody, decreasing amounts of labelled antigen are bound to the antibody as the amount of unlabelled antigen is increased. The radioimmunoassay is incubated and allowed to reach equilibrium, according to the equation:

\[ \text{Ag} + \text{Ab} + \text{Ag} \rightarrow \text{AgAb} + \text{Ab} \]

\( \text{Ag} = \) unlabelled antigen
\( \text{*Ag} = \) radiolabelled antigen
\( \text{Ab} = \) antibody

Separation of the bound from the free antigen is achieved by addition of either dextran-coated charcoal (free label is contained in the charcoal pellet following centrifugation) or using a primary-secondary antibody complex (free label is contained in the supernatant following centrifugation). The secondary antibody is derived from an animal species different from that used to generate the primary antibody. After incubation and separation, the bound and free label are counted in a γ-counter. The data are used to construct a standard curve from which the values of the unknowns can be obtained by interpolation.
Inter-assay variation can be calculated by assaying aliquots of the same sample in each assay performed and comparing the concentrations obtained in each. To measure and correct for baseline drift, tubes with no sample (‘zero’ tubes) are placed at regular intervals throughout the assay and standard curves are performed at the beginning and end of each assay. The general structure of the RIA is outlined in table 9.1, which shows the content of the tubes according to their designation.

The following tubes are important for the assessment and performance of the assay:

**Non-specific binding**: low binding indicates adequate label integrity.

½ X: assesses if greater sensitivity could be achieved by adding half the volume of label.

2 X: assesses if greater sensitivity could be achieved by adding double the volume of label.

Zero tubes: allows assessment of assay drift.

Excess antibody: assesses the immunological integrity of the labelled peptide.

Quality Controls: includes previously aliquoted samples containing high and low levels of the antigen. These tubes allow the assays to be standardised.
<table>
<thead>
<tr>
<th>Tube number</th>
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<tr>
<td>1-2</td>
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<tr>
<td>3-4</td>
<td>$\frac{1}{2} \times$</td>
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<tr>
<td>5-6</td>
<td>$2 \times$</td>
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<tr>
<td>Standard curve</td>
<td></td>
</tr>
<tr>
<td>Final two tubes</td>
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</table>
PYY Radioimmunoassay Protocol

1. The PYY assay is set up in 0.06M phosphate buffer, pH 7.4 (containing 0.05M Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}.2H\textsubscript{2}O, 0.006M KH\textsubscript{2}PO\textsubscript{4}, 0.01M Na\textsubscript{2}H\textsubscript{2}(EDTA).2H\textsubscript{2}O) plus 0.3% bovine serum albumin (BSA).

2. The Iodogen method is used to iodinate human PYY label. Label is high pressure liquid chromatography purified, aliquotted and freeze-dried. On the day of assay, label is made up in required volume in buffer so there is 25-30 Bq/100μl.

3. Rabbit anti-PYY antibody (Y21) is diluted as appropriate to provide correct dilution for 100μl addition.

4. 200μl of sample is added.

5. Human PYY 1-36 standard is made up in buffer to 1pmol/ml concentration. This is used to construct a 10-point standard curve with points at 1, 2, 3, 5, 10, 15, 20, 30, 50, 100μl addition.

6. Label and antibody (see table below for volumes) is added and incubate assay at 4°C for 3 days.

Table showing assay set-up volumes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Blank</th>
<th>Zero</th>
<th>1-20fmol</th>
<th>30fmol</th>
<th>50fmol</th>
<th>100fmol</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>600μl</td>
<td>500μl</td>
<td>500μl</td>
<td>470μl</td>
<td>450μl</td>
<td>400μl</td>
<td>300μl</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200μl</td>
</tr>
<tr>
<td>Std (1pmol/ml)</td>
<td>-</td>
<td>-</td>
<td>1-20μl</td>
<td>30μl</td>
<td>50μl</td>
<td>100μl</td>
<td>-</td>
</tr>
<tr>
<td>Label</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
<tr>
<td>Solution</td>
<td>-</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
</tbody>
</table>

7. To separate the assay add 100μl Pharmacia decanting suspension 3 sheep anti-rabbit to each tube. Shake well, and leave at room temperature for 1hr. Add 500μl 0.1% Triton X-100 to each tube, then spin at 2,500 rpm (approx 1600g) at 4 °C for 25mins. Use a glass pipette to separate supernatant (free) from pellet (bound).

