

To Sarah-Jane

**DETERMINING THE CNS ACTION OF
ANORECTIC GUT HORMONES IN HUMANS
USING FUNCTIONAL MRI**

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Abstract

Understanding how the brain controls appetite offers promising inroads towards developing new therapies for obesity. Peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) are anorectic hormones that are co-released post-prandially by the gut. Both have been shown to physiologically inhibit appetite and food intake when administered to humans. However, the mechanisms by which they regulate appetite through the central nervous system (CNS) remain poorly understood. Functional magnetic resonance imaging (fMRI) is a tool that has recently been used to determine the CNS pathways that mediate the action of appetite-modulating hormones.

This dissertation investigates the action of the anorectic gut hormones PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, infused intravenously over 90 minutes in 15 lean, healthy human subjects following an overnight fast. Using blood oxygen level dependent (BOLD) fMRI, I have determined the effects of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on CNS appetite centres and subsequent food intake, comparing the effects with those seen physiologically following a standard meal.

The combined administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide to fasted human subjects led to similar reductions in BOLD fMRI brain activity in *a priori* regions of interest as observed physiologically following feeding, especially in the insular cortex. Additionally, compared with infusion of saline, co-administration of PYY₃₋₃₆ at 0.3 pmol/kg/min with GLP-1₇₋₃₆ amide at 0.8 pmol/kg/min led to a 27.0% reduction in *ad libitum* energy intake at a buffet lunch served immediately after the infusion. The reduction in *ad libitum* energy intake following the combination infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide was similar to that observed when saline was infused in subjects following consumption of a set breakfast (23.5% reduction in *ad*

libitum energy intake at a buffet lunch served immediately after the infusion). My findings provide direct evidence that the combined action of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide in the brain could explain post-prandial satiety.

Declaration of contributors

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

Chapter 2: In the mouse toxicity studies, histopathology was examined by Professor Gordon Stamp. Human infusion studies were performed with assistance from Dr Victoria Salem, Dr Owais Chaudhri, Dr Thomas Hopkins, Dr Adam Januszewski and Dr George Tharakan. Radioimmunoassay was assisted by Dr Owais Chaudhri and Dr George Tharakan.

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Abbreviations

ACC	Anterior Cingulate Cortex
α -MSH	α -Melanocyte Stimulating Hormone
ANOVA	Analysis of Variance
AP	Area Postrema
AgRP	Agouti-Related Peptide
ARC	Arcuate Nucleus
ASL	Arterial Spin Labelling
AUC	Area under the curve
BMI	Body Mass Index
BOLD	Blood-Oxygen-Level-Dependent
BSA	Bovine Serum Albumin
CART	Cocaine- and Amphetamine-Regulated Transcript
CNS	Central Nervous System
DMN	Dorsomedial Nucleus
DPP IV	Dipeptidyl Peptidase IV
DVC	Dorsal Vagal Complex
DVN	Dorsal Motor Nucleus of the Vagus
ELISA	Enzyme-Linked Immunosorbent Assay
EPI	Echo-Planar Imaging
FEAT	fMRI Expert Analysis Tool
fMRI	Functional Magnetic Resonance Imaging
GHS-R	Growth Hormone Secretagogue Receptor
GLP-1	Glucagon-Like Peptide 1
GLP-1R	Glucagon-Like Peptide 1 Receptor
HPLC	High-pressure liquid chromatography
ICV	Intracerebroventricular
Lep R	Leptin Receptor
LHA	Lateral Hypothalamic Area
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time of Flight
MC4R	Melanocortin Receptor 4
MEMRI	Manganese Enhanced Magnetic Resonance Imaging
MNI	Montreal Neurological Institute
MRI	Magnetic Resonance Imaging
NPY	Neuropeptide Y
NTS	Nucleus of the Tractus Solitarius
OFC	Orbitofrontal Cortex
PEG	Polyethylene glycol
PET	Positron Emission Tomography
PFA	Perifornical Area
POMC	Pro-opiomelanocortin
PP	Pancreatic Polypeptide
PVN	Paraventricular Nucleus
PYY	Peptide YY
QC	Quality Control
RF	Radiofrequency
RIA	Radioimmunoassay
ROI	Region of Interest
SEM	Standard Error of Mean

Abbreviations- continued

SPECT	Single Photon Emission Tomography
TCA	Time Clustering Analysis
TE	Echo Time
TR	Repetition Time
VMH	Ventromedial Hypothalamus
VAS	Visual Analogue Scale
VTA	Ventral Tegmental Area

Table of Contents

Title Page	1
Abstract	2
Declaration of contributors	4
Acknowledgements	5
Abbreviations	6
Table of contents	8
Index of figures	13
Index of tables	15
1. Introduction	16
1.1. Obesity	17
1.2. The importance of understanding appetite regulation	18
1.3. Homeostatic and non-homeostatic controls of appetite and food intake	19
1.4. Role of the hypothalamus	20
1.4.1. Hypothalamic nuclei implicated in appetite control	21
1.5. Role of the brainstem	22
1.6. Hedonic brain regions modulating appetite and food intake	23
1.6.1. Amygdala	24
1.6.2. Insula	25
1.6.3. Striatum	25
1.6.4. Orbitofrontal cortex (OFC)	26
1.7. The role of PYY and GLP-1 in the control of food intake	28
1.7.1. Peptide YY (PYY)	29
1.7.2. Glucagon-like Peptide 1 (GLP-1)	36
1.7.3. Studies investigating combined administration of PYY ₃₋₃₆ and GLP-1 ₇₋₃₆ amide	42
1.8. Other hormones implicated in the control of food intake	45
1.8.1. Ghrelin	45
1.8.2. Insulin	46
1.8.3. Leptin	47
1.9. Neural signals controlling food intake	48
1.9.1. Orosensory and visual stimuli	48
1.9.2. Gastric distension	49
1.9.3. Glucose	49
1.10. The use of imaging techniques to determine CNS neuronal activation in the regulation of feeding	50

1.11. Overview of fMRI	51
1.11.1. fMRI - Manganese enhanced MRI (MEMRI)	54
1.11.2. fMRI - Blood-oxygen-level-dependent (BOLD) fMRI	55
1.12. BOLD fMRI image reconstruction	56
1.13. Principles of fMRI in human appetite studies	56
1.14. fMRI studies in normal weight subjects - comparing the response to food cues in the fasted and fed states	57
1.15. fMRI studies of hormones implicated appetite control	61
1.15.1. PYY and GLP-1	62
1.15.2. Ghrelin	63
1.15.3. Insulin	63
1.15.4. Leptin	64
1.16. Imaging before and after gastric bypass surgery	65
1.17. Summary	67
1.18. Hypotheses and aims	69
1.18.1. Hypotheses	69
1.18.2. Aims	69
2. Investigation of the effects of single and combined administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on appetite and energy intake: dose verification study	70
2.1. Introduction and rationale	71
2.2. Methods	74
2.2.1. Peptides	74
2.2.2. Subjects	75
2.2.3. Design	76
2.2.4. Peptide preparation and delivery	77
2.2.5. Blood sampling and processing	78
2.2.6. Visual analogue scale scores	78
2.2.7. Measurement of haemodynamic parameters and blood glucose	78
2.2.8. <i>Ad libitum</i> test meal	79
2.2.9. Measurement of total PYY	81
2.2.10. Measurement of amidated GLP-1	81
2.2.11. RIA set up for measurement of total PYY and amidated GLP-1	82
2.2.12. Statistical analysis of energy intake, VAS, haemodynamic parameters, blood glucose and gut hormone levels	84

2.3. Results	85
2.3.1. Peptides	85
2.3.2. Energy intake	85
2.3.3. Ratings of meal palatability	86
2.3.4. Hunger, nausea and fullness scores	87
2.3.5. Haemodynamic parameters	90
2.3.6. Blood glucose	91
2.3.7. Plasma PYY levels in Study 1	92
2.3.8. Plasma GLP-1 levels in Study 1	94
2.4. Discussion	96
3. Investigation of the effects of single and combined administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on appetite, energy intake and neuronal activity in brain appetite centres (Study 2)	99
3.1. Introduction and rationale	100
3.2. Methods	101
3.2.1. Peptides	101
3.2.2. Subjects	101
3.2.3. Design	102
3.2.4. Peptide preparation and delivery	103
3.2.5. Blood sampling and processing	103
3.2.6. Visual analogue scale scores	104
3.2.7. Measurement of haemodynamic parameters and blood glucose	104
3.2.8. Study meals	104
3.2.9. Functional MRI scan	105
3.2.10. Measurement of total PYY	108
3.2.11. Measurement of PYY ₃₋₃₆	108
3.2.12. Measurement of amidated GLP-1	109
3.2.13. Measurement of active GLP-1 (GLP-1 ₇₋₃₆ amide and GLP-1 ₇₋₃₇)	109
3.2.14. Statistical analysis of energy intake, VAS, haemodynamic parameters, blood glucose and gut hormone levels	110
3.2.15. fMRI analysis: pre-processing of data	111
3.2.16. fMRI analysis: whole brain activation with presentation of food-salient visual stimuli	112
3.2.17. fMRI analysis: generation of whole brain maps of effect of feeding, PYY ₃₋₃₆ and GLP-1 ₇₋₃₆ amide administration on brain activation by food images	112
3.2.18. fMRI ROI analysis: effect of feeding, PYY ₃₋₃₆ and GLP-1 ₇₋₃₆ amide administration on brain activation by food images	113
3.2.19. fMRI analysis: effect of feeding, PYY ₃₋₃₆ and GLP-1 ₇₋₃₆ amide administration on hypothalamic and brainstem activation by food images	114

3.3. Results	115
3.3.1. Comparison of energy intake between acclimatisation visit and fasted saline visit	115
3.3.2. Analysis of visit order effects on energy intake	116
3.3.3. Effects of feeding, PYY ₃₋₃₆ and GLP-1 _{7-36 amide} administration on subsequent energy intake	117
3.3.4. Ratings of meal palatability	119
3.3.5. Analysis of the effect of feeding and gut hormone infusion on food related VAS parameters	120
3.3.6. Correlation of nausea with energy intake	122
3.3.7. Analysis of the effect of feeding and gut hormone infusion on non-food related VAS parameters	123
3.3.8. Haemodynamic parameters	125
3.3.9. Blood glucose	126
3.3.10. Total PYY levels	127
3.3.11. PYY ₃₋₃₆ levels	129
3.3.12. Amidated GLP-1 levels	131
3.3.13. Active GLP-1 levels (GLP-1 _{7-36 amide} and GLP-1 ₇₋₃₇)	133
3.3.14. Brain activation with presentation of food-salient visual stimuli	135
3.3.15. Effect of feeding, PYY ₃₋₃₆ and GLP-1 _{7-36 amide} administration on brain activation by food images: whole brain analysis	138
3.3.16. Effect of feeding, PYY ₃₋₃₆ and GLP-1 _{7-36 amide} administration on brain activation by food images: ROI analysis	142
3.3.17. Effect of feeding, PYY ₃₋₃₆ and GLP-1 _{7-36 amide} administration on brain activation by food images: analysis of hypothalamic and brainstem responses	154
3.4. Discussion	
3.4.1. Discussion of the effects of single and combined administration of PYY ₃₋₃₆ and GLP-1 _{7-36 amide} on appetite, energy intake and neuronal activity in brain appetite centres	156
3.4.2. Comparison of the effects of PYY ₃₋₃₆ and GLP-1 _{7-36 amide} on neuronal activity related to appetite with those of other appetite-modulating hormones	162
3.4.3. Comparison of the effects of PYY ₃₋₃₆ and GLP-1 _{7-36 amide} on neuronal activity related to appetite with those observed after gastric bypass surgery	165
3.4.4. Limitations of my study	168

4. General Discussion	171
4.1. Summary	172
4.2. Further work: imaging the hypothalamus	173
4.3. Further work: fMRI responses in obesity	175
4.4. Conclusion	179
Appendix 1: Principles of Radioimmunoassay. Principles of Enzyme-Linked Immunosorbent Assay (ELISA)	181
Appendix 2: Results of mouse toxicology study	184
Appendix 3: Publications arising from thesis	189
REFERENCES	190

Index of figures

Figure 1.1: The major determinants of appetite control	20
Figure 1.2: Brain regions implicated in the control of appetite	27
Figure 1.3: An overview of the role of PYY and GLP-1 in the control of appetite and food intake	44
Figure 1.4: T1 and T2 relaxation	53
Figure 2.1: Protocol for Study 1	80
Figure 2.2: Mean <i>ad libitum</i> energy intake in Study 1	86
Figure 2.3: Meal rating following <i>ad libitum</i> meal in Study 1	87
Figure 2.4: Hunger, nausea and fullness ratings during Study 1	88
Figure 2.5: Change in hunger, nausea and fullness ratings during Study 1	89
Figure 2.6: Haemodynamic parameters during Study 1	90
Figure 2.7: Blood glucose during Study 1	91
Figure 2.8: Variation of plasma total PYY levels with time during Study 1	93
Figure 2.9: Variation of plasma amidated GLP-1 levels with time during Study 1	95
Figure 3.1: Protocol for Study 2	107
Figure 3.2: Mean <i>ad libitum</i> energy intake during acclimatisation visit and control saline visit in Study 2	115
Figure 3.3: Visit order effects on energy intake in Study 2	116
Figure 3.4: Mean <i>ad libitum</i> energy intake in Study 2	118
Figure 3.5: Meal rating following <i>ad libitum</i> meal in Study 2	119
Figure 3.6: Food related VAS parameters during Study 2	120
Figure 3.7: Correlation of nausea with energy intake during combined PYY ₃₋₃₆ and GLP-1 ₇₋₃₆ amide infusion during Study 2	122
Figure 3.8: Non-food related VAS parameters during Study 2	123
Figure 3.9: Change in non-food related VAS parameters during Study 2	124
Figure 3.10: Blood pressure and pulse during Study 2	125

