Per Massimo, Anna, Saul e la “Terzolina”......i miei illustri signori.
Investigation of the Effects of the Anorectic Gut Hormones PYY and GLP-1 on Brain Appetite Pathways: A Human fMRI Study.

Thesis submitted for the degree of Doctor of Philosophy from Imperial College London

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ABSTRACT

This thesis explores the roles of the gut hormones Peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) in the modulation of human brain reward pathways utilising functional magnetic resonance imaging (fMRI).

PYY and GLP-1 are co-released post-prandially and both have been shown to reduce appetite and inhibit food intake when administered to humans. They have the potential to be developed into anti-obesity therapies, with the expectation that low-dose combination therapy may provide more effective weight loss and limited side effects. There are currently no safe and effective medications available to treat obesity, and yet this global health crisis continues unabated. In this context, a study of the mechanisms by which gut hormones exert their anorectic effects, may guide the rational development of new drugs.

To date, the effects of GLP-1 alone and the ways in which PYY and GLP-1 combine to modulate brain activity in humans are unknown. This thesis contains a set of functional MRI experiments designed to determine these effects in healthy, fasted, normal-weight human subjects. Results are compared with the changes in brain activation patterns observed physiologically following a meal.

For the first time in humans, I have demonstrated that, in conjunction with a comparable effect on lunchtime energy intake, combined infusion of PYY$_{3-36}$ and GLP-1$_{7-36}$amide to fasted subjects results in a similar modulation of brain activity as observed following a large breakfast. This supports the proposal that these hormones are major physiological mediators of satiety in humans. Both the fed state and the administration of anorectic gut hormones to fasted subjects, reduces activation in multiple brain reward regions in response to visual food-cues. This confirms that circulating gut hormones modulate the hedonic processing of food. The lack of any obvious differential activation pattern between PYY$_{3-36}$ and GLP-1$_{7-36}$amide raises the possibility that they act at corticolimbic structures via a final common pathway.
DECLARATION OF CONTRIBUTORS

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

The Dose Verification and Gut Hormone fMRI studies (volunteer recruitment, study visits and RIA/ELISA analyses) were performed in collaboration with Dr Akila De Silva.

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Dr Christopher Long (image analysis)
Ms Ros Gordon, Mr Mark Tanner and Ms Ineke de Meer (MRI radiographers)
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Chapter Two: Investigation of the Effects of Single and Combined Infusions of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} on Energy Intake in Healthy Humans

2.1 Introduction

2.2 Methods

2.2.1 Peptides

2.2.2 Participants

2.2.3 Dose Verification Study: Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY\textsubscript{3-36} and 0.8 pmol/kg/min GLP-1\textsubscript{7-36amide} on Energy Intake in Healthy Human Subjects

2.2.4 Plasma Hormone Radioimmunoassays

2.2.4.1 Measurement of Total Plasma PYY

2.2.4.2 Measurement of Total Plasma Amidated GLP-1

2.2.5 Statistical Analysis

2.3 Results

2.3.1 Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY\textsubscript{3-36} and 0.8 pmol/kg/min GLP-1\textsubscript{7-36amide} on Energy Intake in Healthy Human Subjects

2.3.2 Visual Analogue Score (VAS) Analysis

2.3.3 Total Plasma PYY Levels in Dose Verification Study

2.3.4 Total Plasma Amidated GLP-1 Levels in Dose Verification Study

2.4 Discussion

Chapter Three: Investigation of the Effects of Single and Combined Administration of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} On Brain Appetite Centres Using fMRI

3.1 Introduction

3.2 Methods

3.2.1 Peptides

3.2.2 Participants

3.2.3 Study Protocol for the Gut Hormone fMRI Study

3.2.4 BOLD fMRI Food Image Task

3.2.5 fMRI Data Acquisition

3.2.6 fMRI Data Analysis

3.2.7 Region Of Interest (ROI) Analysis of fMRI Data

3.2.8 Plasma Hormone Level Analysis

3.2.9 Statistical Analysis

3.3 Results

3.3.1 Participants

3.3.2 Energy Intake

3.3.2.1 Energy Intake During Acclimatisation Visits

3.3.2.2 Energy Intake During fMRI Study

3.3.2.3 Order Effect on Ad Libitum Energy Intake Across Visits

3.3.3 VAS Results

3.3.4 Physiological Measurements

3.3.5 Plasma PYY and GLP-1 Levels Measured in the fMRI Study

3.3.5.1 Plasma Levels of Total PYY and Total (Amidated) GLP-1

3.3.5.2 Plasma Levels of Active PYY and Active GLP-1

3.3.6 fMRI Analysis

3.3.6.1 Whole Brain Activation with Presentation of Food-Salient Visual Stimuli (Effect of Task Analysis)

3.3.6.2 Amalgamated Region of Interest % BOLD Signal Change (food images minus non-food images) by Intervention

3.3.6.3 Individual Region of Interest Analysis by Intervention

3.3.6.4 Regression Analysis of ROI MRI Signal with VAS Scores, Gut Hormone Levels and Energy Intake

3.3.6.5 Whole Brain Activation with Presentation of Food-Salient Visual Stimuli (Effect of Task Analysis)

3.3.6.6 Amalgamated Region of Interest % BOLD Signal Change (food images minus non-food images) by Intervention

3.3.6.7 Individual Region of Interest Analysis by Intervention

3.3.6.8 Regression Analysis of ROI MRI Signal with VAS Scores, Gut Hormone Levels and Energy Intake
3.4 Discussion

3.4.1 The Effects of Combination PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} Infusion on Energy Intake in Humans ................................................................. 109

3.4.2 The Effects of Combination PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} Infusion on Subjective Appetite Ratings .......................................................... 111

3.4.3 The Effects of Combination PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} Infusion on Haemodynamic and Glycaemic Parameters ......................................................... 112

3.4.4 The Effects of Combination PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} Infusion on Brain Appetite Pathways in Humans as Studied Using fMRI ................................................................. 113

3.4.5 Limitations of fMRI ........................................................................................................ 116

4 Chapter Four: General Discussion ................................................................. 118

4.1 Summary of the Effects of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} on Appetite and Brain Reward Pathways Reported in this Thesis ................................................................. 119

4.2 Comparison with fMRI Studies of Hunger and Satiety ................................................................. 120

4.3 Comparison with fMRI Studies of Other Appetite Modulating Hormones ................................................................. 123

4.4 Comparison with fMRI Studies of Appetite Pathways in Obesity ................................................................. 125

4.5 Comparison with fMRI Studies Pre- and Post- Gastric Bypass Surgery ................................................................. 128

4.6 Future Work ........................................................................................................ 129

3.3.1 Clarifying the Effects of Stress on Appetite Pathways as Measured by fMRI ........ 129

3.3.2 Analysing Resting State BOLD and Arterial Spin Labelling Data ......................... 130

3.3.3 Investigating the CNS-Mediated Mechanisms of Weight Loss and Metabolic Improvement Following Bariatric Surgery ................................................................. 131

4.6 Concluding Comment ................................................................................................. 131

6 Appendices ........................................................................................................ 132

6.1 Appendix I: Visual Anlogue Scales ........................................................................ 133

6.2 Appendix II: Radioimmunoassay and ELISA Details .............................................. 136

6.3 Appendix III: Mouse Histopathology Reports ................................................................. 141

6.4 Appendix IV: Information Sheet for Healthy Volunteers ........................................ 145

6.5 Appendix V: Nutritional Breakdown of the Set Breakfast ........................................ 153

Publications and Presentations ........................................................................ 154

References ........................................................................................................ 155
INDEX OF FIGURES

Figure 1.1. Schematic of the Neuroendocrine Network of Appetite Control.................................. 21
Figure 1.2. Schematic of T1 and T2 Relaxation................................................................................. 38
Figure 1.3. The BOLD (Blood Oxygen Level Dependent) Effect....................................................... 40

Figure 2.1. Protocol for Dose Verification Study: Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY₃-36 and 0.8 pmol/kg/min GLP-1₇-₃₆ amide on Energy Intake in Healthy Human Subjects................................................................................................................. 54
Figure 2.2. Analysis of Mean Ad Libitum Energy Intake in Dose Verification Study: Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY₃-36 and 0.8 pmol/kg/min GLP-1₇-₃₆ amide on Energy Intake in Healthy Human Subjects....................................................................................................................... 58
Figure 2.3. Analysis of VAS Ratings During Dose Verification Study.............................................. 60
Figure 2.4. Meal Palatability Rating Following Ad Libitum Lunch in Dose Verification Study........ 61
Figure 2.5. Plasma Total PYY Levels During Dose Verification Study............................................... 63
Figure 2.6. Total Plasma Amidated GLP-1 During Dose Verification Study..................................... 65

Figure 3.01. Protocol for Gut Hormone fMRI study....................................................................... 73
Figure 3.02. Examples of Images Shown During the BOLD fMRI Food Image Task............................. 75
Figure 3.03. Ad Libitum Lunch Energy Intake in fMRI Study by Condition......................................... 83
Figure 3.04. Reduction in Ad Libitum Lunch Energy Intake in fMRI Study for Each Condition Compared with Fasted Saline. ............................................................................................................. 84
Figure 3.05. Order Effect of Visit Number on Ad Libitum Lunch Energy Intake in fMRI Study........ 85
Figure 3.06. Analysis of Nausea Related Visual Analogue Score (VAS) Parameters from t=-60 to t=90 min During each Infusion for the Gut Hormone fMRI Study................................................................. 87
Figure 3.07. Analysis of Food Related Visual Analogue Score (VAS) Parameters from t=-60 to t=90 min During each Infusion for the Gut Hormone fMRI Study................................................................. 88
Figure 3.08. Meal Palatability Rating Following Ad Libitum Lunch in Gut Hormone fMRI Study................................................................. 89
Figure 3.09. Analysis of Non-Food Related Visual Analogue Score (VAS) Parameters from t=-60 to t=90 min during each Infusion for the Gut Hormone fMRI Study................................................................. 90
Figure 3.10. Analysis of Physiological Measurements Taken During Each fMRI Study Visit.............. 92
Figure 3.11. Plasma Levels of Total PYY and Total (Amidated) GLP-1 in the fMRI Study ...............94
Figure 3.12. Plasma levels of Active PYY and Active GLP-1 in the fMRI Study..........................96
Figure 3.13. Thresholded Effect of Task Maps.. ........................................................................98
Figure 3.14. Amalgamated Region of Interest % BOLD Signal Change (food images minus
non-food images) by Intervention..................................................................................................101
Figure 3.15. Modulation of BOLD Signal Across Brain ROIs by Feeding or Either Individual or
Combined Gut Hormone Infusions. .................................................................................................103
Figure 3.16. Summed Reduction in Mean % BOLD Signal Change in ROIs with Individual
Administration of PYY$_{3-36}$ and GLP-1$_{7-36}$amide$_{x}$, Compared with the Reduction in Mean % BOLD
Signal Change Measured after Combined Administration of PYY$_{3-36}$ and GLP-1$_{7-36}$amide$_{x}$........107
INDEX OF TABLES

Table 3.1. Local Maxima where Δ BOLD (when subjects viewed images of food compared with when they viewed images of non-food) was Significantly Greater than Zero............................................................................................................................. 99

Tables 3.2 a-f. Peak Activation Coordinates for Each Comparator Condition by Region of Interest.......................................................................................................................... 104
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MSH</td>
<td>α – Melanocyte stimulating hormone</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;2c&lt;/sub&gt;</td>
<td>5-hydroxytryptamine 2c receptor</td>
</tr>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>B&lt;sub&gt;0&lt;/sub&gt;</td>
<td>External magnetic field produced by the large bore magnet</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood oxygen level dependent</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and Amphetamine Regulated Transcript</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid receptor 1</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DPP IV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>DVC</td>
<td>Dorsal vagal complex of the brainstem</td>
</tr>
<tr>
<td>EPI</td>
<td>Echo planar imaging</td>
</tr>
<tr>
<td>FDA</td>
<td>United Stated Food and Drug Administration</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>FSL</td>
<td>FMRIB Software Library (for MRI image analysis)</td>
</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ME</td>
<td>Median eminence</td>
</tr>
<tr>
<td>MNI</td>
<td>Montreal National Institute (standard brain atlas)</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NMV</td>
<td>Nuclear magnetic vector</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the Tractus Solitarius (brainstem)</td>
</tr>
<tr>
<td>OFC</td>
<td>Orbitofrontal cortex</td>
</tr>
<tr>
<td>OXM</td>
<td>Oxyntomodulin</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-Y gastric bypass</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SNRI</td>
<td>Selective noradrenaline re-uptake inhibitor</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue score</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Y2R</td>
<td>Y2 receptor</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 The Problem of Obesity

The problem of obesity has become a critical and global health concern. In 2005 the World Health Organisation estimated that 1.6 billion adults worldwide were overweight [body mass index (BMI) 25 to 30 kg/m\(^2\)] and at least 400 million obese [BMI >30kg/m\(^2\)]. By 2015 this is projected to rise to 2.3 billion and over 700 million respectively (WHO, 2006). In the UK in 2010, 26.1% of all adults (aged over 16 years) and 16% of all children were classified as obese (Health Survey for England data, UK Department of Health). The medical costs associated with treatment of obesity-related diseases in the NHS are estimated to increase by £1·9–2 billion/year by 2030 (Wang et al., 2011).

It is well established that overweight and obesity are independently linked to an increased risk of cardiovascular disease, and contribute to pre-established risks such as hypertension and dyslipidaemia (Dhaliwal and Welborn, 2009, Dyer et al., 1990). Obesity is the most important risk factor for the development of Type 2 diabetes mellitus, with all of its attendant risks of macrovascular disease, limb loss, blindness and renal failure (Colditz et al., 1995). Obesity has also been causally linked to numerous cancers, obstructive sleep apnoea, musculoskeletal disorders, infertility, depression and many other morbidities (Kopelman, 2000). The most recent epidemiological analysis suggests a reduction in life expectancy of 0.66 years for every BMI increase of 2 kg/m\(^2\) (Stewart et al., 2009).

The fundamental cause of overweight and obesity is an imbalance between calories consumed and calories expended. In normal adults, body weight remains relatively constant over decades, despite large short-term fluctuations in food intake and energy expenditure. Central energy-homeostasis mechanisms sense deviations in nutrient intake or energy stores and act to conserve body weight at a set point, by altering metabolic rate or the sensation of hunger. Overlaid on this is a higher brain reward network, aimed at reinforcing behaviours associated with a survival advantage, such as food consumption, by making them pleasurable. This central
control of appetite is achieved through a complex neural and hormonal signalling network which, according to the “thrifty gene” hypothesis, evolved over millions of years of relative food shortage and is therefore optimised towards energy conservation (Ravussin and Bogardus, 2000, Weinsier et al., 1998). Set against this backdrop of physiological mechanisms designed to resist food deprivation, the global environmental shift towards readily-available energy-dense foods and sedentary lifestyles is fuelling the obesity pandemic.

Lifestyle changes to reduce energy intake and increase energy output can promote weight loss, but the “obesogenic” forces of modernisation have proven much more difficult to overcome (Rennie and Jebb, 2005). For most people, their weight-loss solution is not as simple as “eating less” and “moving more”. This urges scientists toward a better understanding of the regulation of human eating behaviour and energy homeostasis, with the aim of developing new therapies to treat obesity.

1.2 Current Obesity Treatments

1.2.1 Lifestyle

There is compelling evidence that weight loss can reverse or attenuate many of the health consequences of obesity and improve life expectancy. A retrospective matched cohort study of obese subjects undergoing weight loss surgery, revealed a 40% reduction in all-cause mortality, 92% reduction in diabetes deaths, 56% reduction in coronary artery disease deaths and a 60% reduction in cancer deaths secondary to weight loss (Adams et al., 2007, Adams et al., 2009).

Unfortunately, lifestyle interventions alone rarely result in long term weight loss and the vast majority of dieters return to baseline weight within 3-5 years (Weiss et al., 2007). Even patients enrolled in randomised controlled trials have difficulty adhering to weight-reducing diets and typically achieve only very modest weight loss (Dansinger et al., 2005).
Since even small, sustained reductions in weight can significantly improve cardiovascular risk (Pi-Sunyer et al., 2007), the US Food and Drug Administration (FDA) guidance for the approval of weight loss drugs recommends a 5% placebo-corrected weight reduction that should be maintained for at least 12 months after treatment initiation. With a target population for drug therapy set at BMI >30 (or >27 with co-morbidities), there is a huge market for weight-loss drugs. However, there is currently only one licensed drug in the UK for the long term treatment of obesity - orlistat.

Orlistat (Xenical, Roche Pharmaceuticals) irreversibly inhibits intestinal lipases required to hydrolyse dietary triglycerides into free fatty acids (FFAs), and so up to 30% of ingested fat is not absorbed. This is the reason for the most common adverse events, gastrointestinal discomfort and faecal urgency. Meta-analysis of clinical trials indicate a mean weight loss for orlistat-treated patients of 2.89 kg above placebo over 12 months (Rucker et al., 2007). This mix of modest weight loss and often intolerable side effects leads to high attrition rates in users. One survey of 17,000 orlistat users reported rates of ongoing use of less than 10% at one year and 2% at two years (Padwal et al., 2007).

The history of weight-loss drugs is now littered with examples of agents which have been withdrawn due to dangerous side effects. In general, such drugs have either caused an increase in energy expenditure via increased autonomic tone, resulting in raised cardiovascular risk, or have non-specifically targeted brain reward systems, resulting in negative side effects on mood and cognition. The major examples are sibutramine and Rimonabant, which are briefly discussed here.

Sibutramine (Reductil, Abbott Laboratories) is a selective noradrenaline and serotonin re-uptake inhibitor (SNRIs), which centrally modulates appetite and also increases energy expenditure through sympathetic activation. Meta-analysis
revealed an average placebo-corrected weight loss of 4.5 kg in patients taking the drug and it gained widespread approval in the late 1990s (Rucker et al., 2007). A decade later, interim analysis of the Sibutramine Cardiovascular Morbidity/Mortality Outcomes in Overweight or Obese Subjects at Risk of a Cardiovascular Event (SCOUT) trial, revealed a 16% increased risk of cardiovascular events in the sibutramine-treated group (James et al., 2010). By late 2010 the drug had been withdrawn from both the US and European markets. This followed in the wake of the withdrawal of Rimonabant from the European market in 2008. Rimonabant (Acomplia, Sanofi-Aventis), a selective cannabinoid (CB1) receptor antagonist, had weight reducing effects via hedonic modulation of food intake in higher reward centres as well as interaction with the hypothalamic circuitry governing the homeostatic regulation of appetite. In clinical trials, Rimonabant was associated with a 6.6 kg greater weight loss over placebo after a year (Van Gaal et al., 2005). However, within 2 years of post-marketing surveillance in Europe, it was withdrawn due to the discovery of an increased risk of depression and suicide in those taking the drug. It never received FDA approval for usage in the US.

In the light of these serious failures, anti-obesity agents currently in development are facing far more stringent pre-approval assessment. The FDA has recently considered two new anti-obesity agents, following resubmissions from their manufacturers citing extra safety data or more restrictive prescribing guidance. The first is a combination of the amphetamine-like drug phentermine and the anticonvulsant topiramate (Qnexa, Vivus). Phase 3 trials have reported very impressive weight loss benefits of 14.2 kg over a year versus placebo (Gadde et al., 2011). However, there is also a significant increase in cognitive disturbance and an elevation in heart rate seen with the drug, as well as evidence of teratogenicity limiting its use in women of childbearing potential. The 5-HT2c receptor agonist Lorcaserin (Arena Pharmaceuticals) has been shown to produce more modest weight loss benefits of 4.7 kg over a year against placebo, but was associated with a significantly increased incidence of heart valvulopathy (Smith et al., 2010).
1.2.3 Bariatric Surgery

At present, the most effective means of significant and sustained weight loss for obese patients is bariatric surgery. It is also the only treatment that has been shown to reduce mortality (Adams et al., 2007, Sjostrom et al., 2007).

Broadly speaking, there are two major types of surgical intervention. Gastric banding involves the insertion of an adjustable band around the upper portion of the stomach. The degree of constriction can be varied. Weight loss is achieved by limiting the quantity of food that can be comfortably ingested and by increasing satiety (Dixon et al., 2005). Roux-en-Y gastric bypass (RYGB) involves the formation of a small stomach pouch and bypass of the proximal small bowel, with an end-anastomosis of the jejunum to the small gastric pouch. The bypassed remnant drains via jejun-jejunostomy, so that duodenal and pancreatic secretions can still enter the remainder of the small bowel. The RYGB was originally designed to cause malabsorption as well as restricting stomach size. However, the modern procedure allows a sufficient distance of small bowel to remain for fully efficient absorption. Nevertheless, RYGB results in much more weight loss (in the order of 30% of body mass loss) than restrictive procedures such as banding alone (around 20% loss) (Sjostrom et al., 2004, Olbers et al., 2006). The RYGB is associated with other benefits too. After RYGB, patients reduce their relative intake of fat and refined carbohydrates (Halmi et al., 1981, Kenler et al., 1990) and have a reduced preference for sweet foods (Miras et al., 2012), whereas following banding procedures, patients display a greater preference for high-energy liquid foods such as milk-shakes (Brolin et al., 1994, Olbers et al., 2006). Secondly, RYGB results in a rapid improvement in insulin secretory function and often a resolution of Type 2 diabetes, well before significant weight loss has occurred (Kellum et al., 1990, Rubino et al., 2004, Wickremesekera et al., 2005).

These advantages of RYGB are thought to be partly due to an alteration in the release profile of appetite-inhibiting gut hormones, such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), the post-prandial levels of which have been shown to
be elevated following gastric bypass surgery but not after gastric banding (le Roux et al., 2006a, Borg et al., 2006). The incretin effect of GLP-1, which acts at pancreatic beta cells to augment glucose-dependent insulin release, could explain some of the observed improvement in glucose tolerance. More recent studies have also highlighted a vagally-mediated effect to reduce hepatic glucose output following the altered delivery of nutrients to the jejunum in bypass models (Breen et al., 2012).

However, bypass surgery is costly, in many cases irreversible and associated with significant surgical and anaesthetic risks, including a 0.5% mortality rate (Marsk et al., 2008). Despite recent calls for the procedure to be extended to leaner patients as a treatment for Type 2 diabetes and to perform the procedure early after diagnosis, when the chances of long term remission of diabetes are greater (Rubino et al., 2010), the balance of risks and benefits may not ultimately favour surgery for patients with less severe obesity. Furthermore, limitations in financial and human resources restrict its availability, even for those with most to gain.

There is therefore a huge incentive to develop safe pharmacological alternatives to surgery and the tantalising potential of achieving long-term weight loss using medicines based on gut hormones. There is a clear need to improve our understanding of the pathophysiology of obesity and, in particular, the mechanisms by which gut hormones influence appetite, in order to guide the rational development of new drugs.

1.3 Central Energy Balance and the Control of Appetite

The systems governing food intake may be simplistically divided into “homeostatic” and non-homeostatic, or “hedonic”. Energy homeostasis refers to the subconscious stabilization of body weight, by the transmission of information about the body’s energy status and the reciprocal modulation of appetite and metabolism. However, palatability (flavour and appearance), social and emotional influences affect food intake in a manner which may over-ride these homeostatic mechanisms. The non-
homeostatic control of food intake is largely modulated by brain reward pathways, strongly influenced by past experience (memory and learning). Although traditionally studied and described in greater detail here as two separate entities, the modern viewpoint is that these homeostatic and non-homeostatic pathways are in fact part of an overlapping appetitive network (Harrold et al., 2012). This is schematically illustrated in Figure 1.1.

1.3.1 Homeostatic Mechanisms Modulating Food Intake

The crucial areas of the brain co-ordinating energy and body-weight homeostasis are the brainstem and hypothalamus. Here, neural afferents and hormonal signals carrying peripheral information about the body’s energy stores and nutrient availability are integrated to regulate appetite and control energy expenditure (Schwartz et al., 2000).

1.3.1.1 The Brainstem

The brainstem is continuous with the spinal cord caudally. Visceral vagal afferents terminate in its nucleus of the tractus solitarius (NTS). The NTS together with the adjacent area postrema (AP) and dorsal motor nucleus of the vagus, comprise the dorsal vagal complex (DVC) (Fry et al., 2007). The AP is a circumventricular organ, a select area within the CNS containing highly fenestrated microvasculature, which effectively keeps it outside of the blood-brain-barrier (BBB) and therefore a target for circulating hormones (Johnson and Gross, 1993). The AP and NTS share reciprocal innervations. Thus the DVC plays a fundamental role in relaying sensory and metabolic information from the periphery to the remainder of the CNS.