8. Tubes containing the pellet and supernatant are counted using a gamma counter and percentage binding calculated.

9. Plasma PYY level are then determined in Pmol/L.
6.4 Appendix D - Preparation of rat KBB membranes

Rat KBB membranes were prepared by the method of homogenisation and differential centrifugation. Rats were killed by decapitation and kidneys dissected and snap frozen in liquid nitrogen and stored at -70°C. Kidneys were placed in cold 0.5M sucrose and the renal cortices dissected out and weighed. The cortices were finely sliced and minced, and then gently homogenised at a ratio of 1g tissue to 6ml 0.5M sucrose using a 15ml hand-homogeniser (Jencons, UK). Tissue was then further homogenized using 3 strokes of a motorized 15mL homogenizer with a Teflon pestle at 1000rpm. Following this, 1mol/L MgCl\textsubscript{2} was added to a final concentration of 10 mM, and the homogenate incubated for 15 min in the ice bath to ensure aggregation of other subcellular structures by bivalent metal ions. The suspension was then centrifuged at 3000 rpm (Beckman J2-21, rotor JS-13.1) for 20 min at 4°C, the supernatant removed and then centrifuged at 10000rpm (Beckman J2-21, rotor JS-13.1) for 12 min at 4°C. The supernatant was discarded and the pellet resuspended in 100 µL of 300 mmol/L mannitol in 12 mmol/L Tris-Cl (pH 7.4), with 10 mmol/L MgCl\textsubscript{2}. This was then centrifuged at 3700 rpm at 4°C (Sigma Laboratory Centrifuge 3 K18, Sigma Aldrich, UK). The supernatant was resuspended in 300 mmol/L mannitol in 12 mmol/L Tris-Cl (pH7.4), with 10 mmol/L MgCl\textsubscript{2} and centrifuged for 12 min at 10000rpm (Sigma 3 K18 12348 rotor, Sigma Aldrich, UK) at 4°C. The pellet was then resuspended in 300 mmol/L mannitol in 12 mmol/L HEPES (pH7.4) to achieve a protein concentration of 2.5 mg/mL, as measured by Biuret assay (Sigma Aldrich, UK).
6.5 Appendix E – SCOFF Questionnaire

Please circle the response which applies:

- Do you make yourself sick because you feel uncomfortably full? Yes  No
- Do you worry you have lost control over how much you eat? Yes  No
- Have you recently lost more than one stone in a 3 month period? Yes  No
- Do you believe yourself to be fat when others say you are too thin? Yes  No
- Would you say that food dominates your life? Yes  No

1 point given for every ‘YES’ answer

A score of ≥2 indicates a likely case of anorexia nervosa or bulimia nervosa
Volunteer Name: ___________________________ Date: ___________________________

Volunteer No. ___________________________

Please place an (✓) in the box which applies best to each of the numbered statements. All of the results will be strictly confidential. Most of the questions directly relate to food or eating, although other types of questions have been included. Please answer each question carefully. Thank you.

1. If you have put on weight, do you eat less than you usually do?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often
   - Not Relevant

2. Do you try to eat less at mealtimes than you would like to eat?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

3. How often do you refuse food or drink offered because you are concerned about your weight?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

4. Do you watch exactly what you eat?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

5. Do you deliberately eat foods that are slimming?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

6. When you have eaten too much, do you eat less than usual the following days?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often
   - Not Relevant
7. Do you deliberately eat less in order not to become heavier?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

8. How often do you try not to eat between meals because you are watching your weight?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

9. How often in the evening do you try not to eat because you are watching your weight?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

10. Do you take into account your weight with what you eat?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Very Often

11. Do you have the desire to eat when you are irritated?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Very Often
    - Not Relevant

12. Do you have a desire to eat when you have nothing to do?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Very Often
    - Not Relevant

13. Do you have a desire to eat when you are depressed or discouraged?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Very Often
    - Not Relevant

14. Do you have a desire to eat when you are feeling lonely?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Very Often
    - Not Relevant
15. Do you have a desire to eat when somebody lets you down?
- Never
- Seldom
- Sometimes
- Often
- Very Often
- Not Relevant

16. Do you have a desire to eat when you are cross?
- Never
- Seldom
- Sometimes
- Often
- Very Often
- Not Relevant

17. Do you have a desire to eat when you are approaching something unpleasant to happen?
- Never
- Seldom
- Sometimes
- Often
- Very Often

18. Do you get the desire to eat when you are anxious, worried or tense?
- Never
- Seldom
- Sometimes
- Often
- Very Often

19. Do you have a desire to eat when things are going against you or when things have gone wrong?
- Never
- Seldom
- Sometimes
- Often
- Very Often

20. Do you have a desire to eat when you are frightened?
- Never
- Seldom
- Sometimes
- Often
- Very Often
- Not Relevant

21. Do you have a desire to eat when you are disappointed?
- Never
- Seldom
- Sometimes
- Often
- Very Often
- Not Relevant

22. Do you have a desire to eat when you are bore or restless?
- Never
- Seldom
- Sometimes
- Often
- Very Often
- Not Relevant
23. Do you have a desire to eat when you are emotionally upset?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often
   - Not Relevant

24. If food tastes good to you, do you eat more than usual?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

25. If food smells and looks good do you eat more than usual?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

26. If you see or smell something delicious, do you have the desire to eat it?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

27. If you have something delicious to eat, do you eat it straight away?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

28. If you walk past the baker do you have the desire to buy something delicious?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

29. If you walk past a snackbar or a café, do you have the desire to buy something delicious?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

30. If you see others eating, do you also have the desire to eat?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often
31. Can you resist eating delicious foods?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

32. Do you eat more than usual, when you see others eating?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often

33. When preparing a meal are you inclined to eat something?
   - Never
   - Seldom
   - Sometimes
   - Often
   - Very Often