Figure 3.11: Blood glucose during Study 2	126
Figure 3.12: Variation of plasma total PYY levels with time during Study 2	128
Figure 3.13: Variation of plasma PYY ₃₋₃₆ levels with time during Study 2	130
Figure 3.14: Variation of plasma amidated GLP-1 levels with time during Study 2	132
Figure 3.15: Variation of plasma active GLP-1 levels with time during Study 2	134
Figure 3.16: Brain regions where Δ BOLD (when subjects viewed images of food compared with when they viewed images of non-food) was significantly greater than zero in Study 2	135
Figure 3.17: Whole brain map showing attenuation of BOLD fMRI signal with feeding	138
Figure 3.18: Whole brain map showing attenuation of BOLD fMRI signal with infusion of PYY ₃₋₃₆	139
Figure 3.19: Whole brain map showing attenuation of BOLD fMRI signal with infusion of GLP-1 _{7-36 amide}	140
Figure 3.20: Whole brain map showing attenuation of BOLD fMRI signal with combined infusion of PYY ₃₋₃₆ and GLP-1 _{7-36 amide}	141
Figure 3.21: Modulation of BOLD signal across brain ROIs by feeding, or either individual or combined gut hormone infusions	145
Figure 3.22: Modulation of BOLD signal across brain ROIs by feeding, or either individual or combined gut hormone infusions - left and right sided analysis	147
Figure 3.23: Summed reduction in mean % BOLD signal change in ROIs with individual administration of PYY ₃₋₃₆ and GLP-1 _{7-36 amide} , compared with the reduction in mean % BOLD signal change after combined administration of PYY ₃₋₃₆ and GLP-1 _{7-36 amide}	149
Figure 3.24: Modulation of BOLD signal across collective brain ROIs by feeding, or either individual or combined gut hormone infusions	151
Figure 3.25: Correlation of nausea with reduction in % BOLD signal change during combined PYY ₃₋₃₆ and GLP-1 _{7-36 amide} infusion for each ROI	153
Figure 3.26: Modulation of BOLD signal in the hypothalamus and brainstem (midbrain) by feeding, or either individual or combined gut hormone infusions	155

Index of tables

Table 1.1: Important translational human studies involving PYY ₃₋₃₆	35
Table 1.2: Important translational human studies involving GLP-1	41
Table 2.1: Volumes of reactants for addition to PYY and GLP-1 in-house radioimmunoassay	83
Table 3.1: Analysis of food related VAS parameters from t = 0 to t = 90 min during each infusion in Study 2	121
Table 3.2: 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 in Study 2	136
Table 3.3: Cluster peaks within the 6 <i>a priori</i> ROIs where Δ BOLD (when subjects viewed images of food compared with when they viewed images of non-food) was significantly greater than zero in Study 2	142

CHAPTER 1

General Introduction

1.1. Obesity

In 2008, The Health Survey of England classified 24% of adults and 16% of children as obese (defined by a body mass index (BMI) $\geq 30 \text{ kg/m}^2$). In Europe as a whole, it is currently estimated that 17% of the population is obese (von Ruesten et al., 2011). This figure is rising sharply due to the combination of readily available high-calorie food and sedentary lifestyle; extrapolation of recent trends predicts that by 2015, as many as 30% of European adults will be obese (von Ruesten et al., 2011). It is well known that being overweight or obese carries an increased risk of type 2 diabetes, hypertension, ischaemic heart disease, stroke, osteoarthritis, sleep apnoea and cancer. It has even been predicted that the coming decades could see a reversal of twentieth century gains in life expectancy, due to detrimental health consequences arising from the obesity epidemic (Olshansky et al., 2005).

Effective treatment of obesity has been shown to prove worthwhile; a 92% reduction in diabetes deaths, 60% reduction in cancer deaths, 56% reduction in coronary artery disease deaths and 40% reduction in all cause mortality was demonstrated in a retrospective matched cohort study of obese individuals undergoing bariatric surgery (Adams et al., 2007). Bariatric surgery, in particular Roux-en-Y gastric bypass (which involves the formation of a small stomach pouch and bypass of the proximal small bowel), is the most effective current treatment for obesity, leading to sustained weight loss of approximately 30% (Maggard et al., 2005). However, the procedure is not without risk and carries a mortality rate of 0.5% (Marsk et al., 2008).

Lifestyle modification strategies (diet and exercise) currently form the main treatments for obesity. However, results are generally disappointing and the majority of people who attempt

lifestyle modification regain any lost weight within 5 years (Weiss et al., 2007). Drug treatments sibutramine and rimonabant were recently withdrawn due to cardiovascular and neuropsychiatric side effects respectively. The only licensed pharmacological treatment for obesity in the UK is orlistat, an intestinal lipase inhibitor. This produces modest weight loss (in a systematic review of randomized clinical trials, 60% of patients on orlistat achieved >5% weight loss after 1 year of treatment (Hutton and Fergusson, 2004)). Furthermore, as a consequence of its mechanism of action, orlistat often leads to unacceptable gastrointestinal side effects when fat is consumed. Therefore, effective pharmacotherapy to tackle obesity remains an elusive goal, despite an ever-growing need.

1.2. The importance of understanding appetite regulation

Accumulation of excess body fat (and hence development of obesity) occurs when energy intake exceeds energy expenditure in the long term. The relative balance between energy intake and expenditure is an important regulator of appetite; following a meal, appetite is suppressed, whereas following significant energy expenditure, we feel hungry. The intricacies underpinning this fine-tuned regulation of energy balance form part of a complex field that has been the focus of intense research over the past decade, bridging the disciplines of endocrinology, gastroenterology and neurobiology. An enhanced understanding of the physiological mechanisms that regulate appetite is essential for the development of pharmacological strategies to combat obesity. In particular, within the field of neuroendocrinology, identification of hormone-brain interactions that modulate appetite (and subsequent food intake) will aid the manipulation of naturally occurring hormones to develop safe and effective anti-obesity therapies. This strategy, exploiting physiological pathways of appetite control, holds promise for the future treatment of obesity, especially given the shortage of effective pharmacotherapies for obesity in the existing market. In particular,

identification of how hormones that are secreted post-prandially by the gut interact with brain regions that modulate appetite may eventually allow for their manipulation as therapeutic strategies in obese patients. These anorectic gut hormones, amongst others, include peptide YY (PYY) and glucagon-like peptide 1 (GLP-1). These peptide hormones will form the focus of this thesis and will be considered in detail. Alongside this, a general overview of the control of appetite and food intake will be covered in order to better understand the specific roles of PYY and GLP-1 within this field.

1.3. Homeostatic and non-homeostatic controls of appetite and food intake

Traditionally, there has been distinction between 'homeostatic' and 'non-homeostatic' control of appetite and food intake. Homeostatic control refers to the alteration in consumption of food that follows sensing of energy balance. Post-prandially, activation of gut mechanoreceptors, changes in circulating nutrient concentration, and release of anorectic gut hormones all lead to a reduction in subsequent feeding (Delzenne et al., 2010). Longer term adiposity signals of energy balance such as leptin also interact with central nervous system (CNS) circuits to regulate food intake (Farooqi et al., 2007, Morris and Rui, 2009). Appropriately matching food intake with metabolic need is achieved by the interaction of the above inputs with key central neural appetite circuits.

Across species, food intake is also driven by factors other than basic physiological or homeostatic need. Food appearance, flavour, timing and location of meals, social, cultural, emotional, and economic influences affect food intake by non-homeostatic mechanisms. Importantly, there is also modulation of food intake by hedonic and mnemonic neuronal

circuits (Zheng and Berthoud, 2007). Figure 1.1 summarises the major determinants of appetite control.

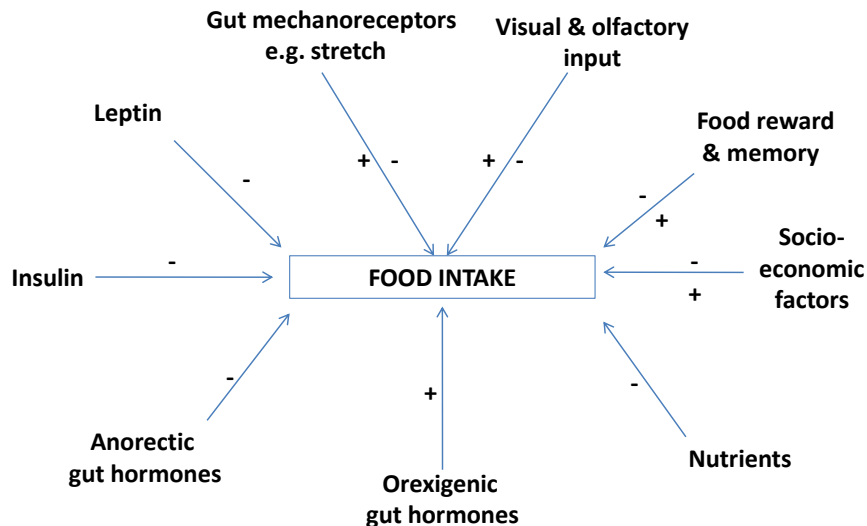


Figure 1.1: The major determinants of appetite control. A variety of homeostatic, hedonic and environmental inputs contribute to the overall control of food intake. ‘+’ indicates factors that increase food intake, whereas ‘-’ indicates factors that reduce food intake.

1.4. Role of the hypothalamus

The hypothalamus is widely recognised as the ‘gate-keeper’ in the control of appetite and food intake. Peripheral signals of energy balance may act directly on the hypothalamus to control food intake and this forms one important focus of current appetite research. Historically, the lateral hypothalamus was thought of as the ‘hunger centre’ and the medial hypothalamus the ‘satiety centre’. This was based on lesioning experiments in animals; damage to the lateral hypothalamus produced anorexia, whereas damage to the medial hypothalamus led to hyperphagia (Mayer and Thomas, 1967). Whilst this view still largely holds true, it has been recently refined by our enhanced understanding of the role of

individual nuclei within the hypothalamus and communication between them. It is also well established that there is a network of communication between the gut, pancreas, adipose tissue, brainstem, and hypothalamus to signal energy balance. Additionally, there is further communication between the hypothalamus and higher cortical centres pertaining to food memory and rewarding aspects of food, resulting in overall co-ordinated control of appetite and food intake.

1.4.1. Hypothalamic nuclei implicated in appetite control

The arcuate nucleus (ARC), replaced by the infundibular nucleus in humans, is thought to be the main hypothalamic area controlling food intake. By virtue of lying close to the median eminence (a circumventricular organ where an incomplete blood-brain barrier is thought to allow peripheral signals to gain access to the CNS), the ARC is ideally positioned to co-ordinate feeding. Lesions of the ARC result in hyperphagia and obesity in mice (Olney, 1969). Within the ARC, two groups of neurons are pivotal in regulating food intake. One group of neurons contains neuropeptide Y (NPY) and the vast majority of these also contain agouti-related peptide (AgRP). Activation of these neurons enhances food intake; i.e. these neurons are orexigenic (Clark et al., 1984). The second group is formed by anorexigenic neurons containing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). POMC is the precursor of α -melanocyte stimulating hormone (α -MSH). α -MSH acts on melanocortin receptors (mainly MC4R) to reduce food intake (Schwartz et al., 2000). AgRP is a competitive antagonist at melanocortin receptors (Rossi et al., 1998). CART is thought to have either orexigenic or anorexigenic effects depending on its site of action; central intracerebroventricular (ICV) administration of CART reduces food intake in rats (Kristensen et al., 1998), whereas administration of CART directly into the ARC increases food intake (Abbott et al., 2001).

Axons from NPY/AgRP and POMC/CART neurons project from the ARC to other areas of the hypothalamus such as the paraventricular nucleus (PVN). Destruction of the PVN leads to hyperphagia and obesity in rats (Leibowitz et al., 1981). In addition to the PVN, axons from the ARC also project to the ventromedial hypothalamus (VMH), dorsomedial nucleus (DMN), lateral hypothalamic area (LHA), and perifornical area (PFA) to modulate food intake.

1.5. Role of the brainstem

The brainstem has a well established role in the sensing of energy balance and modulation of food intake. Within the brainstem, the dorsal vagal complex (DVC) is the main organ responsible for facilitating the communication between peripheral signals of food intake and hypothalamic nuclei (Bailey, 2008). The DVC consists of the nucleus of the tractus solitarius (NTS), the area postrema (AP), and the dorsal motor nucleus of the vagus (DVN). Vagal nerve afferents carry sensory information relaying hunger and satiety from the gut directly to the NTS. Transection of these gut sensory vagal nerve afferents results in increased meal size and duration (Schwartz, 2000). The absence of a complete blood-brain barrier in the AP also allows the brainstem to directly receive metabolic signals of energy balance, such as hormones and nutrients carried by the blood.

The brainstem is then able to process these sensory inputs and relay them to the hypothalamus and higher cortical centres. In keeping with this, it is well established that there are neural projections from the brainstem to the hypothalamus (Ter Horst et al., 1989). There are also descending, efferent pathways from the hypothalamus to the DVN (ter Horst et al., 1984). The DVN modulates efferent vagal nerve activity in the gastrointestinal tract

and can alter gastric emptying, gastric motility, and pancreatic secretions. This points to a role for the brainstem in modulating feeding related activity, as well as sensing energy balance.