1.3.1.2 The Hypothalamus

Alongside numerous other homeostatic functions including thermoregulation, osmoregulation and coordination of the neuroendocrine axis, the hypothalamus has a central role in maintaining energy balance. It is situated symmetrically around the
midline in the floor and walls of the third ventricle. Over forty distinct hypothalamic nuclei and subnuclei have been identified, along with extensive intra- and extrahypothalamic projections (Berthoud, 2002). Broadly speaking, each half of the hypothalamus may be divided in the coronal plane into periventricular, medial and lateral areas. The hypothalamus is in close proximity to and shares extensive neuronal connections with another circumventricular organ, the median eminence, enabling it to respond rapidly to circulating factors. The action of peripheral signals of energy balance on the hypothalamus to control food intake forms an important focus of current appetite research.

The modern integrated hypothesis of central energy homeostasis centres on the crucial role of the arcuate nucleus of the hypothalamus (ARC). This periventricular structure lies particularly close to the median eminence. Two discrete sets of neuronal populations have been described within the ARC which are thought to act in concert to regulate appetite. Orexigenic neurones (ie those stimulating food intake) in the medial ARC express neuropeptide Y (NPY) and Agouti-related protein (AgRP). Anorexigenic neurones (ie those inhibiting food intake) in the lateral ARC express alpha-melanocyte-stimulating hormone (α-MSH) derived from pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) (Cone et al., 2001). The relative tone of orexigenic and anorexigenic hypothalamic neurones is delicately altered by numerous neuroendocrine inputs (Figure 1.1). The ARC projects efferents to the paraventricular nucleus (PVN) of the hypothalamus where responsive effects on energy expenditure arise, including changes in basal metabolic rate (via the thyroid axis), sympathetic outflow and thermoregulation (Cowley et al., 1999). The ARC also shares dense, reciprocal neural connections with the dorsal vagal complex of the brainstem (ter Horst and Luiten, 1986).
Figure 1.1: Schematic of the Neuroendocrine Network of Appetite Control.
Circulating adipostat hormones, which signal long-term energy stores, and prandially released gut hormones, which signal short term nutrient status, are able to directly influence neuronal activity in key brain areas involved in energy homeostasis, the brainstem and hypothalamus. Circulating gut hormones [such as peptide YY (PYY), glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK), pancreatic polypeptide (PP), oxyntomodulin (OXM) and ghrelin] modulate gastrointestinal vagal sensory input into the brainstem. They may also directly access the brainstem’s nucleus of the solitary tract (NTS) and hypothalamic arcuate nucleus (ARC) via areas deficient in blood brain barrier [the area postrema (AP) and median eminence (ME)]. Two major subpopulations of neuron in the ARC influence appetite. Those which co-express neuropeptide Y (NPY) and Agouti-Related Protein (AgRP) are orexigenic, whereas those co-expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) are anorexigenic. AgRP antagonizes the effects of the POMC product, α-melanocyte-stimulating hormone (α-MSH). Orchestrated within corticolimbic brain structures and superimposed on this homeostatic system of appetite control, are hedonic reward drives stimulated by sensory cues and possibly directly modulated by circulating factors too.
A fuller description of appetite regulation must make mention of how higher brain areas associated with reward, motivation, learning and memory interact with homeostatic drives. Food consumption is said to be rewarding, in other words it produces positive emotions that augment the drive to obtain it. In this setting, the salience of an object (or image or situation) is how much it stands out or how emotionally arousing it is. Saliency detection is important for focusing the brain’s limited cognitive resources on the most pertinent of environmental stimuli, and facilitates learning.

In general, reward is divided into two major psychological processes: “liking” (hedonic impact) and “wanting” (incentive salience).

The hedonic impact of an object describes its attractiveness. The concept of reward is generally understood as the subjective sensation of pleasure. However, pleasure is not merely contained in the intrinsic sensation of the stimulus, but the evolved ability of that stimulus to activate brain “liking” systems. It can be shown that hedonic stimuli can subconsciously provoke implicit “liking” reactions (such as positive facial expressions in response to sweet taste) which can be measured physiologically. Furthermore, it is possible to pharmacologically manipulate this hedonic response. In rats, microinjection of mu opioid receptor antagonists into a part of the nucleus accumbens (a corticolimbic brain reward structure) increases the objective “liking” response to sweet taste without altering reward seeking behaviour (Pecina and Berridge, 2005). In short, the nucleus accumbens (NAcc) contains a ‘hedonic hotspot’ where neuronal activity is purely correlated with pleasure perception.

However, “liking” without “wanting” is simply a triggered affective state. Hence pleasure must be translated into “wanting” in order for reward to influence behaviour. “Liking” is closer to a sensory process, “wanting” to motor action and both are needed together for full reward.
In this context, “wanting” is termed incentive salience, and describes the type of motivation that promotes approach toward and consumption of a reward. Incentive salience should be distinguished from more conscious forms of desire. Our general understanding of the word wanting requires the definition of a goal or expected outcome. Instead, incentive salience can be subconscious and, as in the case of addiction, can occur even when cognitively the desire is understood to be irrational or unsafe (Berridge et al., 2009).

Past experience with specific foods forms an important contributor to the reward process. Learning entrains conditioned stimuli, which predict the availability and motivational value of reward and in time themselves become triggers of incentive salience. In humans, vivid imagery of food is one such potent cue and simply imagining food can also elicit cravings (Tiggemann and Kemps, 2005). It is believed that cue triggered urges incrementally contribute to long term overconsumption and ultimately obesity.

1.3.2.1 Corticolimbic Regions of Interest Related to Food Reward Processing

The neural correlates of the reward system are still being delineated. Incentive salience depends largely on a widespread network of subcortical mesolimbic dopamine neurotransmission, with important structures including the dorsal and ventral striata (Ikemoto, 2007). The coding of the reward value of sensory cues depends more on cortical brain areas such as the insula and orbitofrontal cortex (OFC) (Kringelbach, 2005). In contrast, current knowledge suggests that hedonic valence is neurologically organized as islands of mu-opioid and cannabinoid receptor neurones, such as the hotspot previously described within the nucleus accumbens. The specific brain reward regions studied in this work are described in more detail below.
1.3.2.2 Amygdala

The amygdala is a deep cortical structure located within the anterior lobe. It has strong connections with the orbitofrontal, prefrontal and visual cortices, the insula and nucleus accumbens (Amaral and Price, 1984, Morgan et al., 2005). It has a complicated neuronal structure and, as part of the limbic system, is involved in emotion, reward, motivation, learning, memory and attention (Murray, 2007). More specifically in the setting of reward processing, the amygdala encodes the emotional intensity of both pleasant and aversive stimuli (Baxter and Murray, 2002, Zald, 2003). Functional imaging research has shown that the amygdala is reliably activated when viewing or tasting food (LaBar et al., 2001, Killgo re et al., 2003, Fuhrer et al., 2008, Stoeckel et al., 2008, Goldstone et al., 2009).

1.3.2.3 Nucleus Accumbens (NAcc)

The NAcc comprises the main part of the ventral striatum and is a member of the basal ganglia. It has a rich dopaminergic innervation originating from the midbrain and is highly involved in reward processing and addiction. It is particularly activated by the perception of pleasant, emotionally arousing scenes. Whereas amygdala activation correlates with salience per se (positive or negative), the NAcc is selectively activated by stimuli with hedonic valence (Sabatinelli et al., 2007). In the field of appetitive functional imaging, NAcc activity correlates positively with subjective liking ratings of food images in fasted healthy human volunteers (Farooqi et al., 2007).

1.3.2.4 Orbitofrontal Cortex (OFC)

The OFC lies on the ventral surface of the frontal part of the brain. The human OFC plays an important role in encoding food reward. Numerous sensory pathways carrying olfactory, visual, gustatory, and somatosensory information converge on the OFC, where monitoring, learning and memory of the reward value of sensory cues is performed. In line with this, the OFC is closely connected with the hippocampus and
other paralimbic structures involved with memory (Verhagen and Engelen, 2006). Neuroimaging studies frequently implicate the OFC in processing the reward value of food images in the fasted state (Small et al., 2007, Porubska et al., 2006, Stoeckel et al., 2008, Schur et al., 2009). The observation that OFC activation decreases with sensory-specific satiety (when food is repeatedly consumed to a state of aversion) supports its particular role in encoding reward value (Kringelbach and Rolls, 2004).

### 1.3.2.5 Insula

The insula cortex is a cortical structure spanning the temporal and frontal lobes. It is an important gustatory centre, involved in both sensory and reward taste processing, as well as modulating taste aversion (Welzl et al., 2001, James et al., 2009). The insula receives visceral sensory information from the brainstem and projects to the OFC, NAcc and amygdala. Functional MRI studies confirm its role in gustatory processing (Kida et al., 2011) but human fMRI studies also reveal increased activation in the insula in response to many other food cues (St-Onge et al., 2005, Porubska et al., 2006, Fuhrer et al., 2008, Stice et al., 2008, Schur et al., 2009, Siep et al., 2009).

### 1.3.2.6 Caudate and Putamen

The caudate and putamen are two connected structures which together comprise the dorsal striatum. They are the main input nuclei of the basal ganglia, receiving convergent afferents from nearly all parts of the cortex. Historically they have been studied for their role in coordinating motor functions, but there is now mounting evidence for parallel cognitive functions (Grahn et al., 2008).

The caudate nucleus in particular coordinates incentive salience and goal-directed behaviours, particularly where the outcome is perceived as desirable (Balleine and O'Doherty, 2010). Human fMRI studies correlate caudate activity during gustatory stimulation with subsequent hedonic evaluation and reveal that the caudate can be
activated by the expectation of preferred foods (Arana et al., 2003, Pelchat et al., 2004).

The putamen has a greater role in stimulus-response-outcome learning (Haruno and Kawato, 2006). In human fMRI studies, putamen activity correlates with the acquisition of learned associations (Tricomi et al., 2009) and is also modulated by subjective ratings of appetite (Porubska et al., 2006).

There are dense interconnections between the hypothalamus and all of these mesolimbic/cortical reward systems. Exactly how the regulatory (hypothalamic) and reward (corticolimbic) centres interact with one another in the overall control of appetite remains to be fully understood. “Hedonic eating” can override the satiety effect of positive energy balance signals within the hypothalamus (Saper et al., 2002). Conversely, hunger can enhance food reward, in part mediated by hypothalamic projections of orexin neurones on to the nucleus accumbens (Harris et al., 2005). The latest research, supported by finding in this thesis, suggests that circulating satiety hormones, originally discovered for their effects on the brainstem and hypothalamus, can modulate hedonic circuitry too (Malik et al., 2008, Batterham et al., 2007, Farooqi et al., 2007, Dickson et al., 2012).

1.4 Peripheral Signals Regulating Food Intake

Peripheral neural and hormonal feedback signals to the brain transmitting information about the body’s energy status are complex and incompletely understood. In general, the vagus nerve serves as the neural component of the gut-brain axis, conveying information about mechanical distension and the chemical poroperties of gut luminal contents. Peptide gut hormones released around a meal relay information about acute fluctuations in nutritional status, whereas adipostat hormones, related to fat stores, provide information about longer-term energy needs (Figure 1.1).
1.4.1 Adipostat Factors

The observations that dieting and experimental overfeeding induce compensatory responses that tend to restore adiposity to baseline levels are consistent with the adipostat theory, or a set point for the homeostatic control of body fat mass (Havel, 2001). The major adipostat hormones are insulin, which has known centrally mediated anorectic effects and average circulating levels which correlate with fat mass (Woods and Seeley, 2001), and leptin, described in greater detail below.

1.4.1.1 Leptin

The hormone leptin, protein product of the obese \((ob)\) gene (Zhang et al., 1994), is synthesised in white adipose tissue and circulates at concentrations proportional to body fat mass (Maffei et al., 1995). Leptin stimulates anorexigenic neurones and inhibits orexigenic neurones in the ARC. CNS and peripheral administration of leptin to rodents results in inhibition of food intake and decreased body weight (Friedman and Halaas, 1998). Conversely, administration of leptin during food restriction delays other neuroendocrine responses to starvation (Ahima et al., 1996). Thus, leptin is thought to act as a signal of adequate fat stores, with declining levels indicating the onset of negative energy balance.

The \(ob/ob\) mouse is completely leptin-deficient. The behavioural response to perceived starvation in these animals is profound hyperphagia and resultant obesity. Administration of exogenous leptin to \(ob/ob\) mice results in normalisation of food intake and body weight (Halaas et al., 1995, Pelleymounter et al., 1995). Human cases of severe obesity secondary to leptin deficiency are extremely rare but, where identified, have been successfully treated with recombinant leptin, with the principal effect a reduction in hyperphagia (Farooqi et al., 1999).

In contrast, the vast majority of obese patients are appropriately hyperleptinaemic and yet continue to maintain high body fat stores. Treating obese, non-leptin
deficient patients with leptin achieves only modest weight loss (Zelissen et al., 2005). This state of “leptin resistance” remains poorly understood. Several mechanisms may contribute to the phenomenon. The two most credited hypotheses are a failure in the transport of circulating leptin to its targets in the brain (Banks and Farrell, 2003) or downregulation of the intracellular components of the leptin receptor signalling cascade (Munzberg and Myers, 2005). Thus the discovery of leptin produced a huge step in our understanding of energy homeostasis but as yet shows little promise as a therapeutic agent for obesity.

1.4.2 Gut Hormones

Adipostat factors act as signals of long-term energy stores but energy consumption can also be centrally modulated on a meal-by-meal basis by peptide hormones released from the gut. Control of meal size is largely determined by the onset of satiety. There are a number of postprandially secreted hormones, including Peptide YY (PYY), Glucagon-like peptide 1 (GLP-1), oxyntomodulin (OXM), cholecystokinin (CCK) and pancreatic polypeptide (PP), which enhance the sensation of satiety. Conversely, ghrelin is the only known gut hormone to stimulate ingestive behaviour, with peak circulating levels measurable in the fasting state. The two anorectic gut hormones investigated in this project, PYY and GLP-1, are presently discussed in greater detail.

1.4.2.1 Peptide YY (PYY)

PYY is a 36 amino acid peptide hormone originally isolated from porcine intestine and named for the presence of a tyrosine residue at each terminal (Tatemoto and Mutt, 1980). It is produced in the enteroendocrine L cells of the gastrointestinal mucosa (Rindi et al., 2004). PYY is released into the circulation within 15 minutes of ingestion of a meal in proportion to the number of calories ingested. Plasma levels peak 1-2 hours later and remain elevated for up to 6 hours (Adrian et al., 1985).
The major circulating form of PYY is PYY\textsubscript{3-36}. It is formed following cleavage of the amino-terminal tyrosine-proline dipeptide of PYY\textsubscript{1-36} by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) (Eberlein et al., 1989, Mentlein et al., 1993). PYY\textsubscript{3-36} is considered the active circulating form of the hormone, and is much more potent at reducing food intake or delaying gastric emptying in rodents than PYY\textsubscript{1-36} (Chelikani et al., 2004). As with other members of the PP-fold family of peptides [namely, neuropeptide Y (NPY) and pancreatic polypeptide (PP)], PYY binds to the Y-family of G-protein-coupled receptors. PYY\textsubscript{3-36} has a selective (nanomolar) affinity to the Y2 receptor (Y2R) (Berglund et al., 2003).

Endogenous PYY has long been understood to affect gastric motility and secretion, conceptualised as the mediator of the “ileal brake”, which decelerates gastric emptying in response to the arrival of nutrients in the distal small bowel (Pappas et al., 1985, Savage et al., 1987). This paracrine effect to delay gut transit time is partly responsible for the sensation of satiety that is induced by the hormone.

More recently, PYY has additionally been shown to act in a neurohumoral manner on CNS appetite control centres. PYY\textsubscript{3-36} is now accepted as an appetite-inhibitory hormone based on the following evidence: peripherally administered PYY\textsubscript{3-36} at physiological concentrations results in a marked acute inhibition of food intake in multiple species (Batterham et al., 2002, Halatchev et al., 2004, Chelikani et al., 2006, Pittner et al., 2004, Koegler et al., 2005); peripheral injection of PYY\textsubscript{3-36} increases c-fos expression, a marker of neuronal activation, in the arcuate nucleus of the hypothalamus (ARC) and low dose PYY\textsubscript{3-36} administered directly into the ARC has an anorectic effect in rats (Batterham et al., 2002); peripherally administered radiolabelled PYY\textsubscript{3-36} binds at the median eminence in the CNS (Dumont et al., 2007), supporting the notion that circulating PYY gains access to the hypothalamus via this circumventricular organ. In addition, PYY knockout mice are hyperphagic and obese, a phenotype which is reversed by exogenous replacement of the hormone, strongly supporting its physiological role in the control of food intake (Batterham et al., 2006). Y2 receptors probably mediate the actions of PYY\textsubscript{3-36}: Y2 receptor-knockout
mice are unresponsive to the hormone (Batterham et al., 2002), and pre-treatment with a selective Y2 receptor antagonist, either by intra-ARC injection (Abbott et al., 2005b) or by IP injection (Talsania et al., 2005), abolishes the anorectic effects of PYY\textsubscript{3-36} in wild types.

In humans who have been previously fasted from the day before, food intake at a buffet lunch was reduced by 36% following an intravenous infusion of PYY\textsubscript{3-36} that achieved plasma levels similar to those measured physiologically after a meal (Batterham et al., 2002). PYY may indeed be involved in the pathogenesis of obesity in humans. Obese subjects have been reported as having significantly lower levels of PYY compared to people with a normal BMI. This finding has not always been reproduced, although others have reported a blunted postprandial release of PYY\textsubscript{3-36} in obese patients (Stock et al., 2005, le Roux et al., 2006b). Importantly, there is no suggestion that, as for leptin, obesity is a PYY-resistant state (Pfluger et al., 2007). In a separate study, exogenous infusion of PYY\textsubscript{3-36} resulted in a reduction in food intake by 30% in an obese group and 31% in a group of subjects with normal BMI (Batterham et al., 2003).

Chronic exogenous administration of PYY\textsubscript{3-36} to humans has not been reported. However, chronic administration to rodents has not always produced long lasting anorectic effects (Chelikani et al., 2006, Pittner et al., 2004, Reidelberger et al., 2008), although there is some suggestion that the ongoing effects of PYY are masked by counter-regulatory mechanisms to prevent excessive body weight loss (Unniappan and Kieffer, 2008). Nevertheless, there remains significant interest in the therapeutic potential of PYY as a weight-loss agent with the observation that basal and post-prandial levels are elevated in obese patients that have lost weight and experience altered taste preference following Roux-en-Y gastric bypass surgery (le Roux et al., 2006a, Miras et al., 2012). In line with this, some gastrointestinal diseases associated with appetite and weight loss are also characterized by elevated PYY levels (El-Salhy et al., 2002).
Nastech and Merck developed a nasally administered form of human PYY\textsubscript{3-36} to be taken with each meal, which reached phase 2 clinical trials in 2007. The study was terminated after 24 weeks because of significant drop-out rates due to nausea and vomiting, which is a well described dose related adverse effect of PYY (and all other anorectic gut hormones) (Gantz et al., 2007). It remains unclear to what extent the anorectic and nauseating effects of gut hormones are independent processes. However, it is generally understood that satiety in humans involves a spectrum of experience (ranging from extreme hunger at one end, through the onset of satiation during a meal, to feelings of fullness and then nausea at the other end), which is mirrored by the circulating concentrations of anorectic gut hormones. In the Merck nasal PYY study the maximum plasma PYY concentration achieved was 105 pmol/L, which is much higher than the normal postprandial level of 50 pmol/L. Furthermore, the time taken to reach this peak concentration was 18-26 minutes - much shorter than the slower and sustained physiological post-prandial rise. Thus the high drop-out due to nausea and the failure of nasal PYY to induce longer term appetite reduction and weight loss may have been due to the pharmacokinetics of this mode of delivery.

1.4.2.2  Glucagon-like Peptide - 1 (GLP-1)

GLP-1 is a cleavage product of the prohormone preproglucagon, which is expressed in the pancreas, brainstem and enteroendocrine L-cells. Preproglucagon is cleaved in a tissue-specific manner by prohormone convertases 1 and 2 (Mojsov et al., 1986). Glucagon is the principle expression product in the pancreatic islets, whereas GLP-1, GLP-2 and oxyntomodulin are produced in the CNS and intestine.

The stimulus for GLP-1 secretion from the gut is food intake. In humans, there is a biphasic secretion pattern initially within 10-15 minutes of meal ingestion followed by a second plateau phase at 30-60 minutes (Herrmann et al., 1995). The active circulating forms of GLP-1 are GLP-1\textsubscript{7-36amide} and GLP-1\textsubscript{7-37} (Orskov et al., 1993, Orskov et al., 1994). Both are produced by post-translational cleavage of GLP-1\textsubscript{11-37} in the L-
cells. At pancreatic islets, GLP-1 acts as a potent incretin hormone. It physiologically augments glucose-dependent insulin release and inhibits the secretion of glucagon. GLP-1 also modulates digestion by delaying gastric emptying (Naslund et al., 1999b, Kreymann et al., 1987, Gutniak et al., 1992).

As with PYY, GLP-1 additionally acts as a neurohumoral satiety factor, inhibiting food intake in rats after either central or peripheral administration (Turton et al., 1996, Tang-Christensen et al., 1996, Larsen et al., 2001). Central administration of the GLP-1 receptor antagonist Exendin9-39 increases food intake, suggesting a physiological role for endogenous GLP-1 in appetite regulation (Turton et al., 1996). These effects are likely to be centred on the brainstem and hypothalamus, where GLP-1 receptor gene expression is altered by fasting and refeeding (Zhou et al., 2003). Peripherally administered GLP-1 causes neuronal activation in the ARC (Abbott et al., 2005a) and central administration causes neuronal activation in the PVN, nucleus of the tractus solitarius (NTS) and area postrema (AP) (Larsen et al., 1997, Rowland et al., 1997). GLP-1 probably also acts directly at the vagus nerve, whose sensory terminals express the GLP-1 receptor (Nakagawa et al., 2004), since the effect of peripherally administered GLP-1 on both energy intake and activation of ARC neurones is attenuated by either bilateral sub-diaphragmatic truncal vagotomy or bilateral transections of the brainstem-hypothalamus pathway (Abbott et al., 2005a).

In both lean and obese humans, acute IV infusion of GLP-1 dose dependently reduces energy intake at a subsequent buffet meal, although the anorectic effects are not as potent as PYY at doses that mimic normal post-prandial circulating levels (Verdich et al., 2001). Chronic SC administration results in weight loss and improved glycaemic control in patients with type 2 diabetes and after 5 days of subcutaneous GLP-1 injections, obese subjects reduced their cumulative calorie intake by 15% and lost 0.55 kg in weight (Naslund et al., 2004). There is some evidence that altered GLP-1 secretion may contribute to the pathogenesis of obesity in humans, since endogenous levels are inversely correlated with body mass but normalise with weight loss (Ranganath et al., 1996). Higher circulating levels of the hormone are
measured in patients following RYGB, which may help to explain the early post-operative improvement in glycaemic control that is frequently observed (le Roux et al., 2006a).

GLP-1 based therapies have been successfully developed as treatments for Type 2 diabetes, making use of the hormone’s incretin effect. As with other peptide gut hormones, GLP-1 has an extremely short plasma half-life of 2 minutes. In contrast to its effect on PYY\textsubscript{1-36}, DPP-IV N-terminal dipeptide cleavage rapidly and completely inactivates GLP-1, rendering the native hormone itself unviable as a drug (Kieffer et al., 1995). There have been two successful approaches to this problem: degradation resistant GLP-1 receptor (GLP-1R) agonists (incretin mimetics) and DPP-IV inhibitors.

Exendin-4, a peptide with sequence homology to GLP-1 that was first purified from the venom of the Gila monster \textit{Heloderma suspectum}, is a potent agonist at the GLP-1 receptor and is resistant to degradation by DPP-IV. It was developed into the drug exenatide (Byetta, Amylin / Eli Lilly), which was released in 2005 and now has extensive licensing for use as first line treatment in obese patients with Type 2 diabetes mellitus or as an adjunct to all other oral antidiabetic agents and insulin. As well as improvements in glycaemic control, exenatide is the first treatment for diabetes with proven weight loss benefits. A meta-analysis of clinical trials reveals an average weight loss of 2.13 kg in exenatide treated groups compared to placebo, and a 4.76 kg weight loss compared with insulin (Amori et al., 2007). Administration is by twice-daily SC injection, which constituted a barrier to widespread patient acceptance, and prompted the development of once-weekly exenatide (Bydureon\textsuperscript{®}), now also approved for clinical use. A second GLP-1 analogue has recently been introduced into clinical practice: liraglutide (Victoza, Novo Nordisk). Liraglutide is 97% identical to the native human peptide, but is coupled at the position 26 lysine to a palmitoyl side chain, which promotes binding to albumin, thereby lengthening its half-life. In clinical studies, liraglutide shows evidence of slightly improved glycaemic control compared with exenatide and a similar weight loss profile (Feinglos et al., 2005). Currently coming through late-stage clinical trials, GSK have their own incretin mimetic albiglutide and Sanofi are developing lixisenatide, but Roche were
forced to halt the development of their drug taspoglutide, due to the emergence of severe hypersensitivity reactions during Phase III testing.