1.6. Hedonic brain regions modulating appetite and food intake

The word 'hedonic' relates to pleasant (or unpleasant) sensations. It is apparent that visual, smell, and taste signals can override satiety signals to maintain food intake despite neutral or even positive energy balance. For example, the sweet taste of certain foods is associated with positive emotions that motivate animals to find food and continue consumption. These sensory signals are conveyed from visual, smell, and taste receptors to the NTS in the brainstem, then relayed to cortical and limbic 'reward centres' implicated in appetite regulation. Past experience with specific foods also forms an important contributor to continued consumption (if the previous experience was pleasant) or early cessation of intake (if the previous experience was unpleasant), regardless of satiety and energy balance. This is referred to as conditioned preference or conditioned aversion respectively. From an emotional perspective, the evaluation of a stimulus for its motivational significance and cross-reference with the existing working memory occurs in multiple brain regions, particularly the dopaminergic limbic and prefrontal reward areas (Berthoud, 2011). In rodents, pharmacological studies suggest that dopaminergic D2-and D3 receptors play a critical role in reward; mice lacking D2-receptors have reduced sensitivity to the rewarding effects of addictive drugs such as cocaine (Welter et al., 2007) and opiates (Maldonado et al., 1997). Furthermore, administration of D3 receptor antagonists have been shown to reduce cocaine-seeking behaviour and reduce self-administration of nicotine in rats (Heidbreder et al., 2005). Key regions of this dopaminergic reward system are illustrated in figure 2, and include the amygdala, insula, striatum (comprising the nucleus accumbens,

caudate, putamen and globus pallidus), orbitofrontal cortex (OFC), ventral tegmental area (VTA), anterior cingulate cortex (ACC) and pre-frontal cortex. Those regions that form the focus of specific investigation within this thesis will now be introduced further:

1.6.1. Amygdala

This almond shaped limbic structure lies in each anterior temporal lobe. The amygdala is involved in a wide range of emotional processing and is particularly responsive to fear, positive and negative affect (Pessoa and Ungerleider, 2004). In keeping with this, bilateral lesions of the amygdalae in primates produce fearless animals, with impairments in emotional learning, impaired memory for emotional events and deficits in the perception of emotion (Cardinal et al., 2002). The amygdala receives afferent projections from the insular and visual cortices, with efferent projections to the OFC and nucleus accumbens (Amaral and Price, 1984, Morgane et al., 2005). Importantly, some of the functions of the amygdala are carried out in conjunction with the OFC, such as updating the values of expected outcomes stored in the OFC (Holland and Gallagher, 2004). Thus, communication between the amygdala and the OFC is thought to be important in the generation and maintenance of a working memory for food experiences (Verhagen, 2007). Furthermore, in rats trained to associate the availability of food with the presentation of a food cup (resulting in conditioned food intake even when satiated), elimination of amygdala-hypothalamus connections completely abolishes this conditioned food intake (Petrovich et al., 2002). This illustrates the importance of connections between the amygdala and other brain regions (both homeostatic and non-homeostatic) implicated in the control of food intake.

1.6.2. Insula

The insula (or insular cortex) is located within the temporal and frontal cortices. It is comprised of a larger anterior and smaller posterior part. Considered to be the primary gustatory cortex, the insula is thought to be involved in sensory and rewarding aspects of taste processing in addition to taste aversion (James et al., 2009, Welzl et al., 2001). It is also suggested that the insula is responsible for generating representations of previous flavour and reward experience with food (de Araujo et al., 2003). The insula receives afferent inputs from the NTS, and sends efferent projections to the LHA, amygdala, nucleus accumbens and OFC (Van Vugt, 2009), indicating connections with both homeostatic and other reward regions modulating food intake.

1.6.3. Striatum

The striatum can be divided into ventral and dorsal components. The ventral striatum comprises the nucleus accumbens and olfactory tubercle, whereas the dorsal striatum comprises the caudate nucleus, putamen and globus pallidus. The striatum contains GABAergic and cholinergic neurons (Meredith, 1999), expressing D1-, D2- and D3-like dopamine receptors. It is well established that the striatum, in particular the nucleus accumbens, plays a key role in motivation and enabling the behavioural responses necessary for obtaining rewards (Berridge, 1996). Consistent with this, lesions of the nucleus accumbens have been shown to reduce the rewarding effects of addictive drugs such as cocaine and opiates (Kelsey et al., 1989), which normally elevate extracellular concentrations of dopamine in the ventral striatum (Kelley, 2004). There are established projections from the ventral striatum back to the midbrain (Carlezon and Thomas, 2009).

Furthermore, administration of an opioid μ -receptor agonist into the nucleus accumbens increases neuronal expression of orexin (which promotes food intake) in the hypothalamus (Zheng et al., 2007). This evidence implies links between the ventral striatum and homeostatic regions controlling food intake.

1.6.4. Orbitofrontal cortex (OFC)

Occupying the ventral surface of the frontal cortex, the OFC plays a key role in encoding taste, flavour, and food reward (Small et al., 2007). The OFC receives converging sensory input from olfactory, visual, gustatory, and somatosensory signals (Rolls et al., 2003, Verhagen, 2007, Verhagen and Engelen, 2006, de Araujo et al., 2003) and is in close contact with the amygdala, insula and hippocampus (Verhagen and Engelen, 2006), thought to be important in producing an overall co-ordinated response following integration of sensory inputs. The primary role for the OFC in encoding reward value of food is supported by the decrease in activation seen during repeated tasting of a food ('sensory-specific satiety') (Kringelbach and Rolls, 2004). During eating, distinct sensory inputs fuse into an overall perception of flavour; such olfactory and oral somatosensory inputs are thought to be encoded in the OFC (Small et al., 2007).

It is now widely accepted that with regard to appetite control, homeostatic and non-homeostatic systems do not function independently; instead, there is extensive cross-modulation between them, along with a complex integration of inputs before a final decision is made regarding food consumption. Figure 1.2 illustrates some of the major brain regions implicated in appetite control.

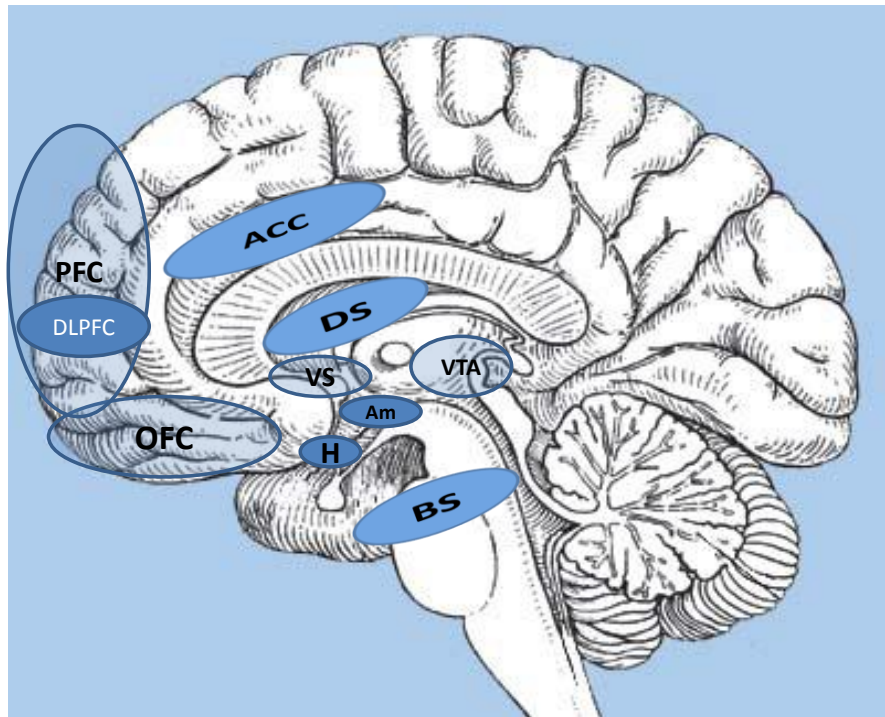


Figure 1.2: Brain regions implicated in the control of appetite: The hypothalamus (H), as a homeostatic gatekeeper, has numerous connections with the brainstem (BS) as well as with higher brain centres which process salience and reward. The hypothalamus transmits to these higher centres information received from the periphery, such as nutritional status signalled via the postprandial release of gut hormones. Important higher brain centres include: amygdala (Am) – emotional and aversive processing, ventral striatum (VS) and dorsal striatum (DS) – anticipatory reward processing, ventral tegmental area (VTA) – numerous dopaminergic projections to other limbic areas, anterior cingulate cortex (ACC) – decision making, orbitofrontal cortex (OFC) – reward encoding, prefrontal cortex (PFC) – translation of external and internal cues into behavioural responses, dorsolateral prefrontal cortex (DLPFC) – self control. Not shown is the insular cortex (a more lateral structure), which is also important in gustatory processing.

1.7. The role of PYY and GLP-1 in the control of food intake

The gut is the largest endocrine organ in the body and secretes over 30 different regulatory peptide hormones. A number of these gut hormones are stimulated by gut nutrient content and interact with receptors at various points in the 'gut-brain axis' to affect short term feelings of hunger and satiety. This thesis will focus on two such hormones - PYY and GLP-1. Both hormones are released together following a meal and are thought to mediate post-prandial satiety.

As discussed previously, gastric bypass surgery is one of the most effective treatments for obese patients, where sustained weight loss results from diminished appetite. Notably, gastric bypass patients demonstrate increased levels of PYY and GLP-1, especially post-prandially (Borg et al., 2006, le Roux et al., 2006, le Roux et al., 2007). Significantly, inhibiting the PYY and GLP-1 responses results in return of appetite and increases food intake (le Roux et al., 2007). Therefore, it is likely that elevated levels of PYY and GLP-1 play a key role in the sustained weight loss observed following gastric bypass surgery. It follows that investigation into the physiological interaction between these hormones and the CNS is of paramount importance in obesity research.

1.7.1. Peptide YY (PYY)

PYY is a 36-amino acid peptide which belongs to the Pancreatic Polypeptide (PP) family. This family of peptides, comprising PP, PYY and NPY, share a common hair-pin-fold motif structure. PP, PYY and NPY bind to G-protein coupled receptors Y_1 , Y_2 , Y_4 , Y_5 and Y_6 , displaying promiscuity in their interactions with these receptors by virtue of their shared hair-pin-fold motif structure.

PYY is produced by the L cells of the gut, with highest concentrations found in the large bowel and the rectum (Adrian et al., 1985). Two endogenous forms, PYY₁₋₃₆ and PYY₃₋₃₆, are released post-prandially into the circulation. PYY₃₋₃₆ is further produced by cleavage of the Tyr-Pro amino terminal residues of PYY₁₋₃₆ by the enzyme dipeptidyl peptidase IV (DPP-IV). PYY₃₋₃₆ acts mainly via the Y_2 receptor (Dumont et al., 1995). Indeed, PYY₃₋₃₆ selectivity for the Y_2 receptor is thought to be conferred by cleavage of the Tyr-Pro amino terminal residues of PYY₁₋₃₆ (Dumont et al., 1995). In the fasted state, PYY₁₋₃₆ predominates in the circulation, whereas post-prandially, PYY₃₋₃₆ is the major circulating form (Grandt et al., 1994). Following a meal, circulating levels of PYY₃₋₃₆ rise within 15 minutes, peak at approximately 90 minutes and remain elevated for up to 6 hours (Adrian et al., 1985). The magnitude of the rise in PYY₃₋₃₆ is in proportion to the calories ingested (Degen et al., 2005). When exogenously administered intravenously, its circulating half-life is approximately 8 minutes (Lluis et al., 1989).

Given that PYY₃₋₃₆ is released within 15 minutes of food intake, this must occur before ingested nutrients reach the distal small intestine and colon (where the greatest concentrations of PYY₃₋₃₆ are found). Therefore, initial post-prandial release of PYY₃₋₃₆ is

likely to be under neural control. Further release of PYY₃₋₃₆ is observed when the nutrients arrive in the distal gut and is particularly stimulated by a high fat diet (Essah et al., 2007). The protein content of the diet is thought to be influential for delayed PYY₃₋₃₆ release approximately 2 hours post-prandially (Helou et al., 2008). PYY₃₋₃₆ is likely to affect appetite via a direct central effect and also via its effects on gut motility – it acts as an ‘ileal brake’ and so leads to a sensation of fullness and satiety (Batterham et al., 2002, Batterham et al., 2003).

Studies by Batterham *et al.* have shown that peripheral administration of PYY₃₋₃₆ to rodents and humans leads to marked inhibition of food intake (Batterham et al., 2002, Batterham et al., 2003). In humans, *ad libitum* food intake at a buffet meal served 2 hours after the completion of a 90 min intravenous infusion of PYY₃₋₃₆ delivered at a dose of 0.8 pmol/kg/min, was reduced by 36% compared with an infusion of saline. The PYY₃₋₃₆ infusion generated plasma levels of PYY₃₋₃₆ similar to those achieved physiologically after a meal (Batterham et al., 2002). Thus, it was suggested that PYY₃₋₃₆ inhibits food intake physiologically. Energy intake measured over the subsequent 24 hours after the infusion of PYY₃₋₃₆ was reduced by 33% compared with saline. This pointed towards a role for PYY₃₋₃₆ in the intermediate control of food intake beyond the study test meal. Significantly, there was no reported nausea in subjects infused with PYY₃₋₃₆.

Some studies have reported that obese individuals have lower basal fasting levels of PYY₃₋₃₆ and have a smaller rise in postprandial levels (Batterham et al., 2003). Obesity does not appear to be associated with resistance to PYY₃₋₃₆, as in obese subjects, there was a 30% in a reduction in *ad libitum* food intake at a buffet meal served 2 hours after completion of a 90 minute infusion of PYY₃₋₃₆ delivered at an unspecified dose based on body surface area (Batterham et al., 2003). This compared with a 31% reduction in food intake in a group of

lean subjects who also received a peripheral infusion of PYY₃₋₃₆. Additionally, there was also a comparable reduction in 24 hour energy intake in both lean and obese subjects following PYY₃₋₃₆ infusion.

A similar study administered a 90 minute intravenous infusion of PYY₃₋₃₆ to lean and overweight human subjects at a dose of 0.8 pmol/kg/min, with 19% reduction in *ad libitum* food intake at a buffet meal served 2 hours after completion of the infusion (Sloth et al., 2007). However, in contrast to the studies by Batterham *et al*, significant nausea was experienced by subjects, culminating in only 4 of the first 9 being able to complete their infusion. This study also found that there was increased thermogenesis, lipolysis, post-prandial insulin and glucose responses in those receiving PYY₃₋₃₆, suggestive of increased sympathoadrenal activity and increased energy expenditure. However, the effect of nausea on these latter findings is a potential confounder.

Significant problems with nausea and vomiting were also encountered when intra-nasal PYY₃₋₃₆ was tested in obese human subjects as a weight-loss treatment (Gantz et al., 2007). In this study, placebo or intra-nasal PYY₃₋₃₆ was administered at a dose of 200 mcg 3 times daily or 600 mcg 3 times daily for 12 weeks. Only 70% of subjects in the low-dose group completed the study and this figure was even lower (26%) in the high-dose group. The mean weight loss from baseline over the 12 weeks was 2.8 kg in the placebo group, 3.7 kg in the 200 mcg PYY₃₋₃₆ group, and only 1.4 kg in the 600 mcg PYY₃₋₃₆ group (Gantz et al., 2007). Pharmacokinetics revealed rapid (C_{max} 20 min) absorption of PYY₃₋₃₆ to pharmacological plasma levels. This is likely to have been a major contributor to nausea.

Oral delivery of peptide hormones has been unsuccessful on the whole, due to the harsh environment of the gastrointestinal tract and thus failure of absorption across the intestinal wall. The subcutaneous route is therefore the established route for peptide hormone delivery; insulin being the classical example. However, it could be argued that oral administration of gut hormones better mimics the physiological post-prandial state (given that these peptides are naturally secreted from the gut, where they may act on local vagal afferents, in addition to being absorbed into the portal circulation before acting systemically). Subcutaneous administration does not allow for these potentially important local physiological gastrointestinal effects. Encouragingly, a recent study of oral delivery of PYY₃₋₃₆ with sodium N-caprylate (Emisphere Technologies, Cedar Knolls, NJ) to 12 healthy human subjects showed that there was effective absorption of the peptide from the gut (Steinert et al., 2010). Supraphysiological plasma levels were achieved, but despite this, no nausea was encountered with administration of oral PYY₃₋₃₆ alone. Energy intake at a buffet meal served 15 minutes later was reduced by 12% compared with placebo, although this was not found to be statistically significant. 24 hour energy intake was unchanged by oral PYY₃₋₃₆ administration. Emisphere's sodium N-caprylate technology uses hydrophobic moieties on carrier molecules, which when in association with a peptide such as PYY₃₋₃₆, allows for the creation of a more lipophilic complex. This facilitates absorption of the peptide across the intestinal wall.

Batterham *et al.* performed a series of experiments in rodents which shed considerable light on the action of PYY₃₋₃₆ on CNS appetite circuits (Batterham et al., 2002). Firstly, it was shown that chronic peripheral administration of PYY₃₋₃₆ resulted in a decrease in food intake and body weight. Furthermore, a single peripheral injection of PYY₃₋₃₆ resulted in induction of expression of the immediate-early gene *c-fos* (a marker of neuronal activation) in the hypothalamic ARC. This suggests that the ARC is an important site of action of the peptide. Furthermore, a single peripheral injection of PYY₃₋₃₆ caused a decrease in expression of

hypothalamic NPY mRNA 6 hours later. A subsequent single injection of PYY₃₋₃₆ directly into the ARC inhibited food intake. Inhibition of food intake was also observed with intra-arcuate administration of a Y₂ receptor specific agonist. Significantly, this effect was absent in Y₂ receptor knock-out mice. Addition of PYY₃₋₃₆ to *ex-vivo* hypothalamic explants inhibited release of NPY and stimulated release of α -MSH. This finding was further validated by electrophysiological studies, which demonstrated that POMC neurons showed disinhibition when exposed to PYY₃₋₃₆. It therefore appears that circulating PYY₃₋₃₆ inhibits appetite by acting directly on the ARC via the Y₂ receptor, increasing the activity of anorexigenic POMC/ α -MSH neurons, whilst suppressing orexigenic NPY neurons.

In contrast to peripheral and intra-ARC injection, administration of PYY₃₋₃₆ into the 3rd ventricle or the PVN is orexigenic in mice (Morley et al., 1985, Stanley et al., 1985). This is thought to be due to action on Y₁ and Y₅ receptors in these regions. In contrast to the anorectic effect produced by Y₂ receptor activation, Y₁ and Y₅ receptor activation promotes feeding (Kanatani et al., 2000).

The Y₂ receptor is abundantly distributed within the hypothalamic ARC, preoptic nucleus and DMN (Gustafson et al., 1997). Given the presence of an incomplete blood-brain-barrier in the hypothalamic median eminence, which lies close to the ARC, it is plausible that circulating PYY₃₋₃₆ accesses the CNS at this level. Furthermore, the Y₂ receptor is found in the NTS of the brainstem (Gustafson et al., 1997). In keeping with this, peripheral injection of PYY₃₋₃₆ activates *c-fos* expression in the AP and the NTS (Blevins et al., 2008). Therefore, circulating PYY₃₋₃₆ may access the brainstem via the incomplete blood brain barrier at the AP. Taking into account the presence of ascending and descending projections between the brainstem and hypothalamus (as discussed earlier), it is possible that there is communication between these areas with regard to PYY₃₋₃₆ action.

Other forebrain areas that express the Y_2 receptor include the posterior hypothalamic nuclei, medial nucleus of the amygdala, substantia nigra and parabrachial area (Dumont et al., 1998). Action on the limbic system (including the amygdala) may be of significance when considering modulation of hedonic pathways by PYY₃₋₃₆.

PYY₃₋₃₆ may also act via the vagus-brainstem-hypothalamic pathway (Koda et al., 2005). Using vagal ligation studies, Koda *et al.* showed that Y_2 receptors are transported to the peripheral terminals of vagal afferent neurons. Furthermore, peripheral administration of PYY₃₋₃₆ led to afferent vagal discharges, while disruption of the vagus-brainstem-hypothalamus pathway abolished its anorexigenic effect.

Table 1.1 summarises some of the major translational human studies investigating the role of PYY₃₋₃₆ in the control of appetite.

Study	Major findings
Batterham <i>et al.</i> 2002	In lean subjects, 36% reduction (compared with placebo) in <i>ad libitum</i> food intake at meal served 2 hours after 90 min intravenous infusion of PYY ₃₋₃₆ . In the same group, 33% reduction (compared with placebo) in 24 hour food intake following 90 min intravenous infusion of PYY ₃₋₃₆ .
Batterham <i>et al.</i> 2003	In overweight subjects, 30% reduction (compared with placebo) in <i>ad libitum</i> food intake at meal served 2 hours after 90 min intravenous infusion of PYY ₃₋₃₆ .
Sloth <i>et al.</i> 2007	In lean and overweight subjects, 19% reduction (compared with placebo) in <i>ad libitum</i> food intake at meal served 2 hours after 90 min intravenous infusion of PYY ₃₋₃₆ .
Gantz <i>et al.</i> 2007	Rapid absorption and significant nausea following prandial, intranasal administration of PYY ₃₋₃₆ to obese subjects over 12 weeks.
Steinert <i>et al.</i> 2010	In lean subjects, effective absorption of oral PYY ₃₋₃₆ when delivered with sodium N-caprylate. 12% reduction (compared with placebo) in <i>ad libitum</i> food intake at meal served 15 min after oral PYY ₃₋₃₆ .

Table 1.1: Important translational human studies involving PYY₃₋₃₆.

1.7.2. Glucagon-like peptide 1 (GLP-1)

Proglucagon is a 160-amino acid prohormone that is produced in the α -cells of the pancreatic islets, the L cells of the gut and within the CNS. Selective post-translational proteolysis of proglucagon by prohormone convertases 1 and 2 results in the tissue-specific production of a number of biologically-active fragments. In the gut and CNS, with respect to the regulation of appetite and food intake, the cleavage product GLP-1 will be the focus of this section.

GLP-1 is an incretin hormone, enhancing endogenous insulin secretion in response to a post-prandial glucose load. GLP-1 is released post-prandially into the circulation from the gastrointestinal L cells in proportion to the calories ingested (Ghatei et al., 1983, Orskov et al., 1994). It has been shown that GLP-1 shows a biphasic response after a meal. As with PYY, the first peak occurs before nutrients enter the distal gut (Delzenne et al., 2010) and has been shown to be augmented by a high-carbohydrate meal (Smeets et al., 2008). This is presumably via the effect of carbohydrates absorbed in the proximal gut. It is thought that the second peak of secretion of GLP-1 is triggered by free fatty acids in the intestinal lumen, via activation of the G-protein coupled receptors GPR 40 (Edfalk et al., 2008), GPR 119 (Chu et al., 2008, Lauffer et al., 2009) and GPR 120 (Hirasawa et al., 2005). The two forms synthesized are GLP-1₁₋₃₇ and GLP-1₁₋₃₆ amide. Further cleavage produces the bioactive fragments GLP-1₇₋₃₇ and GLP-1₇₋₃₆ amide. The major circulating bioactive species of GLP-1 is the truncated form GLP-1₇₋₃₆ amide (Orskov et al., 1994). It is rapidly broken down by DPP-IV and has a half-life of 2 minutes (Kieffer et al., 1995).

GLP-1 acts by binding to the G-protein coupled GLP-1 receptor (GLP-1R). GLP-1₇₋₃₇ and GLP-1₇₋₃₆ amide are equally potent at the GLP-1R (Thorens et al., 1993). The GLP-1R is expressed in pancreatic islets, where GLP-1 functions as an incretin hormone. This allows for enhanced glucose-dependent insulin release post-prandially (Kreymann et al., 1987). GLP-1 also inhibits glucagon secretion and delays gastric emptying (Gutniak et al., 1992, Willms et al., 1996). The GLP-1R is also present in a number of CNS areas implicated in appetite control. These include the ARC and PVN in the hypothalamus and the AP in the brainstem (Wei and Mojsov, 1995, Shughrue et al., 1996). Neurons containing GLP-1₇₋₃₆ amide are located in the AP and NTS. Furthermore, there are projections to the dorsomedial hypothalamus (Larsen et al., 1997).

It is well established that administration of GLP-1₇₋₃₆ amide peripherally or centrally leads to reduced food intake in rodents, whereas co-administration of an antagonist of GLP-1₇₋₃₆ amide (exendin₉₋₃₉) abolishes this effect (Turton et al., 1996). In the same study, single administration of exendin₉₋₃₉ doubled food intake in satiated rats in addition to potentiating the feeding response to NPY administration, suggesting that GLP-1₇₋₃₆ amide regulates appetite physiologically.

A range of studies has demonstrated that peripheral administration of GLP-1₇₋₃₆ amide to humans leads to a dose-dependent reduction in appetite and *ad libitum* energy intake. A meta-analysis of major studies (Verdich et al., 2001) revealed that intravenous infusion of GLP-1₇₋₃₆ amide reduces energy intake dose-dependently in both lean and overweight subjects. The protocols in the individual studies within the meta-analysis varied considerably, in addition to including a mixture of lean, overweight and obese subjects in the studies analysed. The duration of the infusions ranged between 0 and 240 minutes (spanning 60 minutes in the majority of studies). Furthermore, the timing of the *ad libitum* buffet meal with

regard to the infusion varied between studies, such that in some, the meal was served with the infusion still running from the outset (Naslund et al., 1999, Long et al., 1999, Gutzwiller et al., 1999) whereas in others, the infusion was stopped 30 minutes before the meal was served and then restarted at the onset of the meal (Flint et al., 1998, Flint et al., 2001). In one study of 6 obese subjects, the meal was served at the beginning of the infusion (Naslund et al., 1998). Finally, individual protocols varied such that the dose of infused GLP-1₇₋₃₆ amide in individual studies ranged between 0.375 pmol/kg/min and 1.5 pmol/kg/min. Amalgamating the individual study data in the meta-analysis revealed that the mean infusion rate was 0.89 pmol/kg/min across all subjects. In lean subjects, infusion of GLP-1₇₋₃₆ amide led to a 13.2% reduction in energy intake at the *ad libitum* buffet meal compared with saline infusion. When the 6 obese subjects who were served their meal at the beginning of the infusion were excluded from the analysis, the reduction in *ad libitum* energy intake for overweight / obese subjects by GLP-1₇₋₃₆ amide was comparable (10.5%) with that of lean subjects (Verdich et al., 2001). Data on nausea and other well-being scores were only included in 2 of the studies in the meta-analysis. Nausea was not encountered in either of these studies. In addition to the intravenous infusion studies described above, it was found that daily prandial subcutaneous injections of GLP-1₇₋₃₆ amide (76 nmol 4 times daily) to obese human subjects over 5 days led to a 15% mean reduction in energy intake at each meal compared with placebo. Over 5 days, this translated to a mean weight loss of 0.55 kg. (Naslund et al., 2004) However, feelings of sickness were reported in 8 out of 19 subjects receiving prandial subcutaneous injections of GLP-1₇₋₃₆ amide, compared with 2 out of 7 subjects receiving placebo injections.