The suitability of incretin-mimetics as mono-therapy for obesity in the absence of diabetes is under investigation. Liraglutide is currently in late-stage trials to assess its efficacy in this setting. Open-label Phase II results revealed that 75% of non-diabetic obese subjects receiving high dose liraglutide lost greater than 5% body weight and 35% over 10% (Astrup et al., 2009). A 10% drop out rate due to side effects was largely due to nausea rather than severe hypoglycaemia. In fact, at the doses used to treat diabetes, the commonest side effect with all GLP-1 analogues is nausea. The incidence ranges from 33% to 57% with exenatide compared with 0.4% to 9% in patients treated with insulin analogues (Barnett et al., 2007). In general, this side effect can be ameliorated by early dose titration and eventually mitigates with continued use.

In contrast to the incretin mimetics, DPP-IV inhibitors have gained less widespread usage in Type 2 diabetes. In the UK at present there are three such drugs licenced, all available as once daily tablets, with no evidence that any one is more effective that the others at improving glycaemic control. They are sitagliptin (Januvia, Merck & Co), vildagliptin (Galvus, Novartis) and saxagliptin (Onglyza, Bristol Myers Squibb). None of these drugs have weight-loss benefits. This may be because DPP-IV modifies a multitude of peptides. For example, whilst its action is to inactivate GLP-1, it also converts PYY\textsubscript{1-36} to its active anorectic form PYY\textsubscript{3-36}. Thus DPP-IV inhibition has a neutral effect on overall concentrations of active circulating anorectic peptides.

1.4.2.3 The Potential of Combination Gut Hormone Treatments for Obesity

The observations that the natural post-prandial sensation of satiety is mediated by the release of several gut hormones and that the superior weight loss effects of bariatric surgery may be mediated by alterations in numerous neuroendocrine factors, have led to the concept of combining multiple gut hormones or developing
dual agonists of gut hormone receptors as treatments for obesity (Moran and Dailey, 2009). This rationale is further supported by the notion that the dose limiting effects of nausea for each hormone can be offset by administering multiple agents at lower doses.

Oxyntomodulin, a naturally occurring weak dual agonist of both the GLP-1 and glucagon receptors, has already been developed into a potential anti-obesity agent. Chronic administration of the native hormone to healthy overweight and obese volunteers resulted in a 2.3 kg weight loss compared with 0.5 kg weight reduction in placebo-treated controls (Wynne et al., 2005). Its weight-loss effects are mediated both via a reduction in appetite and increase in energy expenditure (Cohen et al., 2003, Dakin et al., 2002). Animal studies with engineered and more potent combined agonists of the GLP-1 and glucagon receptors confirm this dual mode of action (Pocai et al., 2009).

In human studies, administration of PYY_{3-36} and GLP-1_{7-36} amide, at doses that are ineffective on their own, produces a significant reduction in ad libitum food intake when given together (Neary et al., 2005). In the same paper, rodent studies confirmed that the combined peripheral administration of PYY_{3-36} and GLP-1_{7-36} amide led to a reduction in food intake that was greater than that observed when either hormone was infused individually at twice the dose. In the ARC, no increase in c-fos expression was observed after individual low-dose administration PYY_{3-36} or GLP-1_{7-36} amide, but co-administration at the same doses led to significant activation. In another human study, coadministration of PYY_{3-36} and oxyntomodulin reduced energy intake by 42.7% in comparison with the saline control group, a significantly greater effect than that achieved with either hormone alone (Field et al., 2010).
1.5 The Use of Functional MRI to Investigate the Neuroendocrinology of Appetite

The concepts of homeostatic and hedonic control of food intake, and the contribution of circulating satiety signals, represent a huge shift in our understanding of appetite regulation over the past two decades. However, the precise pathways by which anorectic gut hormones exert their appetite-modulating effects, particularly in humans, are technically challenging to research and they remain unclear. Recently, functional magnetic resonance imaging (fMRI) has become a popular and rapidly advancing tool for investigating CNS appetite pathways in humans, allowing for repeated measures in the same subject without exposure to ionising radiation. The earliest fMRI appetite studies explored differences in brain activation patterns between the normal fed and fasted state. This was followed by a cluster of studies exploring differences in food-cue invoked reward area activation between obese and lean populations. Most recently, fMRI studies have begun to investigate hormonal influences on appetitive brain processes and in relation to this, to explore functional neuroimaging outcomes following weight loss interventions.

1.5.1 Principles of Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) utilises the behaviour of hydrogen nuclei, which consist of single protons that possess angular momentum (spin). By virtue of this spin, each proton also possesses a magnetic dipole moment, the axis of which is the same as the spin axis. When an external magnetic field \( B_0 \) is applied, the protons in tissue try to align with it, causing a net magnetic moment (termed the net magnetisation vector, NMV) parallel with \( B_0 \). However, the angular momentum of the protons prevents complete alignment of the NMV with \( B_0 \) and instead the protons precess around the field axis (commonly assigned to the z axis in 3D coordinates).
Generation of MRI images requires application of a radiofrequency (RF) pulse at 90° to $B_0$. This causes the NMV to flip into the transverse (x/y) plane (Figure 1.2 a). When the RF pulse is turned off, the NMV returns to a state of equilibrium with $B_0$ (Figures 1.2 b and c). This ‘relaxation’ results in changes to the magnetic field that can be detected through induction of current in a nearby receiver coil and converted to images. By slightly altering the strength of the magnetic field (and therefore the frequency of the emitted radiation) using gradient coils across the volume to be imaged, spatial information can be inferred. Gradient coils which vary along the x, y and z axes divide the subject being imaged into voxels, or cubic volumes, the dimensions of which define the image resolution.

The T1 relaxation time is a time constant referring to the realignment of the NMV with $B_0$ when the RF pulse is switched off (Figures 1.2 a-c). Simultaneous with this longitudinal relaxation, there is a (much quicker) de-phasing of individual precessing spins in the transverse plane (Figures 1.2 d-f). The time constant relating to this de-phasing is termed T2. In practice, due to localised inhomogeneities in the external magnetic field, measured T2 is usually shorter than predicted for any particular tissue. This apparent T2 is referred to as $T2^*$. T1 and T2 vary depending on the tissue being imaged. The MR signal obtained for a particular tissue therefore depends primarily on three intrinsic parameters: the proton density and the T1 and T2 relaxation times. Contrast in MR images originates from the differences in these properties between adjacent tissues.

The repetition time (TR) is the time elapsed between successive RF excitation pulses. A short TR allows full realignment of the NMV with $B_0$ of tissues with a short T1, but only partial recovery of tissues with a long T1. Full recovery is associated with a greater net signal following further flipping of the NMV by subsequent RF pulses. The echo delay time (TE) is the interval between the RF pulse and the first measurement. A short TR highlights T1 differences between tissues, whereas a short TE minimises T2 effects. Thus, with selection of appropriate TR and TE, it is possible to ‘weight’ the image to accentuate signal from certain tissue types.
Figure 1.2: Schematic of T1 and T2 Relaxation.
MRI utilises the behaviour of tissue protons within varying magnetic fields to produce signals which can be converted into images. The summated magnetic moments of all of the protons trying to align with the main external magnetic field of the scanner (referred to here as B₀ and conventionally shown along the z axis in 3D coordinates), can be considered as a net magnetisation vector, NMV (red arrow). A second magnetic field (in the form of a short radiofrequency RF pulse) is applied, which flips the NMV from alignment in the z direction into the x-y plane (1a). Before application of the RF-pulse the amplitude in the z-axis is maximal while the amplitude in the x-y plane is zero. Just after application of the RF-pulse the amplitude in the z-axis is zero (1a) while the amplitude in the x-y plane is maximal (1d). During relaxation the amplitude in the z-axis will slowly increase (1 b and c) while the amplitude in the x-y plane slowly decreases (1 e and f). T1 relaxation is the time taken for the z vector to regain in strength, whereas T2 relaxation is the time taken for the x-y vector to decay. These changing magnetic vectors invoke their own RF signals, which are picked up by the receiver coils and interpreted into information about the tissues of the subject being scanned.
1.5.2 Principles of Functional Magnetic Resonance Imaging (fMRI)

fMRI utilises adaptations of this classical MRI technique, such that brain activity can be examined rather than structure alone. Virtually all fMRI studies rely on a measure called blood-oxygenation-level dependent (BOLD) contrast, based on the fact that oxygenated and deoxygenated blood possess different magnetic properties. Increased neuronal activity in the brain elicits a local haemodynamic response, which causes an increase in blood flow greater than necessary for tissue demands. This results in a locally reduced ratio of deoxyhaemoglobin to oxyhaemoglobin concentrations and the ensuing differences in local magnetic field inhomogeneities can be detected on a $T_2^*$-weighted imaging protocol (Ogawa et al., 1992). The locally reduced ratio of deoxyhaemoglobin to oxyhaemoglobin leads to a longer $T_2^*$, producing increased image intensity in that region (1.3). BOLD-based protocols typically use magnetic gradients to generate the MR signal (gradient-echo imaging) and, as scanner and computational hardware has advanced, faster sequences have been developed to acquire large numbers of images in short spaces of time. Echo-planar imaging (EPI), for example, is a technique whereby an entire 2-dimensional image can be attained by the rapid alteration of spatial gradients following a single RF pulse. In this way, the entire brain can now be functionally imaged within the same timeframe as the physiological changes of interest.

fMRI image reconstruction results in the selected brain area divided into thousands of voxels, each assigned a signal intensity. Statistical analysis of each voxel, or cluster of voxels, ascertains whether the signal intensity is greater there compared with all others. Whilst this approach may be useful for exploratory studies, most fMRI experiments perform region of interest (ROI) analyses whereby the functional properties of a pre-determined set of voxels (corresponding to a priori anatomical areas of the brain) are analysed. Alternatively, resting state functional connectivity analysis can investigate the activational inter-relationships between different brain regions, allowing for the formation of distinct neuronal networks implicated in a co-ordinated brain response (Biswal et al., 1997).
Figure 1.3: The BOLD (Blood Oxygen Level Dependent) Effect.
The increased metabolic demand from activated neurones results in an increase in oxygen consumption of around 10%. This is physiologically overcompensated for by an increase in regional blood flow by over 30%. As a result, the ratio of deoxygenated to oxygenated haemoglobin (Hb/HbO$_2$) actually decreases in activated brain areas. Oxyhaemoglobin (HbO$_2$) is diamagnetic (ie has the same magnetic properties as surrounding tissue), whereas deoxyhaemoglobin (Hb) is paramagnetic and therefore distorts the local magnetic field. The relative reduction in deoxyhaemoglobin in activated areas means that the magnetic field there is less distorted (longer T2*) and so the MRI signal is stronger. Hence, the BOLD effect results in greater MRI signal from activated brain areas.
1.5.3 Summary of Human fMRI Studies of Appetite to Date

All fMRI studies of human appetite have primarily examined corticolimbic reward areas, with some also reporting on the hypothalamus. The commonest experimental paradigm is to present the subject with images of food or non-food items. It is well understood that whilst taste provides an immediate reward (or punishment) for consumed foods, the visual characteristics of food are quickly learned and become powerful cues, capable of influencing subsequent food-seeking behaviour. In fact, in humans food selection is primarily guided by the visual system (Linne et al., 2002). Thus, showing food images is a useful way of examining appetitive reward circuitry. A subtraction analysis is performed to see whether the difference in regional brain activation between viewing images of food or non-food is altered in different physiological conditions (eg the fasted versus the fed state).

fMRI studies investigating human appetite do indeed reveal greater activation by food than by non-food images in corticolimbic reward areas (van der Laan et al., 2011). However, due to differences in experimental design, concurrence between studies is moderate, with only a few regions consistently reported as task-responsive, particularly the OFC, amygdala, insula and striatum. The most frequently investigated factors that can modulate the brain response to food cues are hunger, the caloric content of the food images and BMI. In general, a satiety-induced reduction in activity in these corticolimbic structures is reported (LaBar et al., 2001, Killgore et al., 2003, St-Onge et al., 2005, Fuhrer et al., 2008). Reward areas are also more strongly activated in response to viewing high calorie versus low calorie foods (Schur et al., 2009, Siep et al., 2009), a difference which is amplified when hungry. Reward area activation, as measured with fMRI, tends to correlate positively with subjective appetite ratings (Porubska et al., 2006, Goldstone et al., 2009). Studies investigating activation patterns in obese subjects have reported a hyper-reactive reward system, particularly to high calorie food cues (Rothemund et al., 2007, Stoeckel et al., 2008). There is also some suggestion that obese individuals show greater responses to anticipated food consumption but weaker activation during actual consumption compared to lean individuals (Stice et al., 2008). Interestingly,
recent imaging studies pre- and post-gastric bypass surgery, by which an average 30% reduction in body weight had been achieved, have suggested that some of these aberrant reward activation patterns can be reversed with weight loss, with the suggestion that such changes may in part be mediated by alterations in post-operative gut hormone levels (Ochner et al., 2011). In line with this, there have been a few fMRI studies which have directly examined the effects of exogenously administered hormones on the CNS.

One study has shown that intranasal insulin administration (that is, direct central administration whilst maintaining euglycaemia) reduces BOLD fMRI activation (with respect to food images versus non-food images) in reward centres in normal adults (Guthoff et al., 2011). This concurs with our understanding of insulin as an anorectic, adipostat factor.

In two congenitally leptin deficient human subjects, daily subcutaneous leptin replacement reduced BOLD fMRI activation (in terms of the difference in activation between viewing food and non-food images) in striatal regions compared with the leptin deficient state (Farooqi et al., 2007). Another study in three leptin deficient patients, suggested regional differences in the way leptin modulates food reward, with some areas, such as the insula, being inhibited and others, like the prefrontal cortex, being activated by leptin (Baicy et al., 2007). In normal, hyperleptinaemic obese subjects, weight loss results in increased activity in reward areas in response to visual food cues, which can be attenuated with leptin administration (Rosenbaum et al., 2008). This suggests that weight loss leads to a state of relative leptin deficiency, which may be responsible for the rebound hyperphagia and subsequent weight gain observed in most dieters. These early fMRI studies of the physiology of leptin were the first to suggest that circulating signals of energy stores interacted with hedonic as well as homeostatic systems.

There are two examples of BOLD fMRI studies investigating the CNS effects of gut hormones. In the first, intravenous infusion of PYY\textsubscript{3-36} to healthy human subjects produced the expected acute reduction in food intake, and increased activity in the
hypothesised hypothalamus and OFC (Batterham et al., 2007). A later study demonstrated that in normal-weight human subjects, intravenous ghrelin infusion increased hunger ratings and also increased the BOLD fMRI signal change (between food and non-food visual cues) in multiple corticolimbic reward areas, compared with saline infusion (Malik et al., 2008).

To date, there has been no fMRI study investigating the effects of GLP-1 infusion on its own, or any combination gut hormone administration on appetitive CNS activity.

1.6 Hypothesis and Aims

PYY$_{3-36}$ and GLP-1$_{7-36}$ amide are both post-prandially released anorectic gut hormones. Previous co-administration studies in humans have shown that their effects on inhibition of food intake are additive. However, the brain pathways which mediate these effects in humans are unknown. The aim of this project from the outset was to utilise BOLD-based fMRI to investigate the CNS effects of PYY$_{3-36}$ and GLP-1$_{7-36}$ amide administration, and to compare and contrast brain activation patterns between the fed and fasted state and following single or combined infusions of these hormones.
I hypothesise that:

1. In subjects fasted overnight, subanorectic doses of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} when infused in combination, will reduce \textit{ad libitum} energy intake at a subsequent buffet meal compared with saline.

2. There will be increased BOLD fMRI signal in the fasted state compared to the fed state when viewing food images versus non-food images in key CNS reward areas.

3. The effect of combined infusion of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} in fasted subjects will reduce the BOLD fMRI signal in key CNS reward areas, similar to that observed in the fed state.

The aims of this project are:

1. To establish doses of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} that are subanorectic alone but anorectic in combination compared with saline.

2. To compare the functional MRI response to food versus non-food images in the fed state and the fasted state during infusion of saline.

3. In the fasted state, to investigate the modulation of the food-salient functional MRI response by the single and combined administration of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide}. 
CHAPTER 2

Investigation of the Effects of Single and Combined Infusions of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} on Energy Intake in Healthy Humans.
2.1 INTRODUCTION

Finding the correct dose of infused hormones to study was of the utmost importance for this neuroimaging study. The primary aim was to find doses of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} that are subanorectic alone but anorectic in combination. This was attempted in order to create an experimental condition which more closely reflected the physiological post-prandial state, whereby satiety without nausea is mediated by the slow and sustained release of multiple gut hormones.

Both PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} are peptide hormones with short circulating half lives in the range of minutes, due to the activity of plasma proteases. PYY\textsubscript{3-36} has a circulating half-life of approximately 8 minutes and that of GLP-1\textsubscript{7-36 amide} is only 2 minutes (Lluis et al., 1989, Kieffer et al., 1995). The therapeutic window for gut peptides is narrow and the anorectic effect and maximum tolerated dose is limited by the initiation of nausea and vomiting. The onset of nausea is thought to be related not only to the peak plasma concentration achieved of the hormone, but also its rate of rise (Gantz et al., 2007). Thus a large bolus dose, giving rise to an immediate sharp peak in plasma levels, is likely to cause vomiting. This is of particular importance given that in the supine position and claustrophobic environment of the MRI scanner, nausea may be accentuated, and vomiting can lead to aspiration. Furthermore, it was important to avoid imaging aversive rather than anorectic brain activation.

A dose verification experiment was therefore performed in volunteers recruited using the same inclusion criteria as for the subsequent imaging study. These subjects would experience an identical gut hormone infusion protocol but not be scanned, and the purpose of this study was simply to ascertain the appropriate doses to be taken forward into the imaging sessions.
An unramped infusion protocol was planned, with the aim of a slow rise in infused hormone plasma concentration until a steady state was achieved. The selection of doses to be tested (0.3 pmol/kg/min PYY$_{3-36}$ and 0.8 pmol/kg/min GLP-1$_{7-36}$ amide) was based on the following review of literature in the field.

Only one study has reported on the co-infusion of PYY$_{3-36}$ and GLP-1$_{7-36}$ amide. Combined administration, each at a dose of 0.4 pmol/kg/min, significantly reduced energy intake by 27% compared to saline at an ad libitum buffet meal served 90 minutes into the infusion. No nausea was reported in the group of 10 lean, healthy subjects. When 0.4 pmol/kg/min GLP-1$_{7-36}$ amide was administered singly, there was a non-significant 5% reduction in energy intake compared to saline at the ad libitum buffet meal. 0.4 pmol/kg/min PYY$_{3-36}$ infusion on its own produced a 15% reduction in food intake (Neary et al., 2005).

I decided to increase the dose of GLP-1$_{7-36}$ amide used in this study to 0.8 pmol/kg/min based on a meta-analysis of single GLP-1$_{7-36}$ amide infusion studies. This revealed that a mean infusion rate of 0.89 pmol/kg/min GLP-1$_{7-36}$ amide across seven studies led to a 13.2% reduction in energy intake at the ad libitum buffet meal compared with saline infusion (Verdich et al., 2001). Although formal assessment of nausea ratings was only made in two of the studies included in the meta-analysis, none reported any nausea. Thus it was predicted that this higher selected dose of GLP-1$_{7-36}$ amide would safely produce a similar reduction in food intake to PYY$_{3-36}$.

Studies reporting the effect of single infusion of PYY$_{3-36}$ suggest it has a narrower therapeutic (ie anorectic but non-nauseating) window. The very first study showing the anorectic effects of PYY$_{3-36}$ in humans reported a 36% reduction in energy intake at an ad libitum buffet meal compared with saline infusion (Batterham et al., 2002). This was measured two hours after the termination of a 90 minute infusion of 0.8 pmol/kg/min PYY$_{3-36}$. No nausea was reported in that study of 12 lean, healthy volunteers. In a different study, no reduction in ad libitum energy intake was seen with an infusion of 0.2 pmol/kg/min PYY$_{3-36}$, whereas a dose of 0.8 pmol/kg/min led to a 19% reduction in ad libitum energy intake (Sloth et al., 2007). However, the
higher dose infusion had to be terminated due to severe nausea and abdominal pain in 5/9 subjects tested. In another PYY\textsubscript{3-36} dose-response study of 16 subjects, 0.2 pmol/kg/min did not produce any nausea, 0.4 pmol/kg/min led to nausea in 2 subjects and a dose of 0.8 pmol/kg/min induced nausea in 4 subjects (Degen et al., 2005). Thus, in order to minimise the risk of nausea in this neuroimaging study, a dose of 0.3 pmol/kg/min PYY\textsubscript{3-36} was chosen.

In planning this study, consideration was also taken over the meal timing. Firstly, the use of an MRI-compatible injector system to deliver the peptide infusion during the scan had to be taken into account, which constrained the protocol to allow for the test meal to be served only after the infusion had ended. This is because the MRI-compatible injector cannot be moved out of the scanner room, switching infusion pumps would have led to pharmacokinetic inconsistencies which such short-lived peptides and, finally, it was not possible to serve a meal in the scanner room. The literature on human PYY\textsubscript{3-36} infusion studies reported an interval of up to 2 hours between termination of the infusion and timing of the test meal, whilst still eliciting a significant reduction in food intake (Batterham et al., 2002). In contrast, many GLP-1\textsubscript{7-36 amide} infusion studies have continued to run the peptide infusion throughout the meal (Naslund et al., 1999a). This is likely to reflect its shorter half-life. I therefore decided to serve the test meal immediately after terminating the 90 minute gut hormone infusion, to ensure that the food intake effects were measured when plasma levels were close to peak.
2.2 METHODS

2.2.1 Peptides

Human PYY$_{3-36}$ and GLP-1$_{7-36}$ amide was purchased from Bachem (St. Helens, UK). The identity and purity of each peptide was confirmed by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, and by high-pressure liquid chromatography (HPLC) (Bachem).

Sterile 0.9% saline was purchased from Bayer (Haywards Heath, UK). Using an aseptic technique in a laminar flow cabinet, the peptides were dissolved in 0.9% saline, aliquoted into vials and freeze dried. Vials were randomly selected for microbiological examination and for the Limulus Amoebocyte Lysate test for pyrogen. The vials were sterile on bacterial and extended fungal culture (Department of Microbiology, Hammersmith Hospital, London) and the Limulus Amoebocyte Lysate tests (Associates of Cape Cod, Liverpool, UK) were within the safe range for human infusion. Further representative vials were randomly selected and sent for amino acid analysis by Alta Bioscience (Birmingham, UK). These results were used to calculate the actual peptide content of the vials and therefore the correction factor when calculating the dose of peptide to be administered to subjects.

Toxicity studies were also performed using PYY$_{3-36}$ and GLP-1$_{7-36}$ amide, at doses greater than 10 times the maximum intended for humans (in pmol/kg body weight), administered by IP injection to 20 mice, alongside a saline-administered control group. The mice were observed for evidence of abnormal behaviour. Half the animals in each group were sacrificed by CO$_2$ asphyxiation after 48 hours, and the other half after 21 days. The lungs, heart, kidneys, liver, brain and reproductive organs of both sexes were dissected, fixed and sent for examination by an independent histopathologist who was blinded to treatment group.
2.2.2 Participants

Healthy, normal-weight, non-smoking adults were recruited for the study by advertisement.

Inclusion criteria for this research were: age 18 years or over, in good health, stable body weight for the preceding three months and BMI 19 – 25 kg/m². Exclusion criteria were: smokers, recreational drug use, substance abuse, significant physical or psychiatric illness, regular medication other than contraceptives, and pregnancy. All subjects were screened and determined to be healthy by full medical history, physical examination, full blood count, urea and electrolytes, liver function tests, bone profile, random glucose, thyroid function tests and 12-lead electrocardiogram. Women of child-bearing age were advised to avoid pregnancy during the studies and underwent a urine test to exclude pregnancy prior to the start of each study visit. All potential subjects were screened to exclude those with disordered eating, as assessed by the SCOFF questionnaire, and those with a high level of restrained eating, as assessed by the standard Dutch Eating Behaviour Questionnaire. Volunteers also completed a three-day diet diary to determine their usual eating habits before acceptance into the studies. During the screening visit, a sample of the study meal was provided, and an assessment of palatability was made using a nine-point hedonic scale. Subjects who disliked the study meal, or rated the study meal as ‘like extremely’ were also excluded.

The research (with the dose verification and imaging arms considered as a single project) was approved by the St Mary’s Research Ethics Committee (reference number 09/H0712/4). All subjects gave written informed consent, and the research was planned and performed in accordance with the Declaration of Helsinki.
2.2.3 Dose Verification Study: Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY\textsubscript{3-36} and 0.8 pmol/kg/min GLP-1\textsubscript{7-36} amide on Energy Intake in Healthy Human Subjects.