The brainstem is thought to be an important site of action of peripheral GLP-1₇₋₃₆ amide. *c-fos* expression occurs in the brainstem only after the peripheral and not central injection of GLP-1₇₋₃₆ amide. (Turton et al., 1996). In contrast, both peripheral and central injection of GLP-1₇₋₃₆ amide induce *c-fos* in the PVN (Baggio et al., 2004). Furthermore, vagotomy or lesions of

projections from the brainstem to the hypothalamus reduce the appetite suppression mediated by peripheral GLP-1₇₋₃₆ amide (Abbott et al., 2005). Therefore, as seems to be the case with PYY₃₋₃₆, peripheral GLP-1₇₋₃₆ amide may either act via the vagus, with projections to the brainstem and then hypothalamus, or alternatively may act directly on the brainstem (gaining access from the circulation at the AP) or hypothalamus (gaining access from the circulation at the median eminence). Furthermore, action on brain food reward regions (such as the pre-frontal cortex) is likely, either directly or through communication via homeostatic centres.

The fact that circulating GLP-1 is rapidly broken down by DPP-IV has been a challenge to developing its use clinically. Exenatide (exendin-4) is a currently licensed DPP-IV resistant GLP-1R agonist that has enjoyed success in producing improved glycaemic control and promoting weight loss when administered subcutaneously to overweight patients with Type 2 diabetes (Ratner et al., 2006, Riddle et al., 2006). Long acting analogues of GLP-1 such as liraglutide are also licensed for subcutaneous use in Type 2 diabetes as adjunctive therapy, following favourable efficacy and safety data (Nauck et al., 2009, Zinman et al., 2009, Garber et al., 2009, Marre et al., 2009). The half-life of liraglutide is prolonged by albumin binding. The fact that exenatide and liraglutide produce an incretin effect is favourable in enhancing endogenous insulin secretion in response to a post-prandial glucose load. Furthermore, their effects of suppressing food intake and causing weight loss (through reduction in gastric emptying and central effects on the CNS as discussed above) makes this line of treatment particularly attractive in treating overweight patients with Type 2 diabetes. However, one major limitation surrounds their well-recognised side effect of nausea. Although it occurs commonly, the incidence of nausea declines with duration of treatment (Ratner et al., 2006, Riddle et al., 2006).

In order to avoid the need for injections, nonpeptidic GLP-1R agonists in oral form have been developed and tested in rodents (Knudsen et al., 2007, Chen et al., 2007). Recently, delivery of oral GLP-1₇₋₃₆ with sodium N-caprylate to healthy human subjects reduced energy intake by 13.6% during a meal served 15 minutes later, with marked effects on glucose homeostasis (Steinert et al., 2010). However, in this study, 24 hour energy intake was unaffected by GLP-1₇₋₃₆.

In addition to GLP-1R agonism, oral DPP-IV inhibitors such as sitagliptin and vildagliptin are also currently licensed as adjunctive therapy in Type 2 diabetes. Although they confer the advantage of being available in oral form, unlike exenatide and liraglutide, they tend to be weight-neutral (Green et al., 2006). This may well be due to the fact that DPP-IV has a role in activating the anorectic gut hormone PYY₃₋₃₆; DPP-IV inhibitors may therefore lead to reduced levels of active PYY₃₋₃₆ and theoretically result in a lesser degree of appetite inhibition. Finally, although currently not licensed, GPR 119 agonists (i.e. GLP-1 secretagogues) are under development for use in Type 2 diabetes (Lauffer et al., 2008).

Outside of the context of Type 2 diabetes, liraglutide has proven successful in causing significant weight loss when administered over 20 weeks to obese, non-diabetic patients (Astrup et al., 2009). Administration of liraglutide subcutaneously at a dose of 3 mg once daily led to a mean weight loss of 7.2 kg, compared with 2.8 kg with placebo and 4.1 kg with orlistat 120 mg orally 3 times daily. 76% of participants on liraglutide 3 mg once daily lost >5% body weight (Astrup et al., 2009). Nausea and vomiting occurred more frequently in the liraglutide group than in the placebo group, but this rarely led to discontinuation of treatment. The results of this randomised, double-blind, placebo-controlled study hold promise for the licensing of GLP-1 analogues in obesity alone.

Table 1.2 summarises some of the major translational human studies investigating the role of GLP-1 in the control of appetite.

Study	Major findings
Verdich <i>et al.</i> 2001 (meta-analysis)	In lean subjects, 13.2% reduction (compared with placebo) in <i>ad libitum</i> food intake at meal served following intravenous infusion of GLP-1 ₇₋₃₆ amide. Comparable 10.5% reduction in <i>ad libitum</i> food intake in overweight/ obese subjects. Individual study protocols within the meta-analysis varied.
Naslund <i>et al.</i> 2004	In obese subjects, 15% mean reduction in <i>ad libitum</i> energy intake at each meal (compared with placebo) following prandial, subcutaneous administration of GLP-1 ₇₋₃₆ amide over 5 days.
Steinert <i>et al.</i> 2010	In lean subjects, effective absorption of oral GLP-1 ₇₋₃₆ amide when delivered with sodium N-caprylate. 13.6% reduction (compared with placebo) in <i>ad libitum</i> food intake at meal served 15 min after oral GLP-1 ₇₋₃₆ amide.
Astrup <i>et al.</i> 2009	In obese, non-diabetic subjects, daily subcutaneous administration of liraglutide over 20 weeks led to a mean weight loss of 7.2 kg (compared with 2.8 kg with placebo)

Table 1.2: Important translational human studies involving GLP-1

1.7.3. Studies investigating combined administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide

During and after a meal, a variety of hormones are released from the gut into the circulation. These are thought to act in concert in producing an overall satiety response, thus facilitating meal termination and increasing the period of time before the next meal is sought. Preliminary research has focused on deciphering the role and mode of action of individual gut hormones in appetite regulation. However, more recently, there has been an interest in investigating how these satiety factors act in concert to regulate appetite. This is particularly relevant when considering that in the physiological fed state, a multitude of gut hormones are released into the circulation. More effectively mimicking the physiological fed state using combination gut hormone analogues may prove promising for the future treatment of obesity.

In the case of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, it was shown (Neary et al., 2005) that in 10 healthy, lean human subjects, co-administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide (each at 0.4 pmol/kg/min over 120 min) produced a 27% reduction in *ad libitum* energy intake (compared with saline) during a buffet meal served 90 minutes into the infusion (the infusion was continued during the meal). Individually, single infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide at the above doses did not produce a statistically significant reduction in energy intake compared with saline. Importantly, no nausea was experienced during any of the infusions, including the combined PYY₃₋₃₆ and GLP-1₇₋₃₆ amide infusion.

In the same paper (Neary et al., 2005), rodent studies were carried out and findings strengthened those of the human study; combination of peripheral PYY₃₋₃₆ and GLP-1₇₋₃₆ amide led to reduction in food intake that was greater than that observed when either hormone was infused individually at twice the dose. In the hypothalamic ARC, no changes were observed

after low-dose PYY₃₋₃₆ or GLP-1₇₋₃₆ amide individually, but co-administration led to *c-fos* expression.

In a recent study of 12 healthy human subjects, co-administration of oral PYY₃₋₃₆ and GLP-1₇₋₃₆ amide in conjunction with sodium N-caprylate lead to a 21.5% reduction in energy intake during a meal served 15 minutes later (Steinert et al., 2010). Admittedly, 2 subjects experienced significant gastrointestinal side effects of nausea, abdominal discomfort and vomiting. Furthermore, 24 hour energy intake was no different compared with placebo.

Figure 1.3 provides an overview of the likely major interactions between PYY, GLP-1, the hypothalamus, brainstem and non-homeostatic brain regions in the regulation of appetite and food intake.

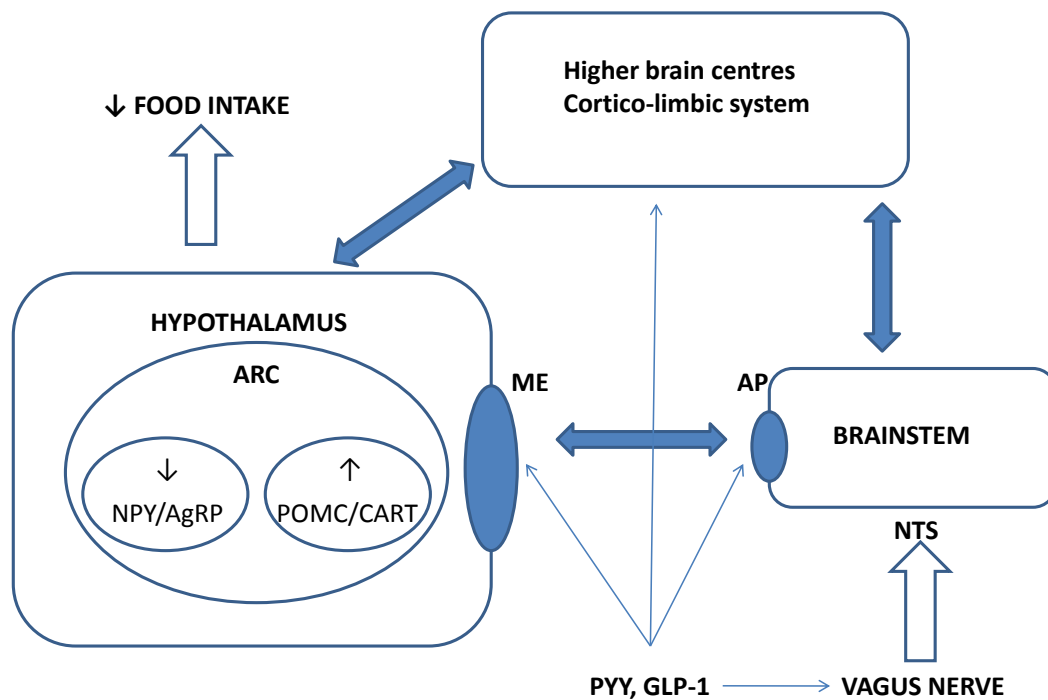


Figure 1.3: An overview of the role of PYY and GLP-1 in the control of appetite and food intake. Gut hormones such as PYY and GLP-1 are released post-prandially into the peripheral circulation. They may interact with the CNS via local vagal afferents that project to the brainstem nucleus of the tractus solitaries (NTS). Alternatively, they may act directly on the brainstem NTS, via an incomplete blood-brain-barrier at the area postrema (AP) or on the hypothalamic arcuate nucleus (ARC), via an incomplete blood-brain-barrier at the median eminence (ME). Within the ARC, NPY/AgRP neurons are predominantly orexigenic, whereas POMC/CART/ α MSH neurones are predominantly anorexigenic. AgRP is a competitive antagonist at central melanocortin receptors. There are connections between hypothalamus, brainstem, reward centres and mnemonic centres, important in the overall regulation of appetite and food intake.

1.8. Other hormones implicated in the control of food intake

This section introduces other hormones, discussed elsewhere in this thesis, that are implicated in the control of appetite and food intake.

1.8.1. Ghrelin

Ghrelin is the only known gut hormone that increases appetite. It was first discovered as an endogenous ligand to the growth hormone secretagogue receptor (GHS-R) in the stomach. It is primarily produced by the A-cells of gastric fundus. Its other actions include increase in gastric motility and stimulation of growth hormone release. Its metabolic effects include increased fat storage and decrease in fat use (van der Lely et al., 2004).

Plasma ghrelin levels rise before meals and decline after eating (Cummings et al., 2001), implicating a role for ghrelin in controlling appetite. Peripheral administration of ghrelin increases food intake in humans (Wren et al., 2001, Druce et al., 2005) and ghrelin is now considered as one of the most powerful physiological orexigenic agents.

Ghrelin mediates its effects on food intake via the GHS-R. GHS-R deficient mice are resistant to diet induced obesity (Zigman et al., 2005). The ARC NPY/AgRP neurons have been implicated in mediating ghrelin's central orexigenic effects. Ghrelin may increase food intake by binding to the GHS-R on vagal afferent nerve terminals or in the neurones of the ARC to alter ARC NPY/AgRP neuronal activity (van der Lely et al., 2004).

Ghrelin levels are inversely correlated with body weight and may constitute a feedback mechanism to reduce appetite in obesity (Chaudhri et al., 2006a). Plasma ghrelin levels are also noted to increase upon weight loss (Chaudhri et al., 2006a). This may be a factor resulting in poor adherence to diets and tendency to regain weight after initial weight loss.

1.8.2. Insulin

Insulin is released by the beta cells of the pancreas in a glucose sensitive manner and binds to the insulin receptor. The primary role of insulin is to increase glucose uptake in peripheral tissues, decrease hepatic glucose production and maintain glucose levels. Its release peaks postprandially in proportion to the glucose influx following a meal. Similar to leptin, insulin levels are also influenced by the amount of adipose tissue in the body, with higher levels in more obese individuals. Therefore, insulin is also considered an adiposity signal (Woods et al., 2006).