In order to verify that the doses of 0.3 pmol/kg/min PYY\textsubscript{3-36} and 0.8 pmol/kg/min GLP-1\textsubscript{7-36} amide were subanorectic when administered individually, anorectic in combination and not nauseating in lean, healthy human subjects, six subjects (2 male, 4 female) were recruited to test their effects on appetite and energy intake.

The subjects attended for 5 study visits, each at least 3 days apart. They were asked to fast (permitted to drink only water) from 22:00h the evening before each study visit. The volunteers standardised their diet (by eating the same evening meal at the same time the night before each study visit), abstained from alcohol and avoided strenuous exercise for 24 hours prior to each visit.

The study was conducted in a single-blinded fashion. On their first visit, subjects always received an infusion of saline after an overnight fast. This protocol was identical to that used for the subsequent visits, and was intended to acclimatise the subject to the clinical environment and to experimental procedures. Results from the first saline visit are not included in the analysis. Following the acclimatisation visit, subjects were assigned to receive each of the following in random order after an overnight fast:

1. Saline infusion (the fasted, control visit)
2. PYY\textsubscript{3-36} infusion at 0.3 pmol/kg/min
3. GLP-1\textsubscript{7-36} amide infusion at 0.8 pmol/kg/min
4. Combined PYY\textsubscript{3-36} + GLP-1\textsubscript{7-36} amide infusion, at 0.3 pmol/kg/min and 0.8 pmol/kg/min respectively.

Subjects arrived for each visit at 09:30 h (t= -60 min), when peripheral venous cannulae (20G) were inserted in both forearms; one for infusions and one for blood sampling. Subjects were encouraged to relax, but not fall asleep, by reading or
watching a DVD. Time cues were removed from the study room to limit the potential effect of time awareness leading to food expectation and appetite alteration.

The intravenous infusion started at 10:30 h (t = 0 min) and was delivered over 90 minutes. All syringes and lines used to deliver peptide were coated in Gelofusin® (B. Braun Medical Ltd, UK) for 20 minutes prior to preparation, to minimise adsorption of peptide to the plastic walls. The actual vehicle for delivering peptide (also used on the “saline” infusion days) was a 10% solution of Gelofusin in 0.9% saline. Vials of peptide were dissolved in 2.5ml vehicle, and drawn up in a single syringe up to a total volume of 60ml. The first 10mls were slowly (over 5 minutes) purged through the delivery line before it was attached to a subject’s infusion cannula. The amount of peptide added to the delivery syringe varied according to the weight-adjusted dose each subject received, such that the infusion rate was kept constant throughout at 20ml/hr (Graseby 3100 syringe driver, SIMS Graseby Ltd, Watford, UK).

Six ml blood samples were collected at t = -30, 0, 15, 30, 45, 60, 75, 90 and 120 min. min, into lithium heparin-coated tubes (International Scientific Supplies Ltd, Bradford, UK) containing 1500 kallikrein inhibitor units (0.15 ml) aprotinin (Trasylol, Bayer Schering Pharma, Berlin, Germany). Samples immediately underwent centrifugation (4000 rpm for 10 minutes at 4°C), after which, plasma was separated immediately and stored at -20°C until analysis. Cannulae were flushed with 5 ml 0.9% saline after collecting each blood sample. Immediately prior to collection of the subsequent sample, 1 ml dead-space blood was collected and discarded.

At t = -30, 0, 15, 30, 45, 60, 75, 90 and 120 min., subjects completed a series of visual analogue scales (VAS) that rated hunger, satiety and nausea. The VAS scales consisted of 100 mm lines with text expressing the most positive and the most negative rating for each variable anchored at either end.
Haemodynamic parameters and blood glucose were measured during the studies. At $t = -30, 0, 15, 30, 45, 60, 75, 90$ and $120$ min. the pulse and blood pressure of each subject was measured and a drop of blood was taken from each blood sample to check blood glucose on an Optimum Exceed blood glucose monitor (Abbott Diabetes Care, Maidenhead, Berks).

The infusion was terminated after 90 minutes. Thirty minutes after stopping the infusion (i.e. $t = 120$ min), subjects were offered a buffet meal and were asked to ‘eat until comfortably full’. The meal, which was provided to excess so that all appetites would be accommodated, was served in private over 20 minutes. It consisted of either a mild chicken tikka curry with rice (628 kJ/100g) or a vegetarian option of tomato and mozzarella pasta (507kJ/100g). Each subject had previously decided which meal they preferred during their screening visit and were then served the same meal for each of their study visits. Food was weighed pre- and post-consumption. Energy intake was calculated from the weight of food consumed. At the end of the meal, subjects were asked to rate the palatability of the food using a VAS. Subjects remained in the study room until $t = 150$ min.

The protocol for the Dose Verification Study is summarised in Figure 2.1
Figure 2.1 - Protocol for Dose Verification Study: Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY$_{3-36}$ and 0.8 pmol/kg/min GLP-1$_{7-36}$ amide on Energy Intake in Healthy Human Subjects.

Following an overnight fast, six healthy, normal-weight subjects each received the following interventions, in random order, over 4 separate study days in a single-blinded fashion:

1. 90 minute saline infusion (fasted saline, control visit)
2. 90 minute PYY$_{3-36}$ infusion at 0.4 pmol/kg/min
3. 90 minute GLP-1$_{7-36}$ infusion at 0.4 pmol/kg/min
4. 90 minute combined PYY$_{3-36}$ and GLP-1$_{7-36}$ infusion, at 0.3 pmol/kg/min and 0.8 pmol/kg/min respectively.

At every visit, an *ad libitum* buffet meal was served immediately after the infusion was discontinued in order to measure energy intake. Blood sampling (for PYY and GLP-1) and assessments of appetite (based on visual analogue scores (VAS)) was performed at the time-points indicated.
2.2.4 Plasma Hormone Radioimmunoassays

2.2.4.1 Measurement of Total Plasma PYY

Total PYY-like immunoreactivity was measured with a specific and sensitive in-house radioimmunoassay, previously established (Adrian et al., 1985). The antiserum (Y21) was produced in rabbits against synthetic porcine PYY coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:50,000. This antibody cross-reacts fully with both PYY\(_{3-36}\) and PYY\(_{1-36}\), but not with pancreatic polypeptide, neuropeptide Y, or other known gastrointestinal hormones. The \(^{125}\)I PYY was prepared by the iodogen method and purified by high pressure liquid chromatography (HPLC). The specific activity of the \(^{125}\)I PYY label was 54 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 (BSA). The assay was incubated for three days at 4\(^\circ\)C before separation of the free and 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 % and inter-assay variation below 10%. All samples were assayed in one assay to avoid inter-assay variation.

2.2.4.2 Measurement of Total Plasma Amidated GLP-1

GLP-1 like immunoreactivity was measured by a specific and sensitive in-house radioimmunoassay, previously established (Kreymann et al., 1987). The antibody was produced in rabbits against synthetic GLP-1\(_{7-36}\text{amide}\) coupled to BSA. The antibody cross reacted 100% with all amidated forms of GLP-1 [GLP-1\(_{1-36}\text{amide}\), GLP-1\(_{7-36}\text{amide}\) and GLP-1\(_{9-36}\text{amide}\)] but did not cross react with extended forms [GLP-1\(_{1-37}\) and GLP-1\(_{7-37}\)] or any other known pancreatic or gastrointestinal peptide. \(^{125}\)I-GLP-1 was prepared by the iodogen method and purified by HPLC. The specific activity of the \(^{125}\)I GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.7 ml of 0.06 M sodium barbitone buffer (pH 8) containing 0.3 % BSA. The assay was incubated for three days at 4\(^\circ\)C before separation of the free and antibody bound label by charcoal absorption. The limit of detection was 7.5 pmol/l, with an intra-assay variation of
5.4% and inter-assay variation below 10%. All samples were assayed in one assay to avoid inter-assay variation.

Full details of all in-house RIA methods are given in Appendix II.

### 2.2.5 Statistical Analysis

Combined data are represented as the mean ± SEM. Comparisons of energy intake and ratings of meal were by repeated measures ANOVA with Tukey’s multiple comparison post-test and Dunnett’s comparison post-test against the fasted saline visit. VAS scores were adjusted for baseline and differences between t= 0 min and t= 90 min compared by repeated measures non-parametric Friedman’s test with Dunn’s multiple comparison post-test. Comparisons of plasma gut hormone levels were by repeated measures ANOVA with Tukey’s multiple comparison post-test. Analyses were performed using Prism version 5.01 software (Graphpad Software, San Diego, CA, USA).
2.3 RESULTS

Microbiological examination and Limulus Amoebocyte Lysate testing of representative peptide vials was unremarkable. Analysis by Alta Bioscience (Birmingham, UK) revealed that a representative 25 nmol vial of PYY$^{3-36}$ actually contained 18.6 nmol and a representative 25 nmol vial of GLP-$^{17-36}$ amide actually contained 25.4 nmol. Toxicity studies in mice were unremarkable (Appendix III).

2.3.1 Effect of Intravenous Infusion of 0.3 pmol/kg/min PYY$^{3-36}$ and 0.8 pmol/kg/min GLP-$^{17-36}$ amide on Energy Intake in Healthy Human Subjects.

Six healthy subjects, 4 male and 2 female, average age 27.8 years and mean BMI 21.4, completed the Dose Verification Study. The mean energy intake during the *ad libitum* buffet lunch was 3478 ± 838.4 kJ when they received saline, 3726 ± 804.5 kJ when they received the single infusion of 0.3 pmol/kg/min PYY$^{3-36}$, 3334 ± 961.3 kJ when they received the single infusion of 0.8 pmol/kg/min GLP-$^{17-36}$ amide and 2359 ± 825.7 kJ when they received the combined infusion of 0.3 pmol/kg/min PYY$^{3-36}$ + 0.8 pmol/kg/min GLP-$^{17-36}$ amide. The difference in mean energy intake was statistically significant (P < 0.05) between the fasted saline and combination gut hormone visits. This is illustrated in Figure 2.2.
Six healthy subjects underwent 90 minute infusions of saline (Fast sal), 0.3 pmol/kg/min PYY\textsubscript{3-36} (P 0.3), 0.8 pmol/kg/min GLP-1\textsubscript{7-36} amide (G 0.8) and combined 0.3 pmol/kg/min PYY\textsubscript{3-36} + 0.8 pmol/kg/min GLP-1\textsubscript{7-36} amide (P 0.3 + G 0.8). All subjects were fasted overnight before each study visit. An \textit{ad libitum} buffet meal was served immediately after the infusion in order to measure energy intake. * denotes P<0.05 for P 0.3 + G 0.8 vs all other groups.
2.3.2 VAS Analysis

Combined infusion of 0.3 pmol/kg/min PYY\textsubscript{3-36} + 0.8 pmol/kg/min GLP-1\textsubscript{7-36 amide} resulted in a mean increase in VAS nausea score by 22.5 mm from t=0 min to t= 90 min. This was statistically significant when compared with the mean change in nausea score between t= 0 min and t= 90 min in other groups (P<0.05). There were no reports of nausea with the single infusions (Figure 2.3 a). Ratings of hunger and fullness changed as expected around the study meal, but were not affected by any of the gut hormone infusions (Figure 2.3 b/c).

In the same study, mean rating (at t= 120 min) of how tasty subjects found the buffet lunch on a 100 mm VAS scale was 61 ± 9.4 mm when they received saline, 65.2 ± 8.7mm when they received the single infusion of 0.3 pmol/kg/min PYY\textsubscript{3-36}, 64.8 ± 7.8 mm when they received the single infusion of 0.8 pmol/kg/min GLP-1\textsubscript{7-36 amide} and 56.7 ± 12.0 mm when they received the combined infusion of 0.3 pmol/kg/min PYY\textsubscript{3-36} + 0.8 pmol/kg/min GLP-1\textsubscript{7-36 amide}. There was no significant difference between visits (P= 0.48, Figure 2.4).
Figure 2.3 a: How nauseous do you feel right now?

Figure 2.3 b: How hungry do you feel right now?

Figure 2.3 c: How full do you feel right now?

Figure 2.3  Analysis of VAS Ratings During Dose Verification Study.

Six healthy subjects underwent infusions of saline (Fast sal), 0.3 pmol/kg/min PYY$_{3-36}$ (PYY 0.3), 0.8 pmol/kg/min GLP-1$_{7-36}$amide (GLP-1 0.8) and combined 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$amide (PYY 0.3 + GLP-1 0.8). All subjects were fasted overnight before each study visit. On each visit, the infusion was administered between $t=0$ and $t=90$ min, and lunch served immediately after. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to nausea, hunger and fullness. Graphs are presented as change in score from baseline at $t = -30$ min. * denotes $P < 0.05$ for P 0.4 + G 0.4 vs all other groups at the given timepoint.
How tasty was the meal?

![Graph showing VAS rating at 120 min (mm) for different treatments]

**Figure 2.4** Meal Palatability Rating Following *Ad Libitum* Lunch in Dose Verification Study

Six healthy subjects underwent 90 minute infusions of saline (Fast sal), 0.3 pmol/kg/min PYY$_{3-36}$ (P 0.3), 0.8 pmol/kg/min GLP-1$_{7-36}$amide (G 0.8) and combined 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$amide (P 0.3 + G 0.8). All subjects were fasted overnight before each study visit. An *ad libitum* buffet meal was served immediately after the infusion and subjects retrospectively rated its palatability on a scale of 0-100.
2.3.3 Total Plasma PYY Levels in Dose Verification Study

Mean plasma concentration of total PYY (from time points t= 15 min to t = 90 min) was 27.48 ± 1.7 pmol/L when saline was infused, 66.78 ± 9.0 pmol/L when 0.3 pmol/kg/min PYY$_{3-36}$ was infused, 20.5 ± 0.4 pmol/L when 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused, and 87.29 ± 8.8 pmol/L when the combination 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused.

Peak mean plasma concentration of total PYY (during the entire study) was 33.26 pmol/L when saline was infused (occurring at t = 30 min), 91.07 pmol/L when 0.3 pmol/kg/min PYY$_{3-36}$ was infused (occurring at t= 90 min), 33.3 pmol/L when 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused (occurring post-prandially at t = 120 min), and 106.5 pmol/L when combination 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused (occurring at t = 90 min).

There was no significant difference between plasma concentration of total PYY achieved between saline and GLP-1$_{7-36}$ amide 0.8 pmol/kg/min infusions. Furthermore, there was no significant difference between mean plasma concentration of total PYY achieved when PYY$_{3-36}$ was infused singly or in combination with GLP-1$_{7-36}$ amide. This was true for repeated measures analysis over the whole study (i.e. t = -60 to t = 120 min) as well as for repeated measures analysis for samples analysed during the infusion period (t= 15 to t= 90 min). Figure 2.5 shows the variation of plasma total PYY levels with time in the Dose Verification Study.
Figure 2.5  Plasma Total PYY Levels During Dose Verification Study.

Six healthy subjects underwent infusions of saline, 0.3 pmol/kg/min PYY_3-36, 0.8 pmol/kg/min GLP-1_7-36amide and combined 0.3 pmol/kg/min PYY_3-36 + 0.8 pmol/kg/min GLP-1_7-36amide. All subjects were fasted overnight before each study visit. On each visit, the infusion was administered between t= 0 and t= 90 min, after which lunch was immediately served.
2.3.4 Total Plasma Amidated GLP-1 Levels in Dose Verification Study

Mean plasma concentration of amidated GLP-1 (from time points t = 15 min to t = 90 min) was 19.8 ± 0.2 pmol/L when saline was infused, 17.2 ± 0.5 pmol/L when 0.3 pmol/kg/min PYY$_{3-36}$ was infused, 58.9 ± 6.0 pmol/L when 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused, and 59.4 ± 5.7 pmol/L when the combination of 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused.

Peak mean plasma concentration of total amidated GLP-1 (during the entire study) was 32.4 ± 4.7 pmol/L when saline was infused (occurring post-prandially at t = 120 min), 26.3 ± 4.0 pmol/L when 0.3 pmol/kg/min PYY$_{3-36}$ was infused (occurring post-prandially at t= 120 min), 73.3 ± 6.7 pmol/L when 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused (occurring at t= 90 min), and 73.0 ± 9.4 pmol/L when the combination of 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$ amide was infused (occurring at t = 90 min).

There was no significant difference between plasma concentration of total amidated GLP-1 achieved between saline and single PYY$_{3-36}$ infusions. There was also no significant difference between mean plasma concentration of total amidated GLP-1 achieved when GLP-1$_{7-36}$ amide was infused singly or in combination with PYY$_{3-36}$. This was true for repeated measures analysis over the whole study (i.e. t = -60 to t = 120 min) as well as for repeated measures analysis for samples analysed during the infusion period (t= 15 to t= 90 min). Figure 2.6 shows the variation of plasma total amidated GLP-1 levels with time in the Dose Verification Study.
Figure 2.6  Total Plasma Amidated GLP-1 During Dose Verification Study

Six healthy subjects underwent infusions of saline, 0.3 pmol/kg/min PYY_{3-36}, 0.8 pmol/kg/min GLP-1_{7-36amide} and combined 0.3 pmol/kg/min PYY_{3-36} + 0.8 pmol/kg/min GLP-1_{7-36amide}. All subjects were fasted overnight before each study visit. On each visit, the infusion was administered between t= 0 and t= 90 min, after which lunch was immediately served.
2.4 DISCUSSION

The combined infusion of 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$ amide led to a statistically significant 32% reduction in ad libitum energy intake during the test meal compared with the control saline visit. This was associated with a small and tolerable rise in nausea ratings – notably only recorded on the written VAS scoresheets and never verbally reported during the infusions by any subject, or severe enough to necessitate discontinuation of the infusion. Single infusions of each of the hormones at these doses appeared to have no effect at all on subsequent energy intake in this small group of healthy volunteers, and also produced no nausea.

A peak mean plasma total PYY concentration of 106.5 pmol/L and total GLP-1 level of 73 pmol/L was achieved during the combined 0.3 pmol/kg/min PYY$_{3-36}$ + 0.8 pmol/kg/min GLP-1$_{7-36}$ amide infusion. In the previously cited paper where combination 0.4 pmol/kg/min PYY$_{3-36}$ + 0.4 pmol/kg/min GLP-1$_{7-36}$ amide were co-infused, the peak circulating PYY and GLP-1 levels reported were 58.4 pmol/L and 62.5 pmol/L respectively (Neary et al., 2005). These discrepancies may be partly due to the fact that the doses used in this study were adjusted for the peptide content of the prepared vials. Furthermore, there is likely to be a degree of inter-assay variation. Circulating plasma hormone levels fell sharply (more so for GLP-1) back towards baseline within 30 minutes of discontinuation of the infusions, adding weight to the decision to serve the test meal immediately after termination of the infusion.
Although limited by small sample size, the results from this dose verification study were reassuring. At the chosen doses of 0.3 pmol/kg/min PYY$^{3-36}$ and 0.8 pmol/kg/min GLP-1$^{7-36}$ amide, single gut hormone infusion was subanorectic, combined gut hormone infusion significantly reduced food intake without inducing serious nausea and the plasma levels achieved were in line with the literature. These doses were taken forward into the functional neuroimaging study described in the next chapter.
CHAPTER 3

Investigation of the Effects of single and Combined Administration of PYY$_{3-36}$ and GLP-1$_{7-36}$ amide on Brain Appetite Centres using fMRI.
3.1 INTRODUCTION

Blood-oxygen-level-dependent (BOLD) functional magnetic resonance imaging (fMRI) has recently been used as a tool to investigate the changes in brain activity associated with differences in nutritional status in humans. It is hoped that a clearer understand of brain appetite pathways will aid the rational design of effective anti-obesity agents. Activity of reward systems in the brain is increased in the fasted state compared to the fed state with presentation of food-relevant stimuli. However, there are only a few reports on the use of fMRI for characterization of human brain activity following the systemic administration of appetite-altering hormones. Intravenous infusion of PYY$_{3-36}$ to human subjects modulates activity in brain regions mediating appetitive behaviour and leads to reduced food intake. However, there have been no human fMRI studies investigating the effects of administration of GLP-1, or co-administration of PYY and GLP-1 on brain activity in humans.

In this work, I have used BOLD fMRI to investigate the changes in brain activity following single or combined administration of PYY$_{3-36}$ and GLP-1$_{7-36}$amide in fasted healthy human subjects and compared the effects to those seen naturally following a meal.

3.2 METHODS

3.2.1 Peptides

The same batch of peptides and corresponding dose calculations were used as for the Dose Verification Study (Section 2.2.1)
3.2.2 Participants

For the imaging study, it was predicted that a sample size of 16 will have 90% power to detect a difference in mean food intake of 950 kJ assuming a standard deviation of differences of 1140, using a paired t test with a 0.05 two sided significance level.

Inclusion and exclusion criteria for the imaging study and the screening protocol were identical to those adhered to for the preliminary Dose Verification Study (described in full detail in Section 2.2.2). A copy of the volunteer information sheet for the study is given in Appendix IV.

3.2.3 Study Protocol for the Gut Hormone fMRI study

As for the dose verification study, volunteers were asked to fast (they were permitted to drink only water) from 22:00h the evening before each study visit. They standardised their diet (by eating the same evening meal at the same time the night before each study visit), abstained from alcohol and avoided strenuous exercise for 24 hours prior to each visit. Study visits were separated by a minimum of three full days.

The fMRI study was conducted in a single-blinded fashion. On their first visit, subjects always received an infusion of saline after an overnight fast. This protocol was identical to that used for the following fasted visits, and was intended to acclimatise the subject to the clinical environment and to experimental procedures. At the start of the acclimatisation visit, the nature of the MRI scan experience was explained to the volunteers. This first scanning session also included an additional structural brain scan (2 minutes duration) to exclude organic disease and to act as an anatomical template for future functional analyses. Functional data from the acclimatisation saline visit are not included in the analysis.
Following the first visit, subjects were randomly assigned to receive each of the following:

1. Overnight fast followed by saline infusion (the fasted, control visit)
2. Overnight fast followed by a set breakfast, then saline infusion (the fed visit)
3. Overnight fast followed by PYY\textsubscript{3-36} infusion at 0.3 pmol/kg/min
4. Overnight fast followed by GLP\textsubscript{1-36 amide} infusion at 0.8 pmol/kg/min
5. Overnight fast followed by combined PYY\textsubscript{3-36} and GLP\textsubscript{1-36 amide} infusion, at 0.3 pmol/kg/min and 0.8 pmol/kg/min respectively

Subjects arrived for each visit at 09:00 h (t= -90 min). Peripheral venous cannulae were immediately inserted in both forearms and subjects could relax and read in a room next to the scanner until the preparation for the study had been completed. At that stage, a 100 cm long extension line was attached to the larger cannula so that blood samples could be taken without disturbing the subject during the scan. During this period all time and food cues were removed from the room.

On their fed visit, subjects received a 730 kcal set breakfast as described in Appendix V, which was served in a private area between 09:30 – 09:50 h (t= -60 to -40 min). At 10:30 h (t= 0 min), the subject was taken into the scanning room and the 90 minute infusion started.

All syringes and lines used to deliver peptide were coated in Gelofusin for twenty minutes as described for the Dose Verification Study. The MEDRAD MR Injector Spectris Solaris EP (Seimens UK, Camberley, Surrey) pump was used to deliver the hormone infusions at a constant rate of 54ml/hr. Vials of peptide were dissolved in 2.5ml vehicle (10% Gelofusin in normal saline) and the amount of peptide added to the delivery syringes varied according to the weight-adjusted dose each subject received.
Six ml blood samples were collected at t= -60, 0, 15, 30, 45, 60, 75, 90 and 120 min and processed and stored as described in Section 2.2.3. Three ml deadspace blood was collected and discarded at each sample point to account for the 100 cm long extension line.

At t= -60, 0, 90, and 120 min, subjects completed a series of 100 mm visual analogue scores (VAS) that rated five food-related sensations (hunger, nausea, pleasantness to eat, how much one could eat, fullness), and four non-food related sensations (sleepiness, irritability, anxiety and warmth) (Appendix I).

The pulse and blood pressure of each subject was measured at t= -60, 0, 15, 30, 60, 75, 90, and 120 min. A drop of blood was taken from the t= -60, 0, 30, 60, 90 and 120 min samples to check blood glucose with an Optimum Exceed blood glucose monitor (Abbott Diabetes Care, Maidenhead, Berks).

Twenty minutes after the start of the infusion (i.e. t= 20 min), the 60 minute fMRI scan began. Subjects lay supine in the scanner, with a specially designed head coil to optimise brain signal acquisition and padding to minimise head movements. Volunteers could verbally communicate with the radiographer, if required, at all times. The subjects were requested to stay awake throughout, and they were asked about their general wellbeing during periodic breaks in image acquisition. At the end of the scan, subjects were helped to sit up slowly, and stayed in the scanner room for a further 10 minutes until the infusion was stopped at t= 90 min. The subject was then immediately led into a nearby private dining area where an *ad libitum* buffet meal (as described in Section 2.2.3) was served. The lunch was provided in excess so that all appetites could be satisfied. Subjects were instructed to “eat until comfortably full”. Subjects were allowed to go home at t = 120 min.

The protocol for the fRMI study is summarised in Figure 3.01.
Figure 3.01 Protocol for Gut Hormone fMRI study

Following an overnight fast, fifteen healthy, normal-weight subjects each received the following interventions, in random order, over 5 separate study days in a single-blinded fashion:

1. 90 minute saline infusion (fasted saline, control visit)
2. Standard breakfast, then 90 minute saline infusion (fed saline)
3. 90 minute PYY$_{3-36}$ infusion at 0.3 pmol/kg/min
4. 90 minute GLP-1$_{7-36}$ infusion at 0.8 pmol/kg/min
5. 90 minute combined PYY$_{3-36}$ and GLP-1$_{7-36}$ infusion, at 0.3 pmol/kg/min and 0.8 pmol/kg/min respectively.

On each visit, subjects underwent a 60 min fMRI scan, which commenced 20 min after the start of the infusion. During the fMRI scan, a picture processing task was performed where images of food and non-food were shown. The mean % change in BOLD signal in pre-specified brain regions of interest when viewing images of food compared to non-food were determined for each study day. An *ad libitum* buffet meal was served immediately after the infusion on all study days in order to measure energy intake. Blood sampling (for PYY and GLP-1) and assessments of appetite (based on visual analogue scores (VAS)) was performed at the time points shown.
3.2.4 BOLD fMRI Food Image Task

During the fMRI scan, subjects were asked to watch and rate a series of colour images. These visual stimuli were viewed via an angled mirror, which reflected images back-projected from a screen positioned in the bore of the magnet behind the subject’s head. There were two sets of 75 images, alternated between each scanning session. The 75 images that were shown during each scan were divided into three classes of 25 exemplars: 25 high-calorie foods, 25 low-calorie foods and 25 non-food items (everyday objects eg watch, chair or scenery) (Figure 3.02). The images appeared in blocks of 5 of the same exemplar, each image shown for 5 seconds, and each 25 second block interspersed with periods of rest, during which time a blue octagon was projected. Images from each category were presented in counterbalanced order across participants for the first three sessions, which was repeated for the final three sessions. Non-food images were chosen as a positive control requiring similar perceptual analysis to food images but without any obvious affective or motivational value. These images were imported from another appetitive fMRI study (Beaver et al., 2006), and revalidation of the salience ratings of these images was not formally repeated in this study group.

The food image task paradigm allows for a subtraction analysis of the fMRI data. In short, certain brain regions will be activated in response to food images more than non-food images. The difference in regional brain activation between viewing images of food or non-food can be studied in different physiological conditions (eg the fasted state, the fed state or in the presence of exogenously administered anorectic hormones). A larger difference entails a greater food-salient activation in that region under that condition.
During the fMRI scan, subjects were asked to watch and rate a series of colour images. The images were divided into three classes of exemplars: high-calorie foods (bottom row), low-calorie foods (middle row) and non-food items (everyday objects eg watch, chair or scenery, top row). Non-food images were chosen as a positive control requiring similar perceptual analysis to food images but without any obvious affective or motivational value. Each image was shown for 5 seconds in blocks of 5 of the same exemplar, interspersed by short periods of rest. 25 images from each category were presented in counterbalanced order across participants for the first three sessions, which was repeated for the final three sessions. In the ROI analysis, mean brain activation in response to presentation of blocks of food minus non-food images was calculated.
3.2.5 fMRI Data Acquisition

T2*-weighted, dual echo, echo-planar images sensitive to BOLD contrast were acquired continuously on a 3T Siemens Tim Trio scanner with a 32-channel head coil (Siemens Healthcare, Erlangen, Germany). Each scan session consisted of 501 volumes of 36-slice acquisition, angled ~30° coronally to the anterior-posterior commissural plane to minimize signal dropout in orbitofrontal and medial temporal regions (TR = 2000 ms; dual TEs = 13 and 31 ms; flip angle = 80°, slice thickness= 3.0 mm, matrix size = 64 × 64, and field of view = 225 × 225 mm² for voxel size of 3.51 × 3.51 × 3.0 mm). High-resolution T1-weighted anatomical scans were acquired with whole-brain coverage (208 slices) for each participant to facilitate fMRI image co-registration, and ROI definition (TR = 3000 ms, TE = 3.66 ms, flip angle = 90°, voxel size = 1 mm³).

3.2.6 fMRI Data Analysis

fMRI data were processed using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of the FSL software package (www.fmrib.ox.ac.uk/fsl/). The following pre-processing was applied: motion correction using MCFLIRT; fieldmap-based EPI unwarping using PRELUDE+FUGUE and non-brain removal using BET (Jenkinson et al., 2002, Jenkinson and Smith, 2001). Data were high-pass filtered and spatially smoothed (5mm Full Width Half Maximum Gaussian kernel) to allow for gyral variability across subjects and to improve signal-to-noise ratio at the intra-subject level. Individual sessions with greater than 3.5mm absolute head motion or severe stimulus-correlated motion (as exhibited by marked rim artefacts in the later intra-subject statistic maps) were excluded from further analysis. The echo planar functional images of each individual were then registered to the high-resolution anatomical (T1-weighted) scan using FLIRT, and overlaid on a standardized brain atlas (Monreal Neurological Institute, Quebec, Canada) in order to allow for comparisons across individuals.
A general linear model (GLM) was applied to detect task-related neural activation. In essence, neural response is modelled by convolution of the haemodynamic response function with a boxcar (food image on/off) stimulus function, and subsequent random effects contrast analysis (high-calorie food vs. non-food, low-calorie food vs. non-food, food vs. non-food). For each voxel, the GLM estimates a parameter, $\beta$, which represents the “goodness-of-fit” of the model at that voxel, relative to noise. The higher the $\beta$, the better the fit and therefore the more likely the measured MRI signal change over time in that voxel correlates with the stimulus. A t-score is then calculated, which for each voxel is $\beta / \text{standard deviation of } \beta$. This is converted to a z-score for whole brain analysis, with conventionally applied thresholds to decide which voxels have $\beta$ scores high enough to qualify as “activations”. Since brain activations tend to happen in clusters of connected voxels, the statistical threshold is actually applied to clusters (ie cluster-corrected).

Thus, the first stage of data analysis was the production of whole-brain z-statistic parametric maps. A statistical threshold of $P<0.001$ uncorrected and cluster extent $>5$ voxels was used, while controlling the family-wise probability of type 1 error at $p<0.05$. The anatomical labelling of activations was checked with reference to neuroanatomical atlases. The overlaid thresholded maps for the fed and fasted visits confirmed which areas of the brain were activated by the food image paradigm (“effect of task”).

At this exploratory stage it was noted that when contrasting BOLD signal between high-calorie food images and low-calorie food images, the effect of task was less pronounced than when contrasting BOLD signal between all food images and non-food images, although similar brain regions were implicated. It was therefore decided that all subsequent ROI analysis be performed contrasting only combined food vs non-food images only.
The *a priori* ROIs chosen for this imaging project [amygdala, caudate, insula, nucleus accumbens (N Acc), orbitofrontal cortex (OFC) and putamen] were based on previous understanding of which parts of the corticolimbic reward network are particularly pertinent to the processing of visual food cues.

All ROIs were defined as the conjunction between the full group main effect of task (food vs. non-food, using parametric testing at the level of spatially contiguous supra-thresholded clusters, while controlling the family-wise probability of type 1 error at p<0.05, corrected – as described above) and the relevant anatomical region on a standard brain atlas.

Using FSL’s ‘featquery’ tool, the mean % BOLD signal change was extracted for each ROI. This is computed by scaling the relevant GLM contrast parameter estimate with the peak-to-peak regressor amplitude and normalising this ‘signal change’ factor with the global image mean. Hence, whilst z-statistics do not convey the amplitude of activation, the extraction of % BOLD signal change allows for the description of how great the stimulus-induced regional activation was. These estimates were averaged over right and left homologous regions, such that the report for each ROI is not lateralised. For each infusion, the null hypothesis tested was that the within-subject difference between fasted saline, fed saline, PYY infusion, GLP-1 infusion or combined PYY + GLP-1 infusion in regional BOLD response to food images vs. non-food images was zero, i.e. [the gut hormone infusion or fed saline BOLD response to food image exposures] – [fasted saline BOLD response to non-food image exposures] = ΔBOLD = 0.
3.2.8 Plasma Hormone Level Analysis

‘Total’ PYY levels (measuring both the active form PYY\textsubscript{3-36}, and its inactive precursor PYY\textsubscript{1-36}) were measured using the in-house RIA described in Section 2.2.4.1 and detailed in Appendix II. A second aliquot was run on a commercial radioimmunoassay (Millipore Corporation, MA, USA) with the following stated specificity: human PYY\textsubscript{3-36} 100% and human PYY\textsubscript{1-36} not detectable. This was in order to measure only ‘active’ PYY levels. Details of the commercial PYY RIA are also given in Appendix II.

‘Total’ amidated GLP-1 levels (that is GLP-1\textsubscript{1-36}amide, GLP-1\textsubscript{7-36}amide and GLP-1\textsubscript{9-36}amide) were measured using the in-house RIA described in Section 2.2.4.2 and detailed in Appendix II. ‘Active’ GLP-1 immunoreactivity was measured using a commercially available ELISA kit (Millipore Corporation, MA, USA), with the following specificities: GLP-1\textsubscript{7-36}amide 100%, GLP-1\textsubscript{7-37} 72%, GLP-1\textsubscript{1-36} OR GLP-1\textsubscript{1-37} <2%. Details of the commercial GLP-1 ELISA kit are reproduced in Appendix II.
3.2.9 Statistical Analysis

Combined data are represented as the mean ± SEM. Comparisons of energy intake and ratings of meal palatability were by repeated measures ANOVA with Tukey’s multiple comparison post-test and Dunnett’s comparison post-test against the fasted saline visit. VAS scores were adjusted for baseline and differences between t= 0 min and t= 90 min compared by repeated measures non-parametric Friedman’s test with Dunn’s multiple comparison post-test. Linear regression analysis was performed to assess correlation between nausea and energy intake over the whole study. Comparisons of plasma gut hormone levels during the infusion period (plasma samples from t=15 - 90 min inclusive) were by repeated measures ANOVA with Tukey’s multiple comparison post-test. The threshold for statistical significance in each case was set at p<0.05. Analyses were performed using Prism version 5.01 software (Graphpad Software, San Diego, CA, USA).

The statistical model for the fMRI study was a repeated measures analysis of difference of means, between control (fasted saline) and each infusion session BOLD data. ROI analysis was performed using repeated measures ANOVA with Tukey’s multiple comparison post-test and Dunnett’s comparison post-test against the fasted saline visit. Simple linear regression analysis was used to examine the relationship (Pearson correlation coefficient, r) between ROI BOLD signal change and VAS ratings, plasma gut hormone levels and energy intake at lunch. The threshold for statistical significance in each case was set at p<0.05. Analyses were performed outside FSL, using Prism version 5.01 software (Graphpad Software, San Diego, CA, USA).
3.3 RESULTS

3.3.1 Participants

16 healthy right-handed subjects (11 male, 5 female, mean age 29.5 years, range 21-36 years, mean body mass index 22.1 kg/m$^2$, range 18.3-25.1 kg/m$^2$) were recruited and completed all 6 scanning sessions. At the analysis stage, one male subject was excluded due to excessive head movement that caused gross image distortion, resulting in a complete dataset from 15 subjects for analysis.

3.3.2 Energy Intake

3.3.2.1 Energy Intake During Acclimatisation Visits

Participants consumed significantly less energy at lunch on their initial acclimatisation visit compared with the subsequent, randomised fasted control visit, despite identical study protocols: [Energy intake at ad libitum buffet lunch (kJ): fasted control, 4039 ± 399 vs. acclimatisation fasted saline, 3715 ± 419, p<0.05].

3.3.2.2 Energy Intake During fMRI Study

Consumption of the standard breakfast by subjects before saline infusion (fed visit) led to a 23.5% ± 8.3% reduction in energy intake during the subsequent ad libitum buffet lunch, compared to when they received only saline infusion (fasted control visit): [Energy intake at ad libitum buffet lunch (kJ): fasted control, 4039 ± 399 vs. fed, 3090 ± 335, p<0.01]. Infusion of PYY$_{3-36}$, GLP-1$_{7-36}$amide or combined PYY$_{3-36}$ and GLP-1$_{7-36}$amide to fasted subjects resulted in 12.3% ± 11.2%, 15.7% ± 7.5% and 27.0% ± 9.4% reductions in energy intake, respectively, compared to the fasted control visit: [Energy intake at ad libitum buffet lunch (kJ): fasted control 4039 ± 399; PYY$_{3-36}$ 3542 ± 452; GLP-1$_{7-36}$amide 3407 ± 302; PYY$_{3-36}$ + GLP-1$_{7-36}$amide 2950 ± 378]. The reduction in energy intake was significantly lower (p<0.001) relative to fasted saline only for the combined administration of hormone (Figure 3.03).
The additive effects of single infusions of PYY$_{3-36}$ and GLP-1$_{7-36}$amide and the measured effects of the combined infusion of PYY$_{3-36}$ and GLP-1$_{7-36}$amide on energy intake at the *ad libitum* lunch were comparable (Figure 3.04).

### 3.3.2.3 Order Effect on *Ad Libitum* Energy Intake Across Visits

The same lunch was provided at every study visit. The caloric value of the food served at this buffet lunch was approximately 3000 kCal, but more food was available if required. None of the subjects consumed all of the food initially provided.

Analysis of the mean energy intake (of the 15 subjects) during the *ad libitum* lunch across the 5 randomised visits is shown in Figure 3.05. Repeated measures ANOVA with Tukey’s post-test confirmed no evidence of habituation to the study meal.
Following an overnight fast, fifteen healthy, normal-weight subjects each received the following interventions, in random order, over 5 separate study days in a single-blinded fashion:

1. 90 minute saline infusion (fasted saline, control visit)
2. Standard breakfast, then 90 minute saline infusion (fed saline)
3. 90 minute PYY$^{3-36}$ infusion at 0.3 pmol/kg/min
4. 90 minute GLP-1$_{7-36}$ infusion at 0.8 pmol/kg/min
5. 90 minute combined PYY$_{3-36}$ and GLP-1$_{7-36}$ infusion, at 0.3 pmol/kg/min and 0.8 pmol/kg/min respectively.

An *ad libitum* buffet meal was served immediately after the infusion in order to measure energy intake on all study days. This graph shows the energy intake in kilojoules (kJ) during the buffet meal following each infusion. Data shown as mean ± SEM. ** denotes p<0.01 vs. fasted saline. *** denotes p=0.0001 vs. fasted saline.

Figure 3.03 *Ad Libitum* Lunch Energy Intake in fMRI Study by Condition
Figure 3.04 Reduction in *Ad Libitum* Lunch Energy Intake in fMRI Study for Each Condition Compared with Fasted Saline.

This shows the reduction in energy intake in kilojoules (kJ) during the buffet lunch for each condition against the fasted saline (control) infusion. The arithmetic sum of the effects of individual infusions of PYY and GLP-1 (sum of PYY and GLP-1) are shown in the chequered box against the actual measured effect of the combined infusion (PYY + GLP-1) at reducing food intake.
Following an overnight fast, fifteen healthy, normal-weight subjects each received the following interventions, in random order, over 5 separate study days in a single-blinded fashion:

1. 90 minute saline infusion (fasted saline, control visit)
2. Standard breakfast, then 90 minute saline infusion (fed saline)
3. 90 minute PYY\textsubscript{3-36} infusion at 0.3 pmol/kg/min
4. 90 minute GLP-1\textsubscript{7-36} infusion at 0.8 pmol/kg/min
5. 90 minute combined PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36} infusion, at 0.3 pmol/kg/min and 0.8 pmol/kg/min respectively.

This graph shows the mean energy intake of the 15 subjects during the ad libitum lunch across the 5 randomised visits, by visit number not condition. Results shown as mean +/- SEM. Repeated measures ANOVA with Tukey’s post-test did not reveal any evidence that subjects ate progressively less during each subsequent visit as a result of habituation to the study meal.
3.3.3 VAS Results

Visual analogue scores (VAS) by subjects confirmed that consumption of the standard breakfast significantly reduced subjective ratings of hunger, perceived pleasantness of eating more food and anticipated future consumption, as well as increasing a sense of “fullness”. Similarly, in fasted subjects, combined infusion of PYY$_{3-36}$ and GLP-1$_{7-36}$amide led to a small but significant reduction in hunger, perceived pleasantness of eating and anticipated food consumption, as well as increased “fullness” (Figure 3.06 A-D).

Eleven out of the fifteen subjects also reported mild nausea with the combined infusion of PYY$_{3-36}$ and GLP-1$_{7-36}$amide (Figure 3.07 A), but there was no correlation between nausea ratings during the infusion and subsequent energy intake during the ad libitum buffet meal (p= 0.23, r$^2$ = 0.02, Figure 3.07 B). No subject verbally reported the nausea during the scan or withdrew from the study as a result of it. By contrast, the single infusions of PYY$_{3-36}$ or GLP-1$_{7-36}$amide were not associated with any significant changes in subjective feelings of hunger, perceived pleasantness of eating, anticipated food consumption, “fullness” or nausea (Figure 3.06 A-D and 3.07 A).

There was no difference in food palatability ratings across all visits, as judged by the VAS score for “How tasty was the meal” taken immediately after lunch (Figure 3.08). Non-food related VAS scores (sleepiness, irritability, anxiety and warmth) did not alter significantly during any study (Figure 3.09 A-D).
Figure 3.06  Analysis of Food Related Visual Analogue Score (VAS) Parameters from t=-60 to t=90 min During each Infusion for the Gut Hormone fMRI Study.
During each study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger (A), pleasantness to eat (B), anticipated food consumption (C), and fullness (D) The change in VAS scores from baseline (t=-60 min) to the end of the infusion (t=90 min) are shown. Data are shown as mean ± SEM. * denotes p=0.03 for PYY+GLP-1 vs. Fed saline. † denotes p=0.02 for PYY+GLP-1 vs. Fed saline. ‡ denotes p=0.007 for PYY+GLP-1 vs. Fed saline. § denotes p=0.0002 for PYY+GLP-1 vs. Fed saline and p<0.01 for PYY+GLP-1 vs. Fasted saline and PYY. p=ns for Fasted saline vs. Fed saline.
Figure 3.07  Analysis of Nausea Related Visual Analogue Score (VAS) Parameters from t=-60 to t=90 min During each Infusion for the Gut Hormone fMRI Study.

During each study subjects completed 100 mm visual analogue scale questionnaires relating to nausea (A). The change in VAS scores from baseline (t=-60 min) to the end of the infusion (t= 90 min) are shown. * denotes p<0.0001 for PYY+GLP-1 vs. all other groups. Across all infusions, there was no correlation between nausea and energy intake (p=0.23, r² = 0.02) (B).
An *ad libitum* buffet meal was served immediately after the infusion at the end of every study visit. Subjects retrospectively rated its palatability on a visual analogue scale of 0-100 in response to the question “How tasty was the meal”. Results shown as mean +/- SEM. Repeated measures ANOVA with Tukey’s post-test did not reveal any evidence of taste aversion.

Figure 3.08 Meal Palatability Rating Following *Ad Libitum* Lunch in Gut Hormone fMRI Study.
Figure 3.09 Analysis of Non-Food Related Visual Analogue Score (VAS) Parameters from t=-60 to t=90 min during each Infusion for the Gut Hormone fMRI Study.

During each study, subjects completed 100 mm visual analogue scale questionnaires relating to warmth (A), irritability (B), anxiety (C), and sleepiness (D) The change in VAS scores from baseline (t=-60 min) to the end of the infusion (t= 90 min) are shown. Data are shown as mean ± SEM.
3.3.4 Physiological Measurements

There were no physiologically significant or sustained changes in systolic and diastolic blood pressure, pulse or blood glucose after hormone or placebo administration (Figure 3.10 A-D). Blood glucose measurements at t=0 (ie at the start of the saline infusion) were non-significantly higher on the fed visit compared with on the fasted visit (mean blood glucose (mmol/L): fasted control visit, 5.49 +/- 0.09 vs fed visit 6.05 +/- 0.28), a difference which became even less pronounced by t=30 min.

No significant adverse events were experienced by any of the subjects.
During each study, regular recordings of blood pressure (BP) and pulse rate were taken. At each blood sampling point, a drop of blood was taken to record the subjects’ blood glucose reading on a blood glucose monitor (D). Data are shown as mean ± SEM.
3.3.5 Plasma PYY and GLP-1 Levels Measured in the fMRI study

3.3.5.1 Plasma Levels of Total PYY and Total (Amidated) GLP-1

The in-house RIA measured total plasma levels of PYY (that is, PYY$_{3-36}$ and PYY$_{1-36}$) and total plasma levels of amidated GLP-1 (that is, GLP-1$_{1-36amide}$, GLP-1$_{7-36amide}$ and GLP-1$_{9-36amide}$) over the course of the study. These are shown in Figure 3.11.

PYY$_{3-36}$ infusion increased total plasma PYY levels to a peak of 78.8 +/- 6.7 pmol/L on the PYY$_{3-36}$ infusion day and 93.5 +/- 8.4 pmol/L on the PYY$_{3-36}$ and GLP-1$_{7-36amide}$ infusion day. There was no significant difference between total plasma concentrations of PYY achieved when PYY$_{3-36}$ was infused singly or in combination with GLP-1$_{7-36amide}$. There was no significant difference between mean total plasma concentrations of PYY during saline or GLP-1$_{7-36amide}$ infusions (Figure 3.11 A).

Analogously, GLP-1$_{7-36amide}$ infusion resulted in an increase in total amidated plasma GLP-1 levels to a peak of 47.2 +/- 5.2 pmol/L on the GLP-1$_{7-36amide}$ infusion day and 51.2 +/- 5.0 pmol/L on the PYY$_{3-36}$ and GLP-1$_{7-36amide}$ infusion day. There was no significant difference between mean total plasma concentrations of GLP-1 achieved when GLP-1$_{7-36amide}$ was infused singly or in combination with PYY$_{3-36}$. There was no significant difference between mean total plasma concentrations of GLP-1 during the saline or PYY$_{3-36}$ infusions (Figure 3.11 B).
Figure 3.11 Plasma Levels of Total PYY and Total (Amidated) GLP-1 in the fMRI Study

On each study visit, the hormone infusion was administered between t= 0 and t= 90 min. Figure 3.11 A shows plasma total PYY levels (that is, PYY$_{3-36}$ and PYY$_{1-36}$). Figure 3.11 B shows plasma total amidated GLP-1 levels (that is, GLP-1$_{1-36}$amide, GLP-1$_{7-36}$amide and GLP-1$_{9-36}$amide) over the course of the study. Data shown as mean +/- SEM.
3.3.5.2 Plasma Levels of Active PYY and Active GLP-1

In order to confirm that the fMRI signal changes were related to active circulating hormone and that the total plasma levels measured on the in-house RIAs described above were not primarily immunoreactive but biologically inactive breakdown products, a second aliquot of plasma from each subject was tested for active forms of both PYY and GLP-1.

The commercial PYY RIA measured only the active circulating form of the hormone (that is, PYY\textsubscript{3-36}). These results are shown in Figure 3.12 A. Peak circulating levels of active PYY reached 59.8 +/- 3.7 pmol/L on the PYY\textsubscript{3-36} infusion day and 63.6 +/- 2.7 pmol/L on the PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} infusion day. There was no significant difference between these peaks. There was also no significant difference between mean active PYY plasma concentrations during saline or GLP-1\textsubscript{7-36amide} infusions.

The commercial GLP-1 ELISA measured only active forms of the hormone (that is, GLP-1\textsubscript{7-36amide} and GLP-1\textsubscript{7-37}). GLP-1\textsubscript{7-36amide} infusion resulted in peak active GLP-1 levels of 13.2 +/- 2.6 pmol/L on the GLP-1\textsubscript{7-36amide} infusion day and 14.5 +/- 2.0 pmol/L on the PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} infusion day. There was no significant difference between these peaks. There was also no significant difference between mean active GLP-1 plasma concentrations during saline or PYY\textsubscript{3-36} infusions (Figure 3.12 B).
Figure 3.12 Plasma levels of Active PYY and Active GLP-1 in the fMRI Study

On each study visit, the hormone infusion was administered between t= 0 and t= 90 min. Figure 3.12 A shows plasma active PYY levels (that is, PYY$_{3-36}$ only). Figure 3.12 B shows plasma active GLP-1 levels (that is, GLP-1$_{1-36}$amide and GLP-1$_{7-37}$) over the course of the study. Data shown as mean +/- SEM.
3.3.6  fMRI Analysis

3.3.6.1 Whole Brain Activation with Presentation of Food-Salient Visual Stimuli (Effect of Task Analysis).

Overlaid whole-brain maps of brain regions activated by food images (compared with non-food images) for the fasted saline and fed saline visits, thresholded at \( z > 2.3 \) and \( p < 0.05 \), are shown in Figure 3.13. A list of the anatomically defined task-activated areas is given in Table 3.1. All of the \textit{a priori} chosen ROIs showed significant food-salient activation on this analysis, and are highlighted at the end of Table 3.1.