The most common effect on appetite mediated by insulin is seen in hypoglycemia following treatment with excess insulin in diabetes. In this situation, there is a marked increase in food intake. This effect is due to low blood glucose rather than due to insulin directly (see Section 1.9.3.). Insulin itself crosses the blood brain barrier in a dose dependant manner and acts on ARC insulin receptors to decrease food intake (Marks et al., 1990). When insulin is centrally administered, a decrease in food intake and body weight is noted (Woods and Seeley, 2001). The opposite is seen when insulin blocking antibodies are injected in the hypothalamus and when insulin receptor expression is selectively decreased in the ARC (McGowan et al., 1992, Obici et al., 2002). Insulin levels also augment the effect of other satiety signals, an effect seen with leptin as well (Woods et al., 2006). Therefore insulin

provides signals to the brain reflecting circulating energy in the form of glucose and stored energy in the form of adipose tissue. These signals interact with other satiety signals to reduce food intake.

1.8.3. Leptin

Leptin is a hormone produced largely in the adipose tissue, with levels proportional to fat stores and increased by overfeeding. Leptin provides a mechanism by which body fat can control food intake via a feedback system on the hypothalamus. This idea was initially proposed by Gordon Kennedy in 1953 as the 'lipostatic theory' (Kennedy, 1953). Friedman and colleagues demonstrated the absence of leptin in the inbred Ob/Ob strain of severely obese mice (Zhang et al., 1994). Subsequently, human congenital leptin deficiency was identified in 2 severely obese cousins (Montague et al., 1997). The peripheral administration of leptin in both Ob/Ob mice and human congenital deficiency, as well as central administration of leptin in Ob/Ob mice reversed the obese phenotype (Friedman and Halaas, 1998, Farooqi et al., 1999). Therefore, leptin is a signal from the adipose tissue to the brain reflecting the state of energy stores and significantly influencing appetite (Farooqi and O'Rahilly, 2009).

Leptin exerts its effects by acting on the leptin receptor (LepR). LepR mutations in mice (Db/Db strain mice) and humans are associated with severe obesity (Farooqi and O'Rahilly, 2009). Leptin acts on ARC LepR to stimulate POMC neurons and inhibits NPY/AgRP neurons to decrease food intake (Farooqi and O'Rahilly, 2009). Downstream signalling of leptin occurs via the MC4R (Farooqi and O'Rahilly, 2009). Leptin also acts on leptin receptors in other parts of the brain and has important effects on reward pathways, energy

expenditure, pubertal development, fertility, and immune function (Farooqi and O'Rahilly, 2009).

Leptin levels are elevated in obese individuals and administration of leptin in common obesity has a variable effect on food intake (Farooqi and O'Rahilly, 2009). This failure of leptin to diminish appetite in obesity is termed leptin resistance and is not completely understood.

1.9. Neural signals controlling food intake

1.9.1. Orosensory and visual stimuli

Orosensory and visual stimuli provide the brain with sensory information regarding the nature of food. These stimuli include appearance, taste, smell and texture. Visual information regarding the appearance of food is relayed via neural signals in the afferent optic fibres of cranial nerve II. Gustatory, olfactory, orosensory information about food in contact with the tongue and palate is transduced into neural signals by the gustatory fibres of cranial nerves VII, IX, and X, olfactory fibres of cranial nerve I, and sensory fibres of cranial nerve V. These neural signals are directly or indirectly relayed to areas of the brain important for taste, reward, flavour, and mnemonic representations, such as the limbic system and OFC (Rolls, 2006). The information is used by the brain to continue or to stop eating. The stimuli of the food (e.g. sweet or bitter) and prior experience with eating the food (conditioned preferences, such as reward, or conditioned aversions, such as nausea) influence the decision to eat or

not to eat. The potency of these stimuli in promoting food intake during a meal is increased by food deprivation as well as other signals (Rolls, 2006).

1.9.2. Gastric distension

Volume related post-prandial gastric distension results in satiety during a meal. This is demonstrated in rats by the use of a chronic gastric fistula. Food intake is increased when gastric contents are continuously drained whilst these rats eat (Smith, 1998). Pyloric cuffs can reversibly close the pylorus, restrict the food to the stomach and prevent the passage of food downstream. Experiments utilising reversible pyloric cuffs in animals provide further support that gastric distension contributes to satiety during a meal (Smith, 1998, Ritter, 2004). These satiety signals arise from mechanical distension of the stomach rather than nutrient sensing (Smith, 1998). Mechanoreceptors in the stomach wall sense stretch, volume, and tension during a meal. This information is transmitted to the brain by afferent fibres of the vagal and spinal visceral nerves (Cummings and Overduin, 2007). Earlier gastric distention and satiety during a meal resulting from a reduced stomach size forms the basis of 'restrictive' bariatric surgical treatments (e.g. laparoscopic adjustable gastric banding) that are used in the management of severe obesity.

1.9.3. Glucose

In addition to glucose exerting its effects on the gastrointestinal system by inducing the secretion of insulin, the level of glucose itself may be sensed by hypothalamic neurons in the brain involved in appetite regulation. This 'glucostatic theory of feeding' was first proposed

by Jean Mayer more than 50 years ago (Mayer, 1952). Although it has been noted for some time that peripheral and central glucoprivation (low glucose) can stimulate feeding, for example during hypoglycaemia, the role of central glucosensing in affecting day to day food intake and the mechanisms involved in regulating this have only been elucidated recently.

Glucose alters the firing rate of neurons in the ARC, LHA, and NTS (Levin, 2006). The cellular influx of glucose alters the ratio of AMP: ATP within the neuronal cell. This may affect ATP-dependant membrane channels that may influence neuronal depolarisation or may alter the activity of important nutrient sensing enzymes, such as AMP-activated protein kinase, that have important roles in cellular processes relevant to energy homeostasis (Jordan et al., 2010). Some neurons, such as ARC POMC neurons, are excited by glucose whereas others, such as ARC NPY neurons, are inhibited by glucose (Levin, 2006). These glucose sensitive neurons also respond to other hormonal and metabolic signals, such as insulin, leptin, lactate, ketone bodies, and free fatty acids (Levin, 2006). Glucose sensitive neurons may represent focal points where signals converge to alter appetite and allow various signals to act in concert to influence the initiation and termination of meals.

1.10. The use of imaging techniques to determine CNS neuronal activation in the regulation of feeding

In humans, there is a need to further investigate the physiology of appetite control within the CNS in a non-invasive fashion. A deeper understanding of CNS pathways governing appetitive behaviour and in particular, shedding further light on interactions between gut hormones and key brain regions implicated in appetite control, holds promise for the development of pharmacotherapies to combat obesity. In recent years, functional neuroimaging techniques have formed important tools to investigate the above. Techniques such as positron emission tomography (PET) and single photon emission tomography

(SPECT) have been used to investigate patterns of brain activation associated with factors that underpin the regulation of feeding, but they have significant limitations in human research. Firstly, the above techniques are associated with exposure to ionising radiation. Additionally, SPECT suffers from low signal/noise ratio and the requirement for access to a cyclotron greatly limits the availability of PET. In addition, the spatial resolution offered by PET and SPECT is limited to several millimetres, which therefore permits the investigation of changes in neuronal activity at a regional level only (Tataranni and DelParigi, 2003). Magnetic resonance imaging (MRI) (and its adaptation to functional magnetic resonance imaging (fMRI)) exhibits clear advantages over PET and SPECT in that it offers better spatial resolution of tens of microns (Tataranni and DelParigi, 2003) and is relatively non-invasive. Furthermore, MRI does not require exposure to ionising radiation, thereby allowing for repeated measures in a given subject.

1.11. Overview of fMRI

MRI is based on the physical phenomenon of nuclear magnetic resonance. Nuclei that have an odd total number of protons and neutrons possess angular momentum (spin). The hydrogen nucleus is one such example, as it consists of a single proton. As soon as an external magnetic field (B_0) is applied, the protons in tissue tend to align with this, causing their spins to precess about a circular path around B_0 . Here, they are in a low-energy state. Generation of MRI images requires application of a radiofrequency (RF) pulse at 90 degrees to B_0 . The protons will then 'tip' to align with the RF pulse. In doing so, they gain energy. After the RF pulse is switched off, the protons re-align with B_0 . Spins return to the low energy state by emitting the absorbed energy, also in the form of a radiowave. The emitted energy can be measured by the 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. The T1 relaxation time is a time constant referring to the realignment of spins with B_0 in the longitudinal plane after the RF pulse is switched off. The T2 relaxation time, on the other hand, is a time constant referring to the de-phasing of spins in the transverse plane after the RF pulse is switched off (figure 1.4). T1 and T2 vary depending on the tissue being imaged. The strength of magnetic resonance signal obtained for a particular tissue depends primarily on the proton density. However, by altering the time between successive RF pulses and therefore the degree of T1 and T2 relaxation, the image can be weighted towards one or the other of these tissue-specific properties. It should be noted that, in practice, due to localised inhomogeneities in the externally applied magnetic field, T2 is shorter than expected for any particular tissue. This apparent T2 is referred to as T2*. fMRI utilises adaptations of this classical MRI technique, such that function of tissues can be analysed rather than structure alone.

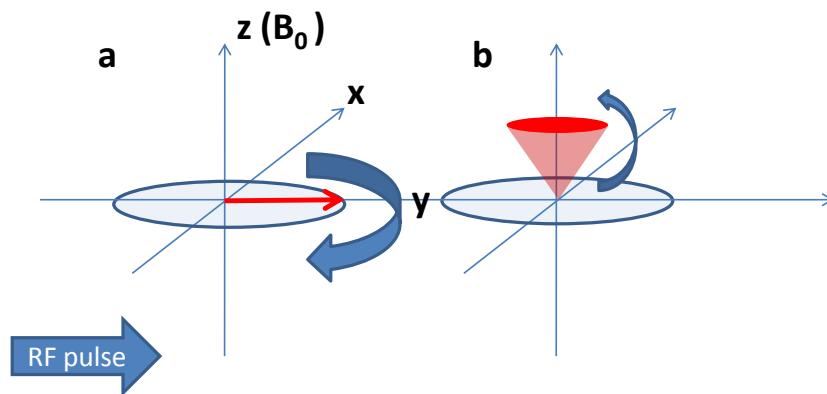


Figure 1.4: T1 and T2 relaxation. MRI utilises the behaviour of protons within varying magnetic fields to produce signals which can be converted into images. Each hydrogen nucleus in the brain can be thought of as a vector (in the z and x-y planes) representing the strength and direction of its magnetic field as it spins on its axis. The spins of the imaged protons try to align with the main external magnetic field of the scanner (referred to here as B_0 and conventionally shown along the z axis in 3D coordinates). A second magnetic field (in the form of a short radiofrequency (RF) pulse) is applied, which flips all of the spins from alignment in the z plane into the x-y plane (figure 1.4 a). Before application of the RF pulse the amplitude in the z-plane is maximal while the amplitude in the x-y plane is zero. Just after application of the RF pulse the amplitude in the z-plane is zero while the amplitude in the x-y plane is maximal (figure 1.4 a). During relaxation (figure 1.4 b) the amplitude in the z-plane will slowly increase while the amplitude in the x-y plane slowly decreases. 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 proton density of the subject being scanned.

1.11.1. fMRI - Manganese-enhanced MRI (MEMRI)

In animals, manganese-enhanced MRI (MEMRI) has been used to determine regions of the CNS activated following gut hormone administration. This technique utilises the fact that unpaired electrons in the outer shell of Mn^{2+} ions alter the relaxation properties of protons in a magnetic field, resulting in an increased T1 weighted signal (Mendonca-Dias et al., 1983). This is proportional to the concentration of Mn^{2+} in the tissue. Mn^{2+} can therefore be used as a contrast agent and has the advantage (compared with chelated gadolinium contrast agents) that its volume of distribution matches the intracellular volume (Crossgrove et al., 2003). Additionally, Mn^{2+} ions biologically mimic Ca^{2+} ions, and are able to enter L-type voltage gated calcium channels. Normal depolarisation of neurons involves entry of Ca^{2+} through L-type voltage-gated calcium channels (Narita et al., 1990). Hence, administration of $MnCl_2$ contrast allows a functional assessment of neuronal activity, given that Mn^{2+} will mimic Ca^{2+} , enter depolarising neurons and increase the T1 weighted signal.

MEMRI without disruption of the blood-brain-barrier has been used to demonstrate an increase in hypothalamic T1 weighted signal in fasted compared with non-fasted rodents in the ARC, PVN and VMH (Kuo et al., 2006). With regard to gut hormone action, MEMRI without blood-brain-barrier disruption showed that peripherally administered ghrelin (an orexigenic gut hormone) increased hypothalamic signal intensity in the ARC and VMH in fed mice. On the other hand, peripherally administered PYY_{3-36} caused a reduction in hypothalamic signal intensity in the same regions (Kuo et al., 2006) in fasted mice. The effect of $GLP-1_{7-36}$ amide has also been studied using MEMRI; there was reduction in hypothalamic signal in the PVN and an increase in the VMH (Chaudhri et al., 2006b). Unfortunately, MEMRI cannot be used to study CNS activation in humans due to the toxicity conferred by Mn^{2+} ions.

1.11.2. fMRI - Blood-oxygen-level-dependent (BOLD) fMRI

Virtually all human 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 T2*-weighted imaging protocol (Kwong et al., 1992, Ogawa et al., 1992); the locally reduced ratio of deoxyhaemoglobin to oxyhaemoglobin leads to a longer T2*, producing an increased image intensity. Such protocols typically use magnetic gradients to generate the MR signal (gradient-echo imaging) and, as scanner and computational hardwares have 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 has thus provided hugely important insights in numerous disciplines. However, its use in the field of neuroendocrinology has been limited due to the inherent weakness of the technique at imaging the hypothalamus. This small (approximately 5-8 mm in diameter) brain structure lies close to the air-tissue boundaries of surrounding sinuses and is susceptible to signal loss due to resultant distortions of the external magnetic field, which is a particular issue for T2*-weighted images (Ojemann et al., 1997).