It was not possible to demonstrate task-responsive signal changes in either the brainstem or hypothalamus, despite the following efforts to improve the probability of acquiring interpretable data (not shown):

- fieldmap-based EPI unwarping during the image pre-processing phase,
- re-analysing the MRI data weighted towards the first echo time (13 ms) rather than the second echo (31 ms), as for the rest of the image analysis,
- reducing the statistical thresholding to \( z>1.6 \) on the effect of task maps.
Figure 3.13  Thresholded Effect of Task Maps

During each infusion, a BOLD fMRI scan was performed, incorporating a picture processing task where images of food and non-food were shown. For the fasted saline and fed saline infusions, a whole brain map of brain regions activated (z-statistics colour coded on the right-hand bar) by food images (compared with non-food images) is shown. Z indicates distance (mm) superior or inferior to the intercommissural plane in standard stereotactic space. Clusters were thresholded by means of parametric testing at the level of spatially contiguous supra-thresholded clusters, simultaneously controlling the family-wise probability of type 1 error at p<0.05, corrected.
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**Table 3.1:** Local Maxima where Δ BOLD (when subjects viewed images of food compared with when they viewed images of non-food) was Significantly Greater than Zero.

Imaging measures (cluster peaks meeting a criterion of p<0.05, z >2.3) for saline infusion (in the fed and fasted state) are shown. For each area, the MNI (Montreal National Institute) atlas co-ordinate where peak activation occurred is listed. The *a priori* ROIs are highlighted in bold in the second half of the table.
3.3.6.2 Amalgamated Region of Interest % BOLD Signal Change (food images minus non-food images) by Intervention.

The effects of feeding, PYY$_{3-36}$, GLP-1$_{7-36}$amide and combined PYY$_{3-36}$ + GLP-1$_{7-36}$amide on changes in mean % BOLD signal in the total brain volume represented by the amalgamated 6 a priori selected ROIs is shown in Figure 3.14. When fasted subjects (during the saline, control visit) viewed images of food compared with when they viewed images of non-food, there was significantly greater MRI signal in all of the ROIs tested (Table 3.1). This % BOLD signal change for food minus non-food images was significantly attenuated (p<0.001) after administration of PYY$_{3-36}$ or after co-administration of PYY$_{3-36}$ + GLP-1$_{7-36}$amide. There was a non-significant reduction in the mean % BOLD signal change in the amalgamated ROIs following feeding or after GLP-1$_{7-36}$amide administration compared to fasted control.
Figure 3.14 Amalgamated Region of Interest % BOLD Signal Change (food images minus non-food images) by Intervention.

The mean % BOLD signal change (food images minus non-food images) across all 6 ROIs studied (amygdala, insula, caudate, nucleus accumbens, orbitofrontal cortex and putamen), is shown for each of the interventions. Data are shown as mean ± SEM. ** p<0.01, *** p<0.001.
3.3.6.3 Individual Region of Interest Analysis by Intervention.

The mean % change in BOLD signal in subjects after feeding, PYY\textsubscript{3-36}, GLP-1\textsubscript{7-36amide} and combined PYY\textsubscript{3-36} + GLP-1\textsubscript{7-36amide} infusion across the individual \textit{a priori} selected ROIs (left and right hemisphere data combined) is shown in Figure 3.15. Following correction for multiple comparisons, only the insula showed a statistically significant reduction in signal compared to the fasted, saline visit for the fed, PYY\textsubscript{3-36} and combined PYY\textsubscript{3-36} + GLP-1\textsubscript{7-36amide} conditions. However, there was general pattern of a reduction in BOLD signal for all conditions in all ROIs against the fasted control visit. Tables 3.2 (a-f) detail peak activations for each of the interventions (feeding, PYY\textsubscript{3-36} infusion, GLP-1\textsubscript{7-36amide} and PYY\textsubscript{3-36}+GLP-1\textsubscript{7-36amide}) for each ROI.

The reduction in mean % BOLD signal change with the combined infusion of PYY and GLP-1 was similar to the arithmetically summed reduction in mean % BOLD signal changes after individual administrations of the two hormones (Figure 3.16).
Figure 3.15 Modulation of BOLD Signal Across Brain ROIs by Feeding or Either Individual or Combined Gut Hormone Infusions.

Fifteen healthy subjects underwent a 90 minute infusion of saline (Fasted saline) as a control. They also had 4 further infusions - saline after a standard breakfast (Figure 3.14 A), PYY after an overnight fast (Figure 3.14 B), GLP-1 after an overnight fast (Figure 3.14 C) and combined PYY + GLP-1 after an overnight fast (Figure 3.14 D). During each infusion, a BOLD fMRI scan was performed, incorporating a picture processing task where images of food and non-food were shown. The mean % BOLD signal change when subjects viewed images of food compared with when they viewed images of non-food is shown for each of the infusions administered as a comparison with the fasted saline infusion (blue bars). Data are shown for individual ROIs (amygdala, insula, caudate, nucleus accumbens, orbitofrontal cortex and putamen), combined for left and right hemispheres and grouped for all 15 subjects, shown as mean ± SEM. * denotes p=0.015 for Fed saline < Fasted saline and p=0.012 for PYY+GLP-1 < Fasted saline. ** denotes p=0.005 for PYY < Fasted saline.
Tables 3.2 (a-f)  Peak Activation Coordinates for Each Comparator Condition by Region of Interest

Imaging measures (cluster peaks meeting a criterion of p<0.05, z >1.6) for all treatment contrasts in each ROI are shown. Cluster peaks where there was greatest difference in mean % change in BOLD signal (when viewing images of food versus non-food) in subjects after feeding, PYY$_{3-36}$, GLP-1$_{7-36}$ amide or combined PYY$_{3-36}$ & GLP-1$_{7-36}$ amide infusion are given for each comparator condition. Where there was no significant ΔBOLD, n/a is inserted. MNI (Montreal National Institute brain atlas) co-ordinates are shown in millimetres. The number of contiguous voxels exceeding threshold activation is given in the first column (voxels) and the z-score (Max z) for that cluster of activation given in the end column.

Table 3.2a

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Table 3.2b

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Figure 3.16 Summed Reduction in Mean % BOLD Signal Change in ROIs with Individual Administration of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide}, Compared with the Reduction in Mean % BOLD Signal Change Measured after Combined Administration of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide}.

The reduction in mean % BOLD signal change when subjects viewed images of food compared with when they viewed images of non-food compared with Fasted saline is expressed as a sum of the effects of individual PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} infusion (lilac bars). This is compared with the actual measured reduction in mean % BOLD signal change compared with Fasted saline for the combination infusion (purple bars). Data are shown for individual ROIs (amygdala, insula, caudate, nucleus accumbens, orbitofrontal cortex and putamen).
3.3.6.4 Regression Analysis of ROI MRI Signal with VAS Scores, Gut Hormone Levels and Energy Intake.

There was no correlation between changes in nausea ratings on the VAS scores over the time course of the infusion and the change in BOLD signal for any of the interventions (fasting, feeding, PYY_{3-36} infusion, GLP-1_{7-36} amide and PYY_{3-36}+GLP-1_{7-36} amide) in any of the 6 ROIs. This was analysed by plotting each subject’s change in nausea VAS score (t=90 mins minus t=0 mins) against their % change in BOLD signal for each ROI and every experimental condition.

There was no correlation between changes in plasma gut hormone levels and the change in BOLD signal for any of the interventions (fasting, feeding, PYY_{3-36} infusion, GLP-1_{7-36} amide and PYY_{3-36}+GLP-1_{7-36} amide) in any of the 6 ROIs. This was analysed by plotting each subject’s area under the curve (AUC) for total and active hormone levels over the course of the infusion against their % change in BOLD signal for each ROI and every experimental condition.

There was no correlation between change in BOLD signal for any of the interventions (fasting, feeding, PYY_{3-36} infusion, GLP-1_{7-36} amide and PYY_{3-36}+GLP-1_{7-36} amide) in any of the 6 ROIs and subsequent energy intake at the buffet lunch. This was analysed by plotting each subject’s *ad libitum* energy intake at lunch against their % change in BOLD signal for each ROI and every experimental condition.
3.4 DISCUSSION

3.4.1 The Effects of Combination PYY$_{3-36}$ and GLP-1$_{7-36}$amide Infusion on Energy Intake in Humans

When analysing the effects of gut hormone infusion on appetite, it is always important to consider two potential confounders before making conclusions about the intervention on energy intake. The first is anxiety, which has been shown to be increased (alongside increased stress hormone levels) on initial study visits (Chandarana et al., 2009). Acute stress can cause an acute anorectic response via inhibition of hypothalamic NPY signalling, an effect which masked many early attempts to recreate the anorectic effects of exogenously administered PYY$_{3-36}$ (Abbott et al., 2006). In support of this, the average energy intake on the acclimatisation visit in this study was 8% lower than that on the fasted saline (control) visit, despite an identical protocol.

It is also important to consider an order effect on food intake across visits, with the possibility of subjects being progressively less enticed by the meal, resulting in lower energy intake at each subsequent visit. Following the initial acclimatisation visit, the remaining visits in this study were randomised in terms of the infusion administered (fasted saline, fed saline, PYY$_{3-36}$, GLP-1$_{7-36}$amide, PYY$_{3-36}$ + GLP-1$_{7-36}$amide). Therefore, one would expect an even distribution of individual interventions across all five study visits, which would counterbalance any possible order effects on average energy intake. Reassuringly, statistical analysis of the mean energy intake (of the 15 subjects) during the ad libitum lunch across the five randomised visits (using a repeated measures ANOVA), revealed no evidence of such an order effect.

This study demonstrated a 27% reduction in ad libitum energy intake during a buffet lunch following co-administration of 0.3 pmol/kg/min PYY$_{3-36}$ and 0.8 pmol/kg/min GLP-1$_{7-36}$amide over 90 minutes, compared with saline-treated controls. Single infusions of these doses of PYY$_{3-36}$ and GLP-1$_{7-36}$amide produce smaller reductions in ad
libitum energy intake. The arithmetically summed effect of the single hormones was similar to the reduction in energy intake measured following combined infusion. Thus, whilst it is not possible to make firm conclusions about whether the effects of dual hormone infusion within this dose range are additive (or, indeed, synergistic), co-administration of PYY$_{3-36}$ and GLP-1$_{7-36}$amide did not appear to lead to loss of effect of either individual hormone.

The doses initially chosen for this study were based on previously reported studies, as described in Chapter 2. However, variations in the technical details of infusion administration results in considerable differences between studies in plasma concentrations and biological effects for a given dose. This is also partly a reflection of the very narrow therapeutic window in which gut hormones are able to exert an anorectic effect without nausea. The earlier dose verification study was not designed to produce exhaustive dose-response information for each of the hormones alone or in combination, but rather to validate the informed choice of doses to safely take forward in the scanning stage.

With this limited information however, it is still possible to suggest that co-administration of low doses of PYY$_{3-36}$ and GLP-1$_{7-36}$amide can avoid the nausea that would result from an equivalently anorectic dose of either hormone alone. It follows that combination treatment may offer greater efficacy than treatment with a single agent. Since PYY$_{3-36}$ is a selective agonist at the Y2 receptor, and GLP-1$_{7-36}$amide at the GLP-1 receptor, it is reasonable to predict that each hormone may have a separate effect on central appetite-regulatory areas. This would allow a significant anorectic effect to occur without elevating plasma concentrations of either hormone to levels sufficient to produce nausea. In this study, the combined gut hormone infusion did produce a significant increase in overall nausea scores by the end of the infusion compared to saline. However, in all cases the nausea was mild and tolerable. In other studies, larger doses of PYY$_{3-36}$ producing comparable reductions in food intake as achieved here with low dose combination infusion, are reported to induce severe nausea and vomiting (Sloth et al., 2007). By comparison, the consumption of a large (but in no circumstance nauseating) breakfast in this study resulted in a 23%
reduction in food intake at lunch. This physiological satiety effect is mediated by the slow, sustained secretion of numerous gut hormones at much lower concentrations than achieved with experimental infusions.

Total hormone concentrations are more commonly reported in the literature and allow for more meaningful comparison with existing relevant data. In this study, combined infusion of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} resulted in a mean total PYY concentration of 70 pmol/l and a mean total GLP-1 concentration of 43 pmol/l. The circulating levels of PYY and GLP-1 following breakfast were 33 pmol/l and 22 pmol/l respectively. In fact, the levels achieved with combined infusion in this study are much more comparable to concentrations measured in patients after bariatric surgery. Le Roux et al. found a mean 90 min post-prandial total PYY level of 40 pmol/l and a mean 30 min post-prandial total GLP-1 level of 47 pmol/l in post-RYGB patients (Le Roux et al., 2006a). Therefore, although circulating levels achieved in this study may be regarded as supraphysiological within the normal population, they may be of greater functional relevance when considering the key role of PYY and GLP-1 in patients who have undergone gastric bypass surgery. This is important when taking into consideration the view that PYY and GLP-1 are implicated in mediating reduced appetite and promoting weight loss following gastric bypass.

### 3.4.2 The Effects of Combination PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} Infusion on Subjective Appetite Ratings

VAS scores provided by each subject revealed that consumption of the standard breakfast significantly reduced subjective ratings of hunger, perceived pleasantness of eating more food and anticipated future consumption, as well as increasing a sense of “fullness” compared to the fasted state. The same subjects when fasted but receiving combined infusion of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36 amide} reported smaller but still significant reductions in hunger, perceived pleasantness of eating and anticipated food consumption, as well as increased “fullness” compared to the fasted saline control visit. These findings support previous reports that nutritional state impacts on the appeal and pleasantness of food (Uher et al., 2006, Stoeckel et al., 2007).
They also provide novel evidence that combination gut hormone infusion can mimic this phenomenon. It has also been previously suggested that the palatability of food is reduced in the fed compared with the fasted state (Berridge, 1991). However, retrospective rating of how tasty the lunch was in this study, revealed that whilst dual gut hormone infusion mirrored the appetite-reducing effects of physiological satiety (i.e. the fed state), it did not produce taste aversion.

Overall, these findings add support to an anti-obesity pharmacotherapy based on the combination of PYY$_{3-36}$ and GLP-1$_{7-36}$amide, which is likely to be efficacious and well-tolerated.

3.4.3 The Effects of Combination PYY$_{3-36}$ and GLP-1$_{7-36}$amide Infusion on Haemodynamic and Glycaemic Parameters.

Throughout every study visit, regular checks of blood pressure and blood glucose were recorded. This was primarily to ascertain patient well-being but also for the following specific considerations:

1. GLP-1 is an incretin hormone, capable of stimulating glucose-induced insulin secretion (Kreymann et al., 1987, Kim and Egan, 2008). Since the exogenous infusion of the hormone was given only to subjects in the fasted state, it was not anticipated that it would incur any significant reduction in plasma glucose concentrations during the study, and this was confirmed on the data analysis.

2. PP-fold peptides, particularly NPY acting via vascular smooth muscle Y1 receptors, have long been known to exert vasoconstrictive effects (Wahlestedt and Reis, 1993). Y2 receptors have also been shown to more modestly potentiate angiotensin II–mediated renal vasoconstriction (Dubinion et al., 2006). There were no effects of exogenous PYY$_{3-36}$ infusion
on blood pressure and pulse during this study, in concurrence with other studies infusing near-physiological concentrations of the hormone (Batterham et al., 2002, Field et al., 2010).

3.4.4 The Effects of Combination PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} Infusion on Brain Appetite Pathways in Humans as Studied using fMRI

For the first time in humans, this study has shown that combined infusion of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36amide} results in a similar modulation of brain activity as observed following a meal. This is consistent with expectations that these hormones are important physiological mediators of post-prandial satiety. In general, both the fed state and the administration of anorectic gut hormones to fasted subjects, reduces activation in brain reward regions in response to visual food-cues. Numerous cross-sectional studies have causally linked lack of breakfast consumption with increased daily fat intake, increased general consumption of high fat snacks and greater weight gain (Rampersaud et al., 2005, Masheb and Grilo, 2006, Timlin et al., 2008). Others have reported that fasting leads to impulsive eating and weight re-gain in obese individuals on weight-reduction programmes (Schlundt et al., 1992). The neuroimaging findings here suggest that fasting heightens brain reward system activity in response to food cues, and that this can be dampened by feeding or the administration of anorectic gut hormones.

Following rigorous statistical correction, the insula cortex was the brain area most significantly modulated by consumption of a meal and also by gut hormone infusion. This is much to do with the fact that the insula is the largest of the ROIs studied, situated at the interface of homeostatic and cognitive systems and involved in gauging the salience of external stimuli and initiating appropriate behavioural reactions. Less robust, yet reproducible reductions in food-cue invoked activation compared to the fasting state were also seen in all of the other ROIs following meal consumption or gut hormone infusion, with the sole exception of the amygdala. In this study, the physiological fed state appeared to make the amygdala more
responsive to food cues compared to when hungry, contrary to the findings of the earliest fed/fasted fMRI studies (LaBar et al., 2001, Morris and Dolan, 2001). This may be the result of a statistical aberration, since with all gut hormone infusions food-invoked activation of the amygdala was attenuated as would be expected.

A comparison of the arithmetically summed results of single infusions of PYY<sub>3-36</sub> and GLP-1<sub>7-36</sub>amide with the measured results from the combined infusion revealed no significant difference in the effect on % BOLD signal change in any ROI. This mirrored the food intake analysis, which also suggested that the combined effects of the hormones were not counteractive. However, only in the amygdala did the combined infusion produce less of a reduction in neuronal activity than the summed effect of the single hormones. This observation did not reach statistical significance, and is likely to be due to normal inter-subject variation. However, it is interesting to consider that these very slight aberrations in the behaviour of the amygdala may be representing the fact that it is playing a role in detecting some of the aversive (that is, nauseating) effects of gut hormone infusion. On the other hand, a formal regression analysis did not show a significant correlation with amygdala activity and the (narrow range of) subjective nausea scores reported by the subjects. This study was not designed to investigate the central mechanisms by which gut hormones switch from being anorectic to nauseating, although this clearly remains an area of great interest to those developing tolerable yet maximally effective pharmacotherapies.

When PYY<sub>3-36</sub> alone was infused in the fasted state, the pattern of signal change in brain reward ROIs was similar to that observed after feeding, with consistent reductions in mean % BOLD signal change across all brain reward ROIs. Reductions in signal change were most prominent (relative to the fed state) in the insula, nucleus accumbens and the OFC, consistent with a previous fMRI study of the effects of single infusion of PYY<sub>3-36</sub>. As previously mentioned, PP-fold peptides have been reported as having vasoconstrictive effects, albeit largely mediated via the Y<sub>1</sub> receptor (to which PYY<sub>3-36</sub> does not bind strongly) and not demonstrated in cerebral vasculature. Nevertheless, the potential of any hormonal or even nutritional...
manipulation to non-specifically alter BOLD response should always be considered. Previous studies have shown that caffeine reduces cerebral perfusion and increases BOLD signal intensity in response to visual stimulation (Laurienti et al., 2002). No such effects have been reported for PYY$_{3-36}$. In fact, in this study, PYY$_{3-36}$ (in line with the fed state) comparatively reduced BOLD signal intensity in all of the ROIs examined and did not produce any difference in activation pattern in the visual (occipital) cortex.

Infusion of GLP-1$_{7-36}$amide alone led to consistent reductions in mean % BOLD signal change compared with fasted saline across all of the selected ROIs, in a pattern that most closely resembled the fed state. Consistent and perhaps more profound reductions in mean % BOLD signal change, relative to infusion of saline alone, across all of the selected ROIs were also observed following combination infusion of PYY$_{3-36}$ and GLP-1$_{7-36}$amide. As before, the insula appeared particularly sensitive to hormonal modulation.

The lack of any obvious differential activation pattern between PYY$_{3-36}$ and GLP-1$_{7-36}$amide within the ROIs I studied here raises the question whether these hormones may be acting at the level of higher reward centres via a final common pathway. It is feasible that, having stimulated Y2 and GLP-1 receptors respectively in the brainstem and/or hypothalamus, circulating PYY$_{3-36}$ and GLP-1$_{7-36}$amide have no direct effect on deeper (corticolimbic) brain structures and that the activation patterns seen represent input by upward projections from the hypothalamus. In this, and most other fMRI studies utilising pictures of food, no effect of task activation was found in the hypothalamus. This is most likely due to the technical limitations of hypothalamic imaging using BOLD-based techniques, which are discussed in full detail in the next section. However, there are some other lines of evidence, to suggest that circulating gut hormones may directly influence reward pathways. In rodents and humans both the Y2 and GLP-1 receptors are expressed in corticolimbic regions, although less densely than in the brainstem and hypothalamus (Gehlert et al., 1996, Stanic et al., 2006, Merchenthaler et al., 1999). In rats, peripheral exendin-4 (a potent and long-lasting GLP-1 mimetic) attenuates motivated (reward-seeking)
behaviour, an effect which is abolished by stereotactic injection of GLP-1 receptor antagonists into the NAcc (Dickson et al., 2012). In other rodent studies, leptin has been shown to bind to amygdala-projecting ventral tegmental area dopamine neurons (the ventral tegmentum or VTA is another corticolimbic reward structure) (Leshan et al., 2010), and intra-VTA injection of ghrelin increases feeding (Skibicka et al., 2011). It also remains untested as to whether, by directly modulating corticolimbic dopaminergic transmission, gut hormones have an effect on the pleasure perception of other rewards. If they do, they may have a potential role in the treatment of drug addiction. If their role is exclusively to modulate the perception of food cues, then the negative side effects on mood which have hampered other anti-obesity agents will not be an issue for gut-hormone based therapies.

3.4.5 Limitations of fMRI

It is pertinent to mention here that there are some inherent limitations when using BOLD-based fMRI to study homeostatic areas. The brainstem is difficult to visualise due to its proximity to large blood vessels and tendency to oscillatory image degradation with the cardiac cycle. The hypothalamus has proven even more elusive due to its small size (approximately 5-8 mm in diameter) and its situation adjacent to the frontal skull sinuses. $T_2^*$-weighted protocols, like BOLD fMRI, are particularly susceptible to signal loss as a result external magnetic field distortions around these air-tissue boundaries (Ojemann et al., 1997). Furthermore, using the hungry, fasted state as an example, the net hypothalamic signal resulting from the activation of orexigenic nuclei and inhibition of anorexigenic nuclei may be very small, as opposing intrahypothalamic signals beyond the spatial resolution of the scanner cancel each other out. It is also possible that the hypothalamus is not responsive to visual cues per se such that even if it were possible to image it, the visual-task paradigm is not an appropriate tool for eliciting differential hypothalamic activation. Thus, the current BOLD fMRI task-based paradigm is limited to the detailed analysis only of higher brain areas. Even in this setting, although fMRI has provided novel
evidence that corticolimbic reward regions are modulated by circulating factors, there remains uncertainty as to which of these structures are directly responsive to the effects of gut hormones and which activations are correlates (as a result of spreading network activation) or consequences (e.g. motor functions related to the food-seeking response to reward).

From the outset of this project, it was opted to perform an a priori ROI analysis of the fMRI data. The major reason for this was to control for Type I error by limiting the number of statistical tests to a few ROIs. The thresholded effect-of-task map in the exploratory analysis was compiled only from the fed and fasted scans. This provided information about which parts of the brain are activated specifically in response to food-image cues in physiological settings. The previously selected ROIs were reassuringly all confirmed as task-responsive. However, it is possible that areas of the brain which are not food responsive in the fed or fasted state, but become so when supraphysiological doses of gut hormones are administered, were missed. Similarly, by looking at the modulatory effects of gut hormone infusion on food-responsive brain activation only in these pre-selected ROIs, it is not possible to conclude the brain-wide effects of PYY$^{3-36}$ and GLP-1$^{7-36}$amide.
CHAPTER 4

GENERAL DISCUSSION
4.1 Summary of the Effects of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36} amide on Appetite and Brain Reward Pathways Reported in this Thesis.

This study has confirmed that co-administration of 0.3 pmol/kg/min PYY\textsubscript{3-36} and 0.8 pmol/kg/min GLP-1\textsubscript{7-36}amide over 90 minutes to subjects fasted overnight, results in similar reductions in *ad libitum* energy intake during a subsequent buffet lunch as in subjects who have been fed a large breakfast. Single infusions of these doses of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36}amide produce smaller reductions in *ad libitum* energy intake. The arithmetically summed effect of the single hormones was similar to the reduction in energy intake measured following combined infusion.

For the first time in humans, this study has also shown that, in conjunction with a comparable effect on appetite and energy intake, combined infusion of PYY\textsubscript{3-36} and GLP-1\textsubscript{7-36}amide results in a similar modulation of brain activity as observed following a meal. Both the fed state and the administration of anorectic gut hormones to fasted subjects, reduces activation in multiple brain reward regions in response to visual food-cues. Simplistically, anorectic gut hormones make food seem less appealing. In all instances (except for the amygdala in the fed state), either feeding or the administration of anorectic gut hormones to fasted individual, resulted in a reduction in neuronal activity in response to visual food cues in all of the reward ROIs studied. The effects of combined gut hormone infusion on brain activation patterns are not significantly different from their arithmetically combined effects when administered alone. Of the *a priori* chosen ROIs, the insula was most significantly modulated by either feeding or gut hormone infusion. There was no obvious differential activation pattern between any of the conditions studied, but a suggestion that the OFC and NAcc are particularly sensitive to the effects of PYY\textsubscript{3-36}.

Since the direct central effects of circulating gut hormones in humans can only be studied using functional imaging, it is useful to compare the findings in this work with other fMRI studies that have explored the neuroendocrinology of appetite. At this point, it is important to point out that direct comparison between studies is not always possible, due to differences in study design. The separate effects of
methodological and physiological factors on between-study variations have yet to be elucidated. A recent meta-analysis reported that concurrence between studies on the brain response to viewing pictures of food was moderate: at best 41% (seven out of seventeen studies) concurred significantly activated clusters for the contrast between food and non-food (van der Laan et al., 2011). fMRI relies on the measurement of small signal changes amongst large inter-subject differences. Appetite pathways and the reward system in general are intricately tuned by numerous genetic, developmental, psychological, environmental and hormonal influences. For example, in the study reported in this thesis, it was not possible to elicit differential neuronal activation patterns between high and low calorie foods. This may be because the food images used in this study were imported from another study into the effects of personality on reward drive (Beaver et al., 2006), and were not separately validated (in terms of palatability ratings) for this specific set of volunteers. With this caveat, the following sections discuss some of the findings of this project in the context of fMRI studies which have investigated the effects of nutritional status, appetite-altering hormones, obesity and reward system responsiveness in patients pre- and post- gastric bypass.

4.2. Comparison with fMRI Studies of Hunger and Satiety

The earliest imaging studies into appetite confirmed the notion that, in humans, images of food can trigger the brain’s reward system and that the motivational potency of this trigger is greater in the fasted state. Such differences in activation patterns have variously been attributed to a number of physiological differences between the fed and fasted state, such as changes in concentrations of gut hormones (PYY, GLP-1, ghrelin, CCK and insulin) and vagal afferents conveying information about gut distension, although none have formally correlated them.

In 2001, LaBar et al reported on 9 healthy subjects, who underwent an initial whole-brain fMRI after an 8 hour fast, followed by another post-meal scan one hour later. A follow up study was performed on 8 subjects who were fed a meal before the first of
the two scans, to rule out the potential of habituation effects in the first group. The subjects were presented with food and non-food images during each scan and subtraction analysis was performed (LaBar et al., 2001). The authors’ hypothesis that variation in the state of hunger would modulate the response of the amygdala and anatomically related corticolimbic structures was borne out in a ROI analysis, which revealed satiety-induced reduction in activity in the amygdala, parahippocampal gyrus and fusiform gyrus. They suggested that the amygdala, with its extensive neural connections with the hypothalamus and higher brain centres, was in a pivotal position for integrating response to visual food stimuli in the context of nutritional status. However, they also noted that their study was under-powered to detect similar satiety-induced changes in many other ROIs studied. As previously mentioned, the findings of amygdala activation in this thesis differ from those reported elsewhere, a likely consequence of normal population variation.

In 2003, Killgore et al used a different approach by scanning satiated recruits who were presented with pictures of high calorie, low calorie and non-food items. Irrespective of calorific content, food images caused greater activation than non-food images in the amygdala, hippocampus and ventromedial prefrontal cortex (Killgore et al., 2003). The authors of that paper pointed out the importance of these areas in the expectation and evaluation of reward. High-calorie foods caused particular activation of the medial and dorsolateral prefrontal cortex, understood to be involved in evaluating stimulus relevance within the current affective state of the individual. In contrast, low calorie foods resulted in lesser activation of these reward areas per se, and greater activation in somatosensory areas, with a suggestion from the authors that this was due to a lesser cephalic phase response by classical conditioning to images of less appealing foods. In 2005, Killgore published a reanalysis of this study data, using an ROI approach (specifically, a sub-analysis of the OFC) and correlated BOLD activity with BMI. It was reported that for the high calorie minus non-food contrast, there was a significant negative correlation between BMI and BOLD signal in the OFC (Killgore and Yurgelun-Todd, 2005). In other words, as BMI increases, activity in the OFC becomes less food-responsive. It was not possible
to extend these findings to the obese population, since the subjects studied were all within the normal weight range.

In 2005 St-Onge et al published the only fMRI study to date where fasted individuals were exposed to four different stimuli: visual food, visual non-food, tactile food and tactile non-food. In an uncorrected whole brain analysis they reported that the anterior cingulate, superior temporal gyrus, hippocampus and insula were significantly activated to a greater extent during the presentation of foods (whether seen or felt) over non-food items (St-Onge et al., 2005).

In 2006, Porubska et al published the results of an fMRI study of 12 normal-weight fasted subjects. Visual food stimuli (in contrast to non-food images) activated the insular and orbitofrontal cortices, with a positive modulation of insula activity induced by subjective ratings of appetite (Porubska et al., 2006). Following on from this, Fuhrer et al in 2008 studied 12 healthy male volunteers undergoing two separate scanning sessions – one when fasted overnight and the other immediately after a large meal. They performed a whole brain, uncorrected analysis of the data and particularly noted significantly enhanced activity within the OFC when hungry (Fuhrer et al., 2008). In 2009, Schur et al performed a study of ten normal weight, fed subjects viewing images of fattening food, non-fattening food and non-food items. These food images were specifically chosen based on whether the food was perceived to be compatible with an effort to lose weight (another example of marked variation in experimental design). In a ROI analysis, this was the first study to report increased hypothalamic activation when viewing pictures of fattening food compared with non-food items, although this finding did not extend to other comparisons (ie fattening vs non-fattening or all food vs non-food). They also found increased activation in the amygdala, insula and OFC when viewing fattening foods compared with non-food items (Schur et al., 2009). Also in 2009, Siep et al. reported increased activity in the amygdala and OFC in response to viewing high calorie versus low calorie food images, but only when their subjects were fasted (Siep et al., 2009). Their experimental protocol allowed for the further observation that this increased
activity in the amygdala and OFC was only evident when participants explicitly evaluated foods but not when their concentration was diverted elsewhere.

4.3 Comparison with fMRI Studies of Other Appetite-Modulating Hormones.

It is perhaps most appropriate to interpret the results presented in this thesis alongside those of similar human fMRI studies investigating neuronal effects of appetite-modulating hormones. Such studies have also tended to measure BOLD signal change when viewing food-related images in the presence or absence of the hormone being studied. There is general concurrence that circulating appetite-altering hormones affect reward processing of food cues, with the directionality of neuronal effects dependent on whether the hormone under scrutiny is anorectic or orexigenic.

In two congenitally leptin deficient human subjects, daily subcutaneous leptin replacement reduced BOLD fMRI activation when viewing food vs. non-food images in the nucleus accumbens, caudate, putamen and globus pallidus (Farooqi et al., 2007). These findings have been recently extended to healthy subjects, in whom there is positive correlation between plasma leptin concentration and fMRI BOLD response in ventral striatal regions during the presentation of visual food cues (Grosshans et al., 2012). Similar activation changes following infusion of PYY_{3-36} and GLP-1_{7-36amide} have been shown in this project. However, there were also notable differences, with PYY_{3-36} and GLP-1_{7-36amide} apparently having much greater effects on the insula and OFC. This implies that leptin, compared with PYY_{3-36} and GLP-1_{7-36amide}, modulates distinct neural networks, which may be related to the longer-term anorectic signalling by the adipokine leptin, relative to the more acute anorectic effects of post-prandially released gut hormones.
There has been one earlier human fMRI investigation of the effects of PYY$_{3-36}$. Batterham et al. showed that in fasted, lean, healthy individuals, energy intake at lunch was predicted by the modulation of OFC signal following PYY$_{3-36}$ infusion, but not with saline infusion, when subsequent food intake was better correlated with changes in hypothalamic signal (Batterham et al., 2007). It was postulated that the presence of PYY$_{3-36}$ switches regulation of food intake from a homeostatic brain region (hypothalamus) to a hedonic one (OFC). However, differences in methodology used (Batterham et al. tested for changes in resting state BOLD fMRI, compared with the task-activated BOLD fMRI data presented here) confound direct comparisons between the studies. Nevertheless, the particular sensitivity of the OFC to PYY in both studies is striking. Other fMRI studies have also demonstrated that compared to low-calorie food pictures, viewing high-calorie food stimuli induces a greater activation in the OFC (Killgore et al., 2003, Siep et al., 2009, Frank et al., 2010) and that the change in appeal bias towards high-calorie foods is positively correlated with OFC activation (Goldstone et al., 2009). An early meta-analysis of neuroimaging data has shown that activity in the OFC is related to the monitoring and learning of reward value of reinforcers (Kringelbach and Rolls, 2004). The latest research has suggested that the OFC can be divided into specific functional subregions. For example, the reward value of a reinforcer is assigned in more lateral parts of the OFC, where it can be modulated by hunger and other internal states (van der Laan et al., 2011).

The best direct comparison can be made with a recent study which investigated the effects of ghrelin, the only known gut hormone which acutely increases food intake, on brain activity in normal-weight humans (Malik et al., 2008). Intravenous ghrelin infusion increased food-image invoked BOLD activation in the amygdala, OFC, insula, visual areas and striatum. The hypothalamus was not reported. Those results are directly mirrored by the negative modulatory effects of PYY$_{3-36}$ and GLP-1$_{7-36}$amide reported here, suggestive of a CNS reward network, which when activated by ghrelin, mediates hunger and when inhibited by the anorectic gut hormones PYY$_{3-36}$ and GLP-1$_{7-36}$amide, mediates satiety.
4.4 Comparison with fMRI Studies of Appetite Pathways in Obesity.

The majority of fMRI studies in overweight volunteers have sought to investigate evidence of aberrant brain appetite signalling with increasing BMI, although it is frequently impossible to tell whether these changes are the cause or effect of obesity. Psychologists have pondered the question of obesity as a consequence of “food addiction” for many years. One concept of addiction holds hypo-dopaminergic functioning (Reward Deficiency Syndrome - RDS) in brain reward pathways as a key risk factor (Blum et al., 2000). As such, food is used as a form of self-medication to boost hedonic capacity. In line with this, nuclear imaging studies have revealed a reduction in striatal dopamine receptor density in overweight subjects (Wang et al., 2001, Volkow et al., 2008). However, tolerance to reward is well known to cause down regulation of dopamine receptors in brain reward regions, and a competing theory is that individuals at risk of obesity actually have a hyper-reactive reward system to start with. These individuals initially experience greater reward from food intake, which drives overeating that subsequently results in a tachyphylaxis of dopamine signalling in response to food intake (Stice et al., 2011b).

In general, fMRI studies investigating the differential reward system activation patterns when looking at high calorie foods between obese and normal weight subjects have shown significantly greater activation in the obese group in several brain regions implicated in food reward. In 2007, Rothemund et al studied 13 obese and 13 normal weight women who had been fasted for at least 90 minutes. Increasing BMI positively predicted BOLD activation of the dorsal striatum (caudate / putamen), anterior insula, claustrum, posterior cingulate, post-central cortex and lateral OFC. This specifically pertained to the contrast between viewing high calorie food images versus non-food images (Rothemund et al., 2007). A further ROI-based study by Stoeckel et al in 12 obese and 12 normal weight women demonstrated that pictures of high calorie foods (versus non-food) produced greater activation in the obese group compared with controls in several brain regions, including the ventral and dorsal striatum, insula, anterior cingulate cortex, amygdala, OFC, hippocampus,
ventral pallidum and medial prefrontal cortex (Stoeckel et al., 2008). A later functional connectivity analysis of the same scans indicated that obese women displayed a relative deficiency in the amgdala’s modulation of the OFC and ventral striatum, along with excessive modulation of the ventral striatum by the OFC (Stoeckel et al., 2009). In 2012, Scharmuller et al confirmed that obese participants showed greater insula activation than the lean participants during the passive viewing of food cues. Moreover, the obese group displayed stronger dorsolateral prefrontal cortex involvement when attempting to attenuate food-elicited craving (Scharmuller et al., 2012). Collectively, these results support enhanced food cue reactivity as playing a role in the pathophysiology of obesity.

Other studies have shown that obese individuals respond differently to satiation compared with normal weight individuals. A ROI-based study by Martin et al measured fMRI BOLD activation pre-and post prandially (following a 500 kCal meal) in response to pictures of food (a combination of high calorie and low calorie images) and non-food in 10 obese and 10 healthy weight adults. During the pre-meal condition, obese subjects showed increased activation in the anterior cingulate and medial prefrontal cortex, compared to healthy weight controls. During the post-meal condition, obese participants also showed greater BOLD activation in the medial prefrontal cortex compared with healthy weight controls (Martin et al., 2010). A recent study by Dimitropoulos et al of 22 overweight / obese males and 16 normal weight males showed that before eating, obese subjects showed greater response to food images (versus non-food images) compared with normal weight subjects in the anterior prefrontal regions. Post-prandially, the obese group demonstrated increased response to all food images (versus non-food) compared with normal weight individuals in frontal, temporal and limbic regions. Specific greater activation to high calorie foods was seen in the obese group compared with normal weight individuals in the lateral OFC, caudate and anterior cingulate cortex (Dimitropoulos et al., 2012).
In 2008, Stice et al used fRMI to compare brain activity in 7 obese and 11 lean adolescent girls during the anticipated receipt of chocolate milkshake, during actual receipt of the milkshake, during anticipated receipt of a tasteless control solution and during actual receipt of the tasteless control solution. In response to anticipated receipt and actual receipt of the chocolate milkshake (versus the tasteless solution), obese adolescent girls showed greater BOLD activation bilaterally in the anterior and mid insula, frontal operculum, parietal operculum and rolandic operculum compared with lean adolescent girls. However, the obese girls displayed lower BOLD activation in the caudate in response to actual consumption of the milkshake (versus the tasteless solution) compared with the lean girls. From these results, the authors suggested that obese individuals show greater salience-associated responses from anticipated food consumption in gustatory and somatosensory regions, but weaker activation in the caudate during actual consumption compared to lean individuals (Stice et al., 2008). It was postulated that this reflects increased anticipatory food reward but reduced consummatory food reward in obesity, which may contribute to overeating. In a later study by the same group, 8 women who gained > 2.5% increase in BMI over a 6 month period showed reduced striatal BOLD fMRI response to chocolate milkshake consumption (versus a tasteless control solution) compared with 12 women who demonstrated stable weight (Stice et al., 2010). However, in their most recent study, it was reported that normal-weight adolescents at high-risk versus low-risk for future obesity showed greater activation in the dorsal striatum in response to palatable food receipt (Stice et al., 2011a). These results reinforce the consensus hypothesis that both reward centre hyper-reactivity and a subsequently reduced sensitivity of striatal reward circuitry (probably due to the down regulation of dopamine D2 receptors as a result of overeating), contribute to the pathophysiology of obesity.
4.5 Comparison with fMRI Studies Pre- and Post- Gastric Bypass Surgery

As previously mentioned, the plasma gut hormone concentrations achieved in this study of 0.3 pmol/kg/min PYY\textsubscript{3-36} and 0.8 pmol/kg/min GLP-1\textsubscript{7-36amide} were comparable to those measured post-prandially in patients who have undergone gastric bypass surgery.

The mechanisms behind the weight loss seen following Roux-en-Y bypass surgery remain to be fully elucidated, but are thought to be due to a number of additional neurohormonal causes (Tadross and le Roux, 2009). These include elevated post-operative levels of endogenous anorectic gut hormones such as PYY and GLP-1 (le Roux et al., 2006a), increases in energy expenditure and metabolic rate (Nestoridi et al., 2012), changes in taste preference away from calorie dense foods (Miras et al., 2012), increased bile acid delivery to the ileum (Patti et al., 2009) and changes in gut microbiota (Li et al., 2011). Recent studies imaging appetite in patients before and after weight loss are important in helping us to understand some of the CNS mediated effects, with the potential translational application of one day being able to identify those patients who will respond best to bypass surgery.

In 2011 van de Sande-Lee et al. performed fMRI studies on 8 lean control subjects and 13 obese patients pre- and post- Roux-en-Y gastric bypass, by which an average 30% reduction in body weight had been achieved (van de Sande-Lee et al., 2011). Using a resting state BOLD fMRI paradigm focusing on a mid-saggital slice only, they reported a high level of functional connectivity between the hypothalamus and the orbitofrontal and somatosensory cortices that was absent in obese subjects, but re-established with post-operative weight loss.

In the same year, a ROI-based study of 10 obese female patients 1 month pre- and post- Roux-en-Y gastric bypass, revealed an attenuation of fMRI BOLD signal (when viewing high-calorie versus low-calorie food images) in the ventral tegmental area, ventral striatum, putamen, lentiform nucleus, posterior cingulate and dorsomedial
prefrontal cortex following the bypass procedure (Ochner et al., 2011). A subsequent analysis of the same cohort of pre- and post- Roux-en-Y gastric bypass patients revealed that of postoperative changes in mesolimbic reward pathway response predicted changes in the desire to eat (wanting), but not liking, for high-versus low-calorie foods (Ochner et al., 2012). Although circulating gut hormone levels were not measured, the authors speculated that post-surgical reductions in the orexigenic hormone ghrelin could have explained their imaging observations. The concurrence with the findings of the work in this thesis, suggest that elevated PYY$_{3-36}$ and GLP-1$_{7-36}$amide levels may also be implicated.

4.6 Future Work

4.6.1 Clarifying the Effect of Stress on Appetite Pathways as Measured by fMRI

In this project, each subject underwent an initial acclimatisation visit, with a scanning protocol identical to that of the subsequent saline control visit. This was performed because acute stress is well documented as having an anorectic effect of its own in appetite studies and, accordingly, data from this first visit was not included in the analysis presented in this thesis.

The exclusion of food intake data taken from the first visit in (non-imaging) appetite studies is now standard. However, it is less common for fMRI studies in other fields to incorporate an acclimatisation visit. This is particularly important when considering the hypothalamus as a ROI, since its paraventricular nucleus plays a major role in generating the adaptive autonomic, behavioural, and hormonal responses to stress (Sawchenko et al., 1996). More generally, stress may also have a non-specific effect on fMRI results, as acute stress has been shown to cause changes in haematocrit (Patterson et al., 1995), which affects the source of the BOLD signal. In addition, the effect of blood sampling on cortisol concentrations, a measure of stress, has been shown to be highly variable (Meeren et al., 1993), which suggests that the effects of stress on the fMRI response will vary between subjects too.
To clarify some of these issues, it will be interesting to analyse the acclimatisation visit data. By comparing the acclimatisation visit activation patterns against those from the proper control saline visit, alongside plasma cortisol values, it will be possible to elicit novel information about the effects of acute stress on activity in brain appetite pathways.

4.6.2 Analysing Resting State BOLD and Arterial Spin Labelling Data

During the 60 minute scanning session performed in this dual gut hormone infusion study, three scanning protocols were applied, of which only the task-based BOLD results have been fully analysed and reported here. An additional resting state BOLD scan and arterial spin labelling (ASL) scan were also performed at every visit. The datasets from these additional scans are currently being analysed and will hopefully continue to provide new information about the central mode of action of PYY and GLP-1 as follows:

1. The resting-state BOLD analysis will provide information about the functional connectivity of specific brain areas in the absence of any particular external stimulus or food cue. Since there is no effect of task analysis required for resting state scans, it is hoped that hypothalamic signal may be interpretable. It is anticipated that the functional connectivity of ROIs that are known to be important in food processing will be altered or modulated in the presence of anorectic hormones.

2. The ASL protocol does not rely on the BOLD effect at all, but rather is an imaging process which magnetically “tags” protons (with a short RF pulse) as they are travelling in the neck arteries, and follows their course into ROIs within the brain. This allows for a quantification of cerebral blood flow, with the assumption that greater flow occurs in activated areas. Should the lack of hypothalamic signal be related to technical limitations of T2*-weighted BOLD imaging, then it is anticipated that a T1-weighted ASL protocol will overcome these (Wong et al., 1997).
4.6.3 Investigating the CNS-Mediated Mechanisms of Weight Loss and Metabolic Improvement Following Bariatric Surgery

An exciting extension of this project will be to further investigate the mechanism of action of gastric bypass surgery. As previously mentioned, the strongest hypothesis at present is that the majority of its positive effects on appetite, energy and expenditure and glycaemic control are as the result of enhanced anorectic gut hormone profiles following the procedure. By comparing groups who have either lost weight via surgery, combined chronic gut hormone therapy or caloric restriction alone, fMRI provides the best means of investigating how the modulation of CNS appetite pathways mediates outcome.

4.7 Concluding Comment

In this thesis, I have characterised the effects of single and combined administration of \( \text{PYY}_{3-36} \) and \( \text{GLP-1}_{7-36 \text{ amide}} \) on brain BOLD fMRI activations in humans and shown for the first time that these hormones modulate brain reward centre activity to an extent and pattern similar to that observed physiologically after a meal. These findings provide direct evidence that the concerted action of gut hormones in the brain could explain post-prandial satiety. It therefore seems plausible that the administration of combined anorectic gut hormones may provide an effective future medical alternative to a surgical gastric bypass procedure to treat obesity. Meanwhile, it remains to be fully elucidated whether the corticolimbic effects of gut hormones are direct or secondary to hypothalamic projection, and many other questions about the brain-wide and dose-response effects of anorectic gut hormones remain unanswered. As imaging technologies continue to evolve, it seems likely that future fMRI studies will continue to help unravel the complicated neuroendocrine network involved in human appetite control.
APPENDICES
APPENDIX 1 – VISUAL ANALOGUE SCALES

How hungry do you feel right now?

NOT AT ALL ——————————————————— EXTREMELY

How nauseous do you feel right now?

NOT AT ALL ——————————————————— EXTREMELY

How pleasant would it be to eat right now?

NOT AT ALL ——————————————————— EXTREMELY

How much do you think you could eat right now?

NOTHING ——————————————————— A LARGE AMOUNT

How full do you feel right now?

NOT AT ALL ——————————————————— EXTREMELY
How tasty was the meal?

NOT AT ALL               EXTREMELY
How warm do you feel right now?

VERY COLD ———— VERY HOT

How irritable do you feel right now?

NOT AT ALL ———— EXTREMELY

How anxious do you feel right now?

NOT AT ALL ———— EXTREMELY

How sleepy do you feel right now?

NOT AT ALL ———— EXTREMELY
All in-house radioimmunoassays (RIAs) used to measure ‘total’ PYY and ‘total amidated’ GLP-1 plasma levels were derived and maintained by Professor MA Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College). All reagents and materials other than peptides were supplied by Sigma.

General Principles of a Radioimmunoassay

RIA is a technique that allows the detection and measurement of biological substances at very low concentrations from tissue or plasma samples. This method relies on the competition between radioactively-labelled and unlabelled antigen for a specific number of antibody binding sites. When the unlabelled antigen in standards or 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 RIA is incubated for at least 72 hours at 4°C in order for this reaction to reach equilibrium, according to the equation:

\[ *Ag + Ab + Ag \times AgAb + AgAb \]

*Ag = unlabelled antigen
*Ag = radiolabelled antigen
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, as for the in-house total amidated GLP-1 assay) or using a primary-secondary antibody complex (free label is contained in the supernatant following centrifugation, as for the in-house total PYY assay). After separation, the bound and free components are measured in a γ-counter and the data used to construct a standard curve. By reference to the standard curve, the concentration of the unknown samples can be calculated.