1.12. BOLD fMRI image reconstruction

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 there is different compared with the signal intensity elsewhere. Whilst this approach may be useful for exploratory studies, most fMRI experiments perform ROI (region of interest) analyses whereby the functional properties of a pre-determined set of voxels (corresponding to a *priori* anatomical areas of the brain) are analysed. ROI analysis, with very many fewer degrees of freedom than whole brain voxel-wise analysis, has a greater chance of finding statistically significant results but entails the inherent risk of missing activated areas of the brain that were not included in the original hypothesis. Resting state functional connectivity analyses 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). Resting state scans are performed when the subject is not performing any purposeful task and have recently reported on blood flow to the hypothalamus as an ROI with or without infusion of anorectic gut hormones (Batterham et al., 2007).

1.13. Principles of fMRI in human appetite studies

A commonly used approach in the area of functional imaging of appetite is to present the subject with images of food (with further subdivision into appetising, bland, high calorie or low calorie food) or non-food items. 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 (e.g. the fasted state, the fed state or in the presence of exogenously administered anorectic hormones). Several studies have utilised this approach;

these will be discussed in depth subsequently. Hypothalamic data remain an elusive goal with such whole brain task-based analyses; in addition to the aforementioned difficulties in hypothalamic imaging, activation of some nuclei within the hypothalamus promotes food intake, whereas other nuclei are appetite-inhibiting (Suzuki et al., 2010). Therefore, using the hungry, fasted state as an example, the overall hypothalamic signal resulting from the activation of orexigenic nuclei and inhibition of anorexigenic nuclei may in fact be negligible, due to the above opposing signals cancelling each other out. In addition to developing stronger magnetic fields to enhance spatial resolution, newer perfusion imaging techniques, which are T1 weighted, may provide better hypothalamic data which is not so affected by signal loss due to magnetic susceptibility artefacts (Wong et al., 1997). Arterial spin labelling (ASL) uses RF pulses to magnetically label protons in blood water molecules before they reach the tissue of interest which lies in the imaging plane from where data is acquired. It has favourable noise properties over BOLD (Wang et al., 2011), so may represent a better method of imaging the hypothalamus.

1.14. fMRI studies in normal weight subjects - comparing the response to food cues in the fasted and fed states

Before moving into the arena of functional brain imaging studies to specifically determine the neuroendocrinology of appetite control, it is useful to look at earlier studies which examined differences in brain activity in hunger and satiety. Such differences may be attributed to a number of physiological differences between the fed and fasted state, such as changes in concentrations of gut-derived neuropeptides (PYY, GLP-1, ghrelin and insulin) and vagal afferents conveying information about gut distension. These early studies into appetitive behaviour have led to our understanding of how images of food can trigger the brain's reward system and how the motivational potency of this trigger is greater in the fasted state.

On the whole, these studies complemented earlier work which utilised PET scanning to assess the effects of hunger and satiety on regional cerebral blood flow differences, and which particularly implicated the OFC as a critical convergence zone for sensory information related to rewarding stimuli (Tataranni et al., 1999, Gautier et al., 2001, Small et al., 2001, Wang et al., 2004).

Most of these fMRI studies have been task-based subtraction analyses, looking at differences in regional brain activation between when viewing 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 secondary reinforcers, capable of influencing subsequent food-seeking behaviour. Thus, showing food images is a useful way of examining the appetitive reward circuitry.

In the first study of its kind 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, it was noted that this study was under-powered to detect similar satiety-induced changes in many other ROIs studied.

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 point 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 food minus non-food contrast, there was a significant negative correlation between BMI and BOLD signal in the OFC (Killgore and Yurgelun-Todd, 2005). This highlights the more subtle role of parts of the OFC not just in simply assigning reward value to certain stimuli, but in modifying stimulus-reward associations and accordingly redirecting feeding-related behaviour in response to new learning (Kringelbach, 2005). Of course, in this particular study, 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 insula and OFC, with a positive modulation of insular activity induced by subjective ratings of appetite (Porubska *et al.*, 2006). These findings are consistent with the recognition of the insula as being an important region in establishing salience. 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, again with reference to our understanding of this area as subjectifying the perceived pleasantness of food (Fuhrer *et al.*, 2008). Furthermore, in 2009, Goldstone *et al.* reported the findings from a fMRI study of twenty individuals in both the fed and fasted state; subjects viewed pictures of high calorie, low calorie and non-food items whilst rating the appeal value of these images. They found that when fasted, there was significantly greater activation to high calorie over low calorie food items in the ventral striatum, amygdala, anterior insula and OFC. They found that high calorie foods were consistently rated as more appealing, that this was augmented with fasting, and that the increase in appeal rating bias for high calorie over low calorie foods in the fasted state was positively correlated with activity in the OFC (Goldstone *et al.*, 2009). In the same year, 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. 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 versus non-fattening or all food versus non-food). In concordance with other studies, 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 agreement with previous findings were the results of Siep *et al.*, who in 2009 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.

Recently, it was demonstrated (Fletcher et al., 2010) that the amygdala, hypothalamus and ventral striatum are all specifically responsive to viewing images of high calorie foods versus low calorie foods in the fasted state. Interestingly, the difference in ventral striatal activity between viewing high and low calorie food images was attenuated in the fed state, whereas the difference in hypothalamic and amygdala responses were unaffected by satiety.

1.15. fMRI studies of hormones implicated in appetite control

Having discussed the first generation of fMRI studies exploring the physiological responses to feeding, I will now turn to focus on research that has investigated the effects of appetite modulating hormones derived from the gastrointestinal tract and adipose tissue on the fMRI neuronal response. As alluded to earlier, such hormones are thought to be key mediators of both short term and longer term energy balance; in the post-prandial state, anorectic hormones (including PYY, GLP-1 and insulin) predominate, and in the longer term, high circulating levels of leptin and insulin correlate with nutritional status. In contrast, in the fasted, hungry state, the orexigenic hormone ghrelin is dominant amongst gut hormones. It is therefore interesting to observe the effects of exogenous administration of such hormones on the fMRI response, and compare these results with outcomes from earlier studies comparing the physiological fasted and fed states. It is worth noting that thus far, the majority of fMRI studies coupling the exogenous administration of appetite modulating hormones

(with the exception of studies of leptin) have been conducted in healthy, normal weight subjects, in order to investigate the action of such hormones in normal physiology.

1.15.1. PYY and GLP-1

PYY and GLP-1 are anorectic hormones, released by the enteroendocrine L cells of the gut following a meal; they lead to marked inhibition of food intake when administered to fasted human subjects (Verdich et al, 2001, Batterham et al. 2002; Sloth et al. 2007). In healthy human subjects, following intravenous infusion of PYY₃₋₃₆, there was not only reduced food intake, but increased activity in the hypothalamus and OFC, as assessed by BOLD fMRI (Batterham et al., 2007). This study was conducted with subjects at rest, without engagement in a visual food-cue based task. Furthermore, during saline infusion visits, subjects' caloric intake correlated positively with BOLD signal change in the hypothalamus, whereas this switched to a negative correlation between caloric intake and OFC signal on study visits when PYY₃₋₃₆ was infused. It was postulated that the presence of PYY₃₋₃₆ switched regulation of food intake from a homeostatic brain region (hypothalamus) to a hedonic region (OFC).

There have been no human studies to date investigating fMRI response to administration of GLP-1. A PET study has previously revealed that the endogenous post-prandial GLP-1 response is positively associated with changes in neuronal activity in the prefrontal cortex and hypothalamus (Pannacciulli et al., 2007).

1.15.2. Ghrelin

Ghrelin is a potent orexigenic gut hormone released from the stomach in response to fasting; administration of ghrelin to humans increases food intake (Wren et al., 2001, Druce et al., 2005). A study by Malik *et al.* 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) compared with saline infusion in the amygdala, OFC, insula, visual areas and striatum (Malik et al., 2008).

1.15.3. Insulin

The central anorectic effect of insulin in normal physiology (Porte and Woods, 1981) must be distinguished from the secondary effect of hunger resulting from hypoglycaemia that may follow the exogenous administration of insulin (the associated over-compensatory eating may provide some explanation as to why patients with diabetes treated with insulin tend to gain weight). Therefore, human studies investigating the direct physiological action of insulin in the CNS have had to overcome the hurdle of maintaining euglycaemia during insulin administration, for otherwise, it is the effect of hypoglycaemia (rather than the effect of insulin *per se*) that would manifest. Furthermore, to effectively study the action of insulin in the CNS, peripheral effects of the hormone should ideally be eliminated, hence even the use of a euglycaemic hyperinsulinaemic clamp technique is unsatisfactory in this regard. One group has used intranasal insulin administration as a method of overcoming this technical difficulty, demonstrating that insulin resulted in reduced BOLD fMRI activation (with respect to food images versus non-food images) in 9 healthy, normal weight adults compared with placebo in the right and left fusiform gyrus, the right hippocampus, right temporal superior

cortex, and the right frontal middle cortex (Guthoff et al., 2010). Notably, peripheral plasma glucose did not change following the intranasal insulin administration, thus validating their technique of observing the central effects of insulin.

1.15.4. Leptin

Leptin deficient individuals are obese and hyperphagic; replacement of leptin in these individuals reduces food intake (Williamson et al., 2005). However, the well recognised phenomenon of leptin resistance in obesity has precluded its development from a therapeutic viewpoint. However, fMRI studies of leptin administration are nevertheless interesting to shed further light on the physiological regulation of appetite.

In 2 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 the nucleus accumbens-caudate and putamen-globus pallidus regions compared with the control, leptin deficient state. Furthermore, before leptin treatment, activation in the nucleus accumbens-caudate correlated positively with liking of food images in both the fed and fasted states, whereas after leptin treatment, activation in the nucleus accumbens-caudate correlated positively with liking of food images only in the fasted state (Farooqi et al., 2007). Another study that year demonstrated that in 3 congenitally leptin deficient human subjects, daily subcutaneous leptin replacement reduced the difference in BOLD fMRI activation (between viewing high calorie and low calorie food images) in regions implicated in the insula, temporal and parietal cortex compared with when subjects were not receiving leptin. On the other hand, leptin replacement increased the difference in BOLD fMRI activation (between viewing high calorie and low calorie food

images) in the prefrontal cortex compared with when subjects were not receiving leptin (Baicy et al., 2007). The authors postulated that the insula, temporal cortex and parietal cortex are hunger regions, therefore are inhibited by leptin, whereas the prefrontal cortex is a satiety region and is thus inhibited by weight loss and activated by leptin.

Following this, a study by Rosenbaum *et al.* in 2008 showed that in 6 leptin replete obese human subjects who achieved an initial 10% weight loss (between 36 and 62 days) through dietary means, there was increased BOLD fMRI signal difference (between comparing food images and non-food images) in several regions including the brainstem and parahippocampal gyrus compared with the baseline state before weight loss. These changes were reversed by 5 weeks of subcutaneous leptin administration. Interestingly, other regions, including the hypothalamus and cingulate gyrus responded in the opposite direction following weight loss, but these changes remained sensitive to reversal by 5 weeks of subcutaneous leptin administration (Rosenbaum et al., 2008). This study reinforced the idea that weight loss leads to a state of relative leptin deficiency, which may be responsible for the rebound hyperphagia and subsequent weight gain in many dieters.

1.16. Imaging before and after gastric bypass surgery

The most effective treatment for obesity available at present is Roux-en-Y gastric bypass surgery, leading to sustained weight loss of approximately 30% and often a very prompt resolution of Type 2 Diabetes (Maggard et al., 2005). Although the mechanisms behind the weight loss seen following Roux-en-Y bypass surgery remain to be fully elucidated, they are unlikely to be solely due to restrictive or malabsorptive means alone. Roux-en-Y results in greater weight loss than adjustable gastric banding and this is thought to be due to a number of additional neurohormonal causes (Tice et al., 2008). These include elevated post-

operative levels of endogenous anorectic gut hormones such as PYY and GLP-1 (Borg et al., 2006, le Roux et al., 2006, le Roux et al., 2007), increases in energy expenditure and metabolic rate (Nestoridi et al., 2012) changes in taste preference away from calorie dense foods (Bueter et al., 2011, le Roux et al., 2011), 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.

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). They demonstrated that there was a reduction in hypothalamic signal seen 5-10 minutes following glucose ingestion in normal controls, but that was diminished in the obese (pre-operative) group. However, a recovery of this impaired hypothalamic response was seen post-operatively when the obese group had lost weight following gastric bypass surgery. Interestingly, they also reported in lean subjects, a high level of functional connectivity between the hypothalamus and the orbitofrontal and somatosensory cortices. This was not seen in obese subjects, but post-operative weight loss was reported to re-establish these connectivity patterns (van de Sande-Lee et al., 2011).

In the same year, a further ROI-based study of 10 obese female patients 1 month pre- and post- Roux-en-Y gastric bypass, there was attenuation of fMRI BOLD signal (when viewing high-calorie versus low-calorie food images) in the VTA, ventral striatum, putamen, lentiform nucleus, posterior cingulate and dorsomedial prefrontal cortex following the bypass procedure (Ochner et al., 2011). In this study, hunger was also reduced after surgery. 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. This would certainly be consistent with the previous fMRI findings (Malik et al., 2008) following exogenous ghrelin administration. The same imaging group have also recently published the results from a further fMRI study (Ochner et al., 2012), where 14 females were assessed 1 month pre- and one month post- Roux en-Y gastric bypass surgery. Results from this study confirmed significant postoperative reductions in striatal neural response, along with reductions in wanting and liking for high calorie relative to low calorie food cues.