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. Samples of plasma with known spiked quantities of peptide (low, medium and high concentration) are also added for quality control. The general structure of the RIA is outlined in the table below, which shows the content of the tubes according to their designation.
<table>
<thead>
<tr>
<th>TUBE NUMBER</th>
<th>DESIGNATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>3-4</td>
<td>½ X</td>
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<tr>
<td>5-6</td>
<td>2 X</td>
</tr>
<tr>
<td>7-8</td>
<td>Zero</td>
</tr>
<tr>
<td>9-10</td>
<td>Zero</td>
</tr>
<tr>
<td>11-12</td>
<td>Standard 1</td>
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<tr>
<td>13-14</td>
<td>Standard 2</td>
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<td>15-16</td>
<td>Standard 3</td>
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<td>Standard 9</td>
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<td>29-30</td>
<td>Standard 10</td>
</tr>
<tr>
<td>31-34</td>
<td>Zero</td>
</tr>
<tr>
<td>35-...</td>
<td>Samples</td>
</tr>
<tr>
<td>Zeros</td>
<td>Two zeros between each new subject/visit</td>
</tr>
<tr>
<td>Standard curve</td>
<td></td>
</tr>
<tr>
<td>Final two tubes</td>
<td>Excess</td>
</tr>
</tbody>
</table>

Quality control is essential in RIA. The following tubes are important for the assessment of the quality for the label, antibody and overall 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.
Detailed Methods for Preparation of the Iodinated Radioactive Labelled Tracers

All tracers were made in house by Prof M. Ghatei (Dept. of Investigative Medicine, Imperial College London). Peptides were iodinated using the iodogen method (Wood et al., 1981) and purified by HPLC using a C₁₈ column (Waters, Milford, CT, USA). In brief, the iodogen method adds a $^{125}$I molecule onto a tyrosine residue in an oxidative reaction. Iodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; Pierce Chemical Co., Rockford, IL, USA) is reacted with Na$^{125}$I to oxidise the $^{125}$I to the reactive iodine state, which subsequently incorporates into available tyrosine residues. The iodogen method requires the reaction (peptide in 0.2 M phosphate buffer, 37 MBq Na$^{125}$I, 23 nmol iodogen reagent) to be incubated at 22°C before the reaction products are purified by reverse-phase HPLC using a NovaPak C₁₈ column (Millipore, Milford, MA, USA). The column was equilibrated for 10 minutes with 15% (v/v) acetonitrile (AcN) solution in water containing 0.05% (v/v) trifluoroacetic acid (TFA), at a flow rate of 1 ml/min. The concentration of AcN was then gradually increased to 20-45% over the following 80 minutes and maintained at the final concentration of 45% for the next 10 minutes. Fractions were collected every 1.5 minutes into tubes containing 1 ml 20 mM HEPES, pH 11.0 (to neutralise the acidity of the collected fractions) and 0.3% (v/v) bovine serum albumin (BSA) (ICN Biochemicals Inc, Costa Mesa, CA, USA). The fractions were tested in an RIA and used at 1500 cpm/tube.

Details of the In-House ‘Total’ PYY Assay

Plasma PYY$_{3-36}$ was measured using a specific and sensitive RIA. The assay was performed using 0.06 M phosphate buffer with 0.3% BSA at a total volume of 700μl per tube and a sample volume of 1–100μl. $^{125}$I-PYY was prepared by the iodogen method (detailed above) and purified by HPLC. The antiserum (Y21) was raised in rabbits against synthetic porcine PYY$_{1-36}$ (Bachem Ltd. U.K), coupled to BSA, and used at a final dilution of 1:50,000. The Y21 antibody is specific for the C-terminal of PYY and reacts fully with human PYY$_{1-36}$ and PYY$_{3-36}$ and does not cross-react with PP, NPY or other known gut hormones. A Standard concentration of 1pmol/ml was used for PYY$_{3-36}$. The intra- and inter-assay coefficients of variation were 6% and 10% respectively.

Details of the In-House ‘Total Amidated’ GLP-1 Assay

GLP-1- like immunoreactivity was measured by a specific and sensitive in-house radioimmunoassay. The antibody was produced in rabbits against GLP-1 coupled to bovine serum albumin. The antibody cross reacted 100% with all amidated forms of GLP-1 but did not cross react with glycine extended forms [GLP-1$_{1-37}$ and GLP-1$_{7-37}$] or any other known pancreatic or gastrointestinal peptide. $^{125}$I-GLP-1 was prepared by the iodogen method (detailed above) and purified by HPLC. The specific activity of the $^{125}$I GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.7 ml of 0.06 M sodium barbitone buffer (pH 8) containing 0.3 % BSA. The assay was incubated for three days at 4°C before separation of the free and antibody bound label by charcoal absorption. The limit of detection was 7.5 pmol/l with an intra-assay variation of 5.4% and inter-assay variation less than 10%.
Commercial Assays Used

In order to ensure that the signal changes detected on BOLD fMRI were related to changes in the concentration of circulating active hormone levels, a second aliquot of plasma was tested on commercial kits to measure active PYY (ie PYY\textsubscript{3-36}) and active GLP-1 (ie GLP-1\textsubscript{7-36amide} and GLP-1\textsubscript{7-37}) only.

These kits were acquired from Merck Millipore (Billerica, MA, USA). Weblinks are provided for access to the manufacturer’s full guide, and a shortened summary of the product characteristics are reproduced below.

**MILLIPORE HUMAN PYY\textsubscript{3-36} SPECIFIC RIA KIT**

http://www.millipore.com/userguides/tech1/proto_pyy-67hk

**I. INTENDED USE**

Millipore’s PYY (3-36) Radioimmunoassay (RIA) Kit utilizes an antibody, which recognizes only the 3-36 form of Human PYY (3-36). Sensitivity of 20 pg/mL can easily be achieved when using a 100μl serum or plasma sample in a two-day, disequilibrium assay.

**II. PRINCIPLES OF PROCEDURE**

In radioimmunoassay, a fixed concentration of labelled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 40%-50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labelled tracer and unlabelled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabelled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labelled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The Millipore PYY (3-36) assay utilizes 125I-labeled PYY and a PYY (3-36) antiserum to determine the level of PYY (3-36) in serum, plasma or tissue culture media by the double antibody/PEG technique.
MILLIPORE Glucagon-Like Peptide-1 (Active) ELISA Kit

http://www.millipore.com/userguides/tech1/proto_eglp-35k

I. INTENDED USE
This kit is for non-radioactive quantification of biologically active forms of Glucagon-Like Peptide-1 [i.e. GLP-1 (7-36 amide) and GLP-1 (7-37)] in plasma and other biological media. It is highly specific for the immunologic measurement of active GLP-1 and will not detect other forms of GLP-1 (e.g., 1-36 amide, 1-37, 9-36 amide, or 9-37). The GLP-1 sequence is highly conserved between the species, with no sequence variation occurring at all in mammals.

II. PRINCIPLES OF PROCEDURE
This assay is based, sequentially, on: 1) capture of active GLP-1 from sample by a monoclonal antibody, immobilized in the wells of a microwell plate, that binds specifically to the N-terminal region of active GLP-1 molecule, 2) washing to remove unbound materials, 3) binding of an anti GLP-1-alkaline phosphatase detection conjugate to the immobilized GLP-1, 4) washing off unbound conjugate, and 5) quantification of bound detection conjugate by adding MUP (methyl umbelliferyl phosphate) which in the presence of alkaline phosphatase forms the fluorescent product umbelliferone. Since the amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1.
APPENDIX III – MOUSE HISTOPATHOLOGY REPORTS FOR PYY AND GLP-1 TOXICOLOGY STUDY

Toxicity studies were performed using the prepared batches of PYY_{3-36} and GLP-1_{7-36} amide, at doses greater than 10 times the maximum intended for humans (in pmol/kg body weight), administered by IP injection to 20 mice, alongside a saline-administered control group. The mice were observed for evidence of abnormal behaviour for 4 hours post injection. Half the animals in each group were sacrificed by CO_{2} asphyxiation after 48 hours, and the other half after 21 days. The lungs, heart, kidneys, liver, brain and reproductive organs of both sexes were dissected, fixed and sent for examination by an independent histopathologist, blinded to treatment group.

GROUP 1 – SALINE TREATED

1. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

2. Normal lungs, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

3. Normal lungs, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

4. Normal lungs, fragment of normal thymus gland, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

5. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

6. Normal lungs, normal trachea, normal oesophagus, normal heart, the brain has prominence of both lateral ventricles although the third ventricle is not dilated. This may be an incidental finding. There is no cellular reaction. The choroid plexus appears normal. Normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

7. Normal lungs, normal trachea, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. There may be subtle ventricular dilatation in the brain although preparation is suboptimal.
8. Normal lungs, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. There is possible mild bilateral ventricular dilatation.

9. Normal lungs, normal trachea, normal oesophagus, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. There is possible ventricular dilatation although orientation is not optimal and the lateral ventricles were not well visualised.

10. Normal lungs, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

GROUP 2 - PYY\textsubscript{3-36} TREATED

11. Normal lungs with normal hilar lymph node, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

12. Normal lungs, normal trachea with normal thyroid gland, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

13. Normal lungs, normal thymus gland, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

14. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

15. Normal lungs and normal hilar lymph node, normal trachea, normal oesophagus, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. The brain may have slight assymmetric ventricular dilatation although the fourth ventricle appears relatively normal.

16. Normal lungs and normal hilar lymph node, normal trachea, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

17. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.
18. Normal lungs, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. There appears to be mild bilateral ventricular dilatation.

19. Normal lungs with normal hilar lymph node, normal trachea, normal heart, normal brain (although preparation is suboptimal and it's impossible to exclude a minor degree of ventricular dilatation), normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

20. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

GROUP 3 – GLP-17-36amide TREATED

21. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

22. Normal lungs, normal bronchi, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

23. Normal lungs with normal hilar lymph node, normal trachea with normal thyroid and parathyroid glands, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

24. Normal lungs with normal hilar lymph node, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

25. Normal lungs, normal trachea, normal oesophagus, normal heart, the brain appears to have an asymmetric dilatation of lateral ventricle although this may be a consequence of orientation. Normal will spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

26. Normal lungs, normal trachea, normal oesophagus, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. There appears to be mild dilatation of the lateral and third ventricle although there is no cellular reaction. Brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. Is possibly subtle dilatation of the lateral ventricles although the third ventricle appears relatively normal.
27. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon. There is possibly subtle dilatation of the lateral ventricles although the third ventricle appears relatively normal.

28. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

29. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

30. Normal lungs, normal thymus gland, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunners glands, normal pancreas with normal islets, normal colon.

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation. Thank you for reading this.

WHAT IS THE PURPOSE OF THE STUDY?

Healthy people release a variety of different hormones from the gut into the blood stream. The timing of these hormones being released depends on whether you are hungry or have recently had a meal. Peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) are two examples of hormones which are naturally released after a meal. We know that these hormones signal to the brain to reduce hunger. This study is designed to see just how these hormones interact with the brain to signal appetite, using a safe brain scan called functional magnetic resonance imaging (fMRI). It will be an important step towards understanding how we regulate our food intake and will aid the development of medicines to treat obesity.
WHY HAVE I BEEN CHOSEN?

Healthy individuals are being recruited to take part in this study.

You should **not** take part in this study if you:
1) have any illnesses which we feel make you unsuitable
2) take any medication which we feel makes you unsuitable
3) are pregnant or breast feeding
4) have given blood in the last three months

It is important that you **should not become pregnant** during the course of the study or for one month following the study, as the effects of gut hormones in pregnancy are not known. It is therefore important that you have adequate and reliable contraception during this period.

DO I HAVE TO TAKE PART?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

**Screening visit**
If you agree to take part in this study, you will be invited for a screening visit. Prior to the screening visit, you will be asked to complete a few short questionnaires about your eating habits, personality and an MRI safety checklist. Please bring these with you to the screening visit.
During the screening visit itself, you will first be asked some questions regarding your general health and medications you may be taking. You will then have your height and weight taken, be examined by one of the research doctors and have a blood test (no more than 20ml blood - equivalent to just over a tablespoon), a pregnancy test (if female) and a heart recording (ECG). You will also be asked to taste a sample of the lunch that you will be receiving on the study days.
As long as these medical checks are satisfactory and you are still happy to participate, you will then be asked to attend for the first study visit.

**Number of visits**
You will be asked to attend as an outpatient the GSK-Imperial College Clinical Imaging Centre at Hammersmith Hospital on another 6 occasions following your screening interview, each separated by at least 3 days. These will be completed on dates convenient to you and the investigators, but should usually be completed within a maximum of 3 months.
**Study design**

We are interested in studying the effects of the gut hormones glucagon-like peptide 1 (GLP-1) and peptide YY (PYY). Both of these hormones are normally released from the gut after a meal and are known to reduce appetite. These naturally occurring hormones have been given to hundreds of human volunteers in the past with no reports of serious adverse events. These two hormones have also been safely given in combination to human volunteers and it was found that their appetite-inhibitory effects were additive.

On each study visit you will be asked to have nothing to eat and only water to drink from 10 pm on the evening before the study. You will be asked to attend at 9 o’clock in the morning and each visit will last around 3.5 hours. You will be asked to abstain from alcohol and strenuous exercise for 24 hours before the visit. We will also ask you to keep a record of all food and drink consumed for one day before the visit, the day of the visit and for one day afterwards. You will also have a pregnancy test on each visit (if female).

When you arrive on the morning of the visit you will have a small plastic cannula tube inserted into a vein in each arm. A vein is the type of blood vessel commonly used for taking blood samples. You may feel some discomfort whilst the cannulas are being inserted. After they have been inserted, one will be used to take blood samples. The other cannula will be used to administer the infusion of hormone or saline as a drip.

At the start of the visit we will ask you to score how you are feeling (e.g. rating your hunger) by placing a mark on a line called a visual analogue scale. You will then either be given a moderate size breakfast to eat over 20 mins (on one of the study visits), or asked to continue fasting until lunchtime (on the other five study visits).

Over the course of each study visit, blood samples will be taken painlessly from one of the cannula tubes. The total amount of blood taken on each study visit will not be more than 80ml (about 5 tablespoonfuls). The total amount of blood taken over all your visits will not be more than 500 ml (a little less than a pint and the same amount taken when making a single donation of blood for blood transfusion). Whilst waiting for the scan you will also be asked to complete further visual analogue scales to rate how you are feeling. During these visits you will be seated or lying on a couch, and can read or watch television when not having the scan.

Approximately 90 mins after arriving, the infusion drip will begin. 20 mins after starting the infusion, you will have a magnetic resonance imaging (MRI) brain scan. We envisage that the scan will take approximately 60 mins. The scan will enable us to look at the structure and activity of your brain. During the functional brain scans we look at the activity of the brain at rest and while you look at a variety of pictures on a screen in front of you. We will also ask you to perform simple tasks like pressing a button, reading, listening, speaking, recalling, thinking about words or numbers. You will be asked to respond to images projected on the screen in front of you.

We will make every attempt to ensure that you are comfortable and relaxed inside the scanner. You will be able to communicate directly with us throughout the entire scan. You
will be given a chance to get used to lying inside the scanner before we start. During the scan you will be allowed to take a break at any stage should you feel like one.

The first study visit will help you acclimatise to the experience of the scan. During this visit, the infusion will be a saline solution only. The saline is a placebo or dummy treatment that is commonly used in studies of this nature. It contains no active ingredient and is not expected to alter your appetite.

On the following five visits you will receive one each of these infusions (in random order):

a. An infusion of saline only.

b. An infusion of saline only.

c. An infusion of the hormone PYY. The PYY infusion will commence 20 mins before the start of the scan and will continue for 90 mins.

d. An infusion of the hormone GLP-1. The GLP-1 infusion will commence 20 mins before the start of the scan and will continue for 90 mins.

e. An infusion of the hormones PYY and GLP-1 together. This combined infusion will commence 20 mins before the start of the scan and will continue for 90 mins.

On one of these five visits you will receive a breakfast. On all of the other visits, you will be asked to remain fasting until after the scan.

This is a randomised, single-blind trial. This means that after the initial acclimatisation visit, the remaining five visits can occur in any random order. Furthermore, on any given study day you will not know whether you are being given hormone or saline. The research doctor will know what you are receiving and will be monitoring your heart rate and blood pressure throughout the study.

At the end of the brain scan you will be taken back to the nearby seating area. At the end of the infusion, you will be presented with a buffet meal and you will be asked to eat as much as you want of the meal until you feel comfortably full. Before you leave you will also be asked to perform a recall exercise. We will show you a selection of pictures and ask you which ones you recognise as being shown to you when you were in the scanner.

Approximately 10 mins after the buffet meal, a final blood sample will be taken, after which you will be free to go home (about 3.5 hours after the cannulas were originally inserted). At the end of the final visit you will also be asked to score how much you usually like to eat the foods in the pictures we showed you during the scans.
WHAT DO I HAVE TO DO?

The only restrictions on your lifestyle are that you will be asked to have nothing to eat and only water to drink from 10pm on the night before the study days. You will need to keep a record of all food and drinks consumed in the three days before every study and for the twenty-four hours afterwards. For twenty-four hours before each study meal you will be asked to refrain from taking strenuous exercise and drinking alcohol.

Female volunteers should have adequate contraception for the period of the study and for one month afterwards as the effects of gut hormones in pregnancy are not known. Pregnancy tests will be carried out to confirm that women of child bearing age are not pregnant on the morning before each study infusion.

WHAT IS THE DRUG THAT IS BEING TESTED?

PYY and GLP-1 are found naturally in the circulation of all healthy people. Both of these gut hormones have been used in several other studies in our department and worldwide, in men and women, without harmful effects. We have calculated the doses of PYY and GLP-1 that you will be given as the smallest possible dose known to induce changes in appetite, based on previous studies. You will not be given a dose of hormone that will cause the level in your body to exceed levels found naturally.

The MRI scans are being performed at the GSK Clinical Imaging Centre at Hammersmith Hospital. This unit has been funded by GlaxoSmithKline in conjunction with Imperial College London, alongside a ten-year research agreement for medical imaging. This particular study is being run by doctors and scientists from Imperial College London.

WHAT ARE THE SIDE EFFECTS OF TAKING PART?

From our previous studies we do not expect any significant side effects. Rarely, volunteers have experienced nausea after receiving these hormones. This effect subsides very quickly when the drip is stopped. You will always be able to communicate with one of the research doctors, even whilst you are in the scanner. You can ask for the study to stop at any time, even without giving a reason.

It is also possible, although very unlikely, that an unforeseen side effect occurs. If this happens, the study will be stopped. During the study, at least one experienced doctor will monitor you closely at all times. If you suffer from any ill effects during the study you should report them to the doctor monitoring you immediately. If you suffer from any ill effects afterwards you should report them to one of the research doctors on the 24 hour contact number given to you or when you next see them.

MRI is a diagnostic body scanning technique, which is used in hospitals worldwide to create images of the inside of the body. MRI has been used safely for several decades and has no known side-effects.
WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?

Gut hormones have been administered in several studies by our laboratory and therefore we do not anticipate any problems with the infusions. Insertion of the cannula (drip) into your arms on each of the study days may cause minor discomfort or superficial bruising.

Magnetic resonance imaging (MRI) is a procedure that allows doctors to look inside the body by using a scanner that sends out a strong magnetic field and radio waves. MRI does not use X-rays. This procedure is used routinely for medical care and is very safe for most people, but you will be monitored during the entire MRI scan in case any problems do occur. The risks of having an MRI scan are:

- The MRI scanner contains a very strong magnet. Therefore, you will not be able to have the MRI if you have any type of metal implanted in your body, for example, any pacing device (such as a heart pacemaker), any metal in your eyes, or certain types of heart valves or brain aneurysm clips. Someone will ask you questions about this before you have the MRI.

- There is not much room inside the MRI scanner. You may be uncomfortable if you do not like to be in close spaces (“claustrophobia”). During the procedure, you will be able to talk to and hear the MRI staff through a speaker and earphone system, and, in the event of an emergency, you can tell them to stop the scan immediately. You will be closely monitored and repeatedly checked on to make sure you are as comfortable as possible. While your head is in the scanner, we will support it, so you can’t move it. If this upsets you, you will be able to signal and speak to the investigator and stop the scan through the use of a radio system and a signalling button. You will have the opportunity during the first MRI scan to ensure that you can tolerate having the scan before the next five scans are done.

- The MRI produces a “hammering noise”. You will wear earplugs and headphones to prevent discomfort or damage to hearing. The headphones will also allow you to be able to hear us talk to you.

- You will be fully awake during the MRI scan and will not be sedated at any time. We will make every effort to ensure your comfort during this experiment. You will be communicating with us all the time you are in the scanner and you can ask for a break from the scanner at any time.

It should be noted that the MRI brain scan cannot be viewed as a comprehensive health screening procedure. However, very rarely, unexpected information can be detected which may warrant further investigation. In this event, you will be informed and a report will be sent to your GP, who will arrange further tests and coordinate your further care. In the rare event that we find a significant abnormality on your structural brain scan on the first visit this may exclude you from continuing with the rest of the study.

It is possible that if the treatment is given to a pregnant woman it will harm the unborn child. Pregnant women must therefore not take part in this study; neither should women who plan to become pregnant during the study. Women who are at risk of pregnancy will be asked to have a pregnancy test before taking part, to exclude the possibility of pregnancy.
Women who could become pregnant must use an effective contraceptive during the course of this study. Any woman who finds that she has become pregnant while taking part in the study should immediately tell her research doctor.

**WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?**

The information that we get from this study will help us to better understand normal appetite regulation and may help us to better treat future patients who suffer from being overweight.

If any of the screening questionnaires or blood tests reveal any medical problems (e.g. depression, diabetes, high cholesterol, thyroid, kidney or liver problems), your GP will be informed so that they can coordinate your further care, arrange any further tests, and refer you on to Hospital Doctors if necessary.

**WHAT IF NEW INFORMATION BECOMES AVAILABLE?**

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

**WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?**

Once the study has finished, the results of the study can be made available to you and/or your GP should you wish. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided below.

**WHAT IF SOMETHING GOES WRONG?**

Imperial College London holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone’s negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (see contact details at the end). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Clinical Research Governance Office.
WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

It is a requirement that your GP is informed, with your consent, of your participation in this study, at the start of the study.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results are likely to be published in the year following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP should you wish.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

This study is being organised and funded by the Department of Investigative Science of Imperial College School of Medicine.

PAYMENT

You will receive a fixed payment to cover expenses including travel costs. This sum of £100 for each injection visit will be paid when you have completed your visits.

WHO HAS REVIEWED THE STUDY?

This study has been reviewed by the St Mary’s Hospitals Research Ethics Committee. Reference number 09/H0712/4um,

CONTACT FOR FURTHER INFORMATION

If you experience any problems during the study, you may withdraw at any stage. The doctors involved in the study, Dr Salem and Dr de Silva, will be available by telephone during working hours (020 8383 2820). The hospital switchboard (020 8383 1000) has full contact details for all the doctors involved in the study and can reach them at any time outside normal working hours.

If you agree to take part in the trial, you will also be given the mobile phone numbers of the doctors.
APPENDIX V – DETAILED NUTRITIONAL BREAKDOWN OF THE SET BREAKFAST SERVED ON ALL “FED” VISITS

All participants were asked to eat the breakfast in its entirety and there was no instance (any subject on any “fed” visit) of this request not being complied with.

<table>
<thead>
<tr>
<th>WEIGHT (g)</th>
<th>MENU</th>
<th>Protein</th>
<th>Fat</th>
<th>CHO</th>
<th>Kcal</th>
<th>KJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>Bran Flakes (Kellogs)</td>
<td>4</td>
<td>0.8</td>
<td>26.8</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>Semi-skimmed milk (Sainsburys)</td>
<td>5.78</td>
<td>2.89</td>
<td>8.5</td>
<td>83.3</td>
<td>350.2</td>
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<tr>
<td>220</td>
<td>Orange Juice</td>
<td>1.1</td>
<td>0</td>
<td>20.02</td>
<td>92.4</td>
<td>391.6</td>
</tr>
<tr>
<td>80</td>
<td>Wholmeal Bread (Hovis)</td>
<td>8</td>
<td>2.16</td>
<td>30.24</td>
<td>172.8</td>
<td>730.4</td>
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<tr>
<td>20</td>
<td>Margarine (flora original)</td>
<td>0</td>
<td>11.8</td>
<td>0</td>
<td>106</td>
<td>436</td>
</tr>
<tr>
<td>20</td>
<td>Jam (Robertson strawberry)</td>
<td>0</td>
<td>0</td>
<td>13.4</td>
<td>53.6</td>
<td>227.8</td>
</tr>
<tr>
<td>25</td>
<td>Cheese (2 x Leerdamer original slices)</td>
<td>6.75</td>
<td>7</td>
<td>0</td>
<td>89.5</td>
<td>372.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>25.63</td>
<td>24.65</td>
<td>98.96</td>
<td>727.6</td>
<td>2508.5</td>
</tr>
<tr>
<td>% Composition</td>
<td></td>
<td>14.1</td>
<td>30.5</td>
<td>51.0</td>
<td></td>
<td></td>
</tr>
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</table>
PUBLICATIONS AND PRESENTATIONS RELATED TO THE WORK IN THIS THESIS


Salem V*, De Silva A*, Matthews PM and Dhillo WD. Imaging the neuroendocrinology of appetite. Adipocyte 1:1, 1–5; January 2012 * joint first authorship

De Silva A*, Salem V*, Matthews PM and Dhillo WD. The use of functional MRI to study appetite control in the CNS. Exp Diabetes Res. 2012:764017; May 2012 * joint first authorship


Oral Presentation at Society for Endocrinology BES 2011.


Poster presentation nominated for Presidential Poster Competition at American Endocrine Society ENDO 2011.

Poster presentation winner of Diabetes and Endocrinology section, Academy of Medical Sciences/RCP meeting for Clinician Scientists 2012.
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