1.17. Summary

The overall control of appetite and food intake is complex, resulting from integration of a variety of inputs in both homeostatic and hedonic brain regions. Obesity arises following an imbalance of energy homeostasis, where there is a relative excess of food intake in relation to energy expenditure. At present, there are no effective pharmacotherapies to tackle obesity that are licensed in the UK, and the only effective treatment of obesity is bariatric surgery, which is not without risk. Anorectic gut hormones, which are released post-prandially and known to exert effects on appetite centres in the brain, remain promising therapeutic targets. Two such hormones are PYY and GLP-1, both of which are secreted by the gut in response to a meal are thought to be important mediators of the satiety response. Acute administration of PYY₃₋₃₆ and GLP-1_{7-36 amide} (the active form of the hormones) to humans is well established to inhibit appetite and reduce food intake, when the hormones are administered both singly and in combination. Thus, clarifying the neuroendocrinology of anorectic gut hormones such as PYY₃₋₃₆ and GLP-1_{7-36 amide}, acting both independently and together, is hugely important for the development of effective obesity treatments.

In humans, fMRI offers an opportunity to safely study the effects of these hormones on the brain. fMRI studies have already established important differences in brain activation in response to food cues between the fed and fasted states in a variety of hedonic brain regions, although homeostatic brain regions have been more difficult to study. This has been followed by further fMRI studies investigating the action of orexigenic (ghrelin) and anorectic (leptin, insulin, and PYY₃₋₃₆) hormones on brain regions implicated in appetite control. To date however, there have been no published human studies investigating the action of GLP-1₇₋₃₆ amide on brain regions implicated in appetite control, nor any studies investigating the combined action of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on the brain fMRI response. Notably, following Roux-en-Y gastric bypass surgery, patients have elevated post-prandial levels of both PYY and GLP-1, and the combined action of these peptides is thought to be a key contributor to the greater and sustained weight loss observed following the intervention. In light of interesting recent fMRI data in patients pre- and post- bariatric surgery, it is all the more compelling to study the combined action of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on the CNS in humans using fMRI.

1.18. Hypotheses and aims

1.18.1. Hypotheses:

I hypothesise that

1. In subjects fasted overnight, mildly anorectic doses of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, when infused in combination, will reduce *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 PYY₃₋₃₆ and GLP-1₇₋₃₆ amide in fasted subjects will reduce the BOLD fMRI signal in key CNS reward areas, similar to that observed in the fed state.

1.18.2. Aims

1. To establish doses of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide that are mildly anorectic alone but anorectic in combination compared with saline.
2. To compare the BOLD fMRI 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 BOLD fMRI response to PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, infused singly and in combination at doses determined in Aim 1.

CHAPTER 2

**Investigation of the effects of single and
combined administration of PYY₃₋₃₆ and
GLP-1₇₋₃₆ amide on appetite and energy intake:**

Dose verification study

(STUDY 1)

2.1. Introduction and rationale

PYY₃₋₃₆ and GLP-1_{7-36 amide} are well established to reduce food intake when administered intravenously to humans (Verdich et al., 2001, Batterham et al., 2002, Neary et al., 2005, Sloth et al., 2007). Varying degrees of nausea were encountered in the above studies. I therefore decided to perform a dose verification study in order to confirm doses of PYY₃₋₃₆ and GLP-1_{7-36 amide} that when administered in combination, would lead to a significant reduction in food intake without causing nausea which would prevent subjects from continuing in the study. I performed this initial dose verification study in order to guide doses of PYY₃₋₃₆ and GLP-1_{7-36 amide} to be used in the subsequent fMRI study.

Previous work by Neary *et al.* (Neary et al., 2005) had demonstrated that combined infusion of PYY₃₋₃₆ & GLP-1_{7-36 amide}, each at a dose of 0.4 pmol/kg/min, reduced energy intake by 27% compared to saline at an *ad libitum* buffet meal served 90 minutes into the infusion. Significantly, no nausea was reported in the group of 10 subjects. When GLP-1_{7-36 amide} was administered singly, there was only a 5% reduction in energy intake compared to saline at the *ad libitum* buffet meal. Taking this study by Neary *et al.* as an initial guide, I firstly decided to change the dose of infused GLP-1_{7-36amide} from 0.4 pmol/kg/min to 0.8 pmol/kg/min. This is based on data from the meta-analysis by Verdich *et al.* in which the mean infusion rate of GLP-1_{7-36 amide} was 0.89 pmol/kg/min across all subjects in all studies and this led to a 13.2% reduction in energy intake at the *ad libitum* buffet meal compared with saline infusion (Verdich et al., 2001). A formal assessment of nausea ratings seems to have been made in only 2 of the studies included in Verdich's meta-analysis; however, reassuringly, nausea was not observed in either of these individual studies.

Secondly, I decided to reduce the infused dose of PYY₃₋₃₆ from 0.4 pmol/kg/min (used in the study by Neary *et al.*) to 0.3 pmol/kg/min in order to limit nausea. In the study by Neary *et al.*, when PYY₃₋₃₆ was administered singly, there was a 15% reduction in energy intake compared to saline at the *ad libitum* buffet meal. Other 90 minute infusion studies of PYY₃₋₃₆ have used varying doses between 0.2 pmol/kg/min and 0.8 pmol/kg/min. In one study (Sloth *et al.*, 2007) found there to be no reduction in *ad libitum* energy intake with a dose of 0.2 pmol/kg/min. In contrast, a dose of 0.8 pmol/kg/min led to a 19% reduction in *ad libitum* energy intake compared with saline in the subjects that managed to complete the study; in 5/9 subjects, nausea and abdominal discomfort was so severe that the studies had to be terminated. This is in contrast to studies performed by Batterham *et al.*, where a PYY₃₋₃₆ dose of 0.8 pmol/kg/min was reported to produce no nausea in 12 subjects (Batterham *et al.*, 2002). A further PYY₃₋₃₆ dose-response study (Degen *et al.*, 2005) in 16 subjects revealed that administering a dose of 0.2 pmol/kg/min did not produce nausea, whereas a dose of 0.4 pmol/kg/min led to nausea in 2 subjects. Increasing the dose to 0.8 pmol/kg/min induced nausea in 4 subjects. As mentioned earlier, Neary *et al.* reported no nausea when PYY₃₋₃₆ 0.4 pmol/kg/min was co-infused with GLP-1₇₋₃₆ amide at a dose of 0.4 pmol/kg/min. The therapeutic window for anorectic gut hormones, particularly that of PYY₃₋₃₆, is narrow and the method of dose adjusting for the actual known peptide content of each vial had not been performed in previously published studies. This may form one source of discrepancy between the above studies in terms of induction of nausea with infusion of PYY₃₋₃₆.

It should be noted that Neary *et al.* continued the peptide infusion during the meal for a further 30 minutes. Although this is possible in a clinical investigation unit, I faced the additional practical issue of using an MRI-compatible injector system to deliver the peptide infusion during the subsequent fMRI study. This injector system cannot be easily moved out of the scanner room and furthermore, it would not possible for subjects to be served their test meal at the end of the study in the MRI scanner room. Hence, continuation of the

peptide infusion during the meal would have necessitated a change of injector systems during the infusion, which would have lead to inconsistency in peptide delivery and would have also required preparing a new infusion mid-way through the study.

I was therefore initially guided by previous studies that administered individual infusions of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide to determine the timing of the test meal in relation to the peptide infusion as well as the duration of the infusion itself. Previous PYY₃₋₃₆ infusion studies (Batterham et al., 2002, Sloth et al., 2007) had left an interval of 2 hours between termination of the infusion and timing of the test meal, yet demonstrated a significant reduction in food intake in response to the peptide. The duration of PYY₃₋₃₆ infusion was 90 minutes in both these studies. Historically, GLP-1₇₋₃₆ amide infusion studies have allowed a shorter interval (if any at all) between termination of the infusion and serving the test meal. Some studies served the test meal whilst continuously running the peptide infusion (Naslund et al., 1999, Long et al., 1999, Gutzwiller et al., 1999), whereas in others, the infusion was stopped 30 minutes before the meal was served and then restarted at the onset of the meal (Flint et al., 1998, Flint et al., 2001). The duration of the infusion in many of these studies has been in the order of 60 minutes. The difference in protocols used between PYY₃₋₃₆ and GLP-1₇₋₃₆ amide infusion studies in terms of the timing of the study meal probably stems from the differing circulating half-life of the two peptides; PYY₃₋₃₆ has a circulating half-life of approximately 8 minutes (Lluis et al., 1989), whereas that of GLP-1₇₋₃₆ amide is only 2 minutes (Kieffer et al., 1995). Given that my protocol was to use a combination of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, I decided to serve the test meal immediately after the termination of the 90 minute infusion. This was to better take into account the extremely short circulating half-life of GLP-1₇₋₃₆ amide.

2.2. Methods

2.2.1. Peptides

Human PYY₃₋₃₆ and GLP-1₇₋₃₆ amide were 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; PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, each at a dose greater than 10 times the maximum intended for humans (in pmol/kg body weight), were administered by intra-peritoneal injection to 20 mice, and saline was administered to a control group of 10 mice. Half the animals in each group were sacrificed by CO₂

asphyxiation after 48 hours, and the other half after 14 days. The heart, lungs, kidneys, liver, spleen, pancreas, brain and gut were dissected, fixed and sent for examination by an independent histopathologist.

2.2.2. Subjects

Six healthy right-handed subjects (2 male and 4 female) of mean age 31.5 years (range 21 – 45 years) and mean BMI 21.7 kg/m² (range 19.1 - 24.6 kg/m²), were recruited by newspaper 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, substance abuse; significant physical or psychiatric illness; regular medication other than contraceptives, and pregnancy. All subjects were screened and determined to be healthy by medical history, physical examination, full blood count, urea and electrolytes, liver function tests, bone profile, 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. Potential subjects were screened to exclude those with disordered eating, as assessed by the SCOFF questionnaire (Morgan et al., 1999), and those with a high level of restrained eating, as assessed by the standard Dutch Eating Behaviour Questionnaire (Van Strien et al., 1986). A score of 1 point or greater on the SCOFF questionnaire, or a total score of 25 points or greater on the first 8 questions of the Dutch Eating Behaviour Questionnaire warranted exclusion from participation in the study. Furthermore, women who reported significant alteration of eating behaviour during different phases of the menstrual cycle were excluded from participation. Subjects 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 was approved by the St Mary's Research Ethics Committee (reference number 09/H0712/4). The research was planned and performed in accordance with the Declaration of Helsinki. Full, informed consent was obtained from all subjects prior to enrolment in the study.

2.2.3. Design

Six healthy right-handed subjects (2 male and 4 female) each attended the clinical investigation unit for 5 study visits, each at least 3 days apart. Subjects fasted (they were permitted to drink only water) from 22:00 h the evening before each study visit. They were asked to standardise their diet (by eating the same evening meal at the same time the night before each study visit), abstain from alcohol and avoid 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 an identical protocol to that used for the subsequent fasted 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 first saline visit, subjects were randomly assigned to receive each of the following after an overnight fast:

1. Saline infusion (control visit)
2. PYY₃₋₃₆ infusion at 0.3 pmol/kg/min
3. GLP-1_{7-36 amide} infusion at 0.8 pmol/kg/min
4. Combined PYY₃₋₃₆ + GLP-1_{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), then had peripheral intravenous cannulae inserted in both forearms; one for infusions and one for blood sampling. Subjects were studied up to a maximum of two per room and were separated by a screen when meals were served. They 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.

2.2.4. Peptide preparation and delivery

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

2.2.5. Blood sampling and processing

Six ml blood samples were collected for gut hormone measurement at $t = -30, 0, 15, 30, 45, 60, 75, 90$ and 120 min into lithium heparin-coated tubes (International Scientific Supplies Ltd, Bradford, UK) containing 1500 kallikrein inhibitor units (0.15 ml) aprotinin (Trasylo1, 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 6 ml 0.9% saline after collecting each blood sample. Immediately prior to collection of the subsequent sample, 1 ml deadspace blood was collected and discarded.

2.2.6. Visual analogue scale scores

At $t = -30, 0, 30, 60, 90$, and 120 min, subjects completed a series of visual analogue scales (VAS) that rated food-related sensations (hunger, nausea, fullness). 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 (Flint et al., 2000).

2.2.7. Measurement of haemodynamic parameters and blood glucose

The pulse and blood pressure of each subject was measured at $t = -30, 0, 15, 30, 45, 60, 75, 90$, and 120 min. A drop of blood was taken from the $t = -30, 0, 30, 60, 90$ and 120 min samples to check blood glucose on an Optimum Exceed blood glucose monitor (Abbott Diabetes Care, Maidenhead, Berks). Haemodynamic parameters and blood glucose were measured primarily to ensure subjects' safety during the studies.

2.2.8. *Ad libitum* test meal

The infusion was terminated after 90 minutes. Immediately after stopping the infusion (i.e. $t = 90$ min), subjects were offered an identical buffet meal at each visit that was provided in excess. Subjects were asked to 'eat until comfortably full'. Water was freely available. The meal was served with the curtains drawn around each subject for 20 minutes and consisted of 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 on their choice of meal during their screening visit; they were subsequently served the same meal during 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 VAS. Subjects remained in the study room until $t = 120$ min.

The protocol for Study 1 is summarised in figure 2.1 below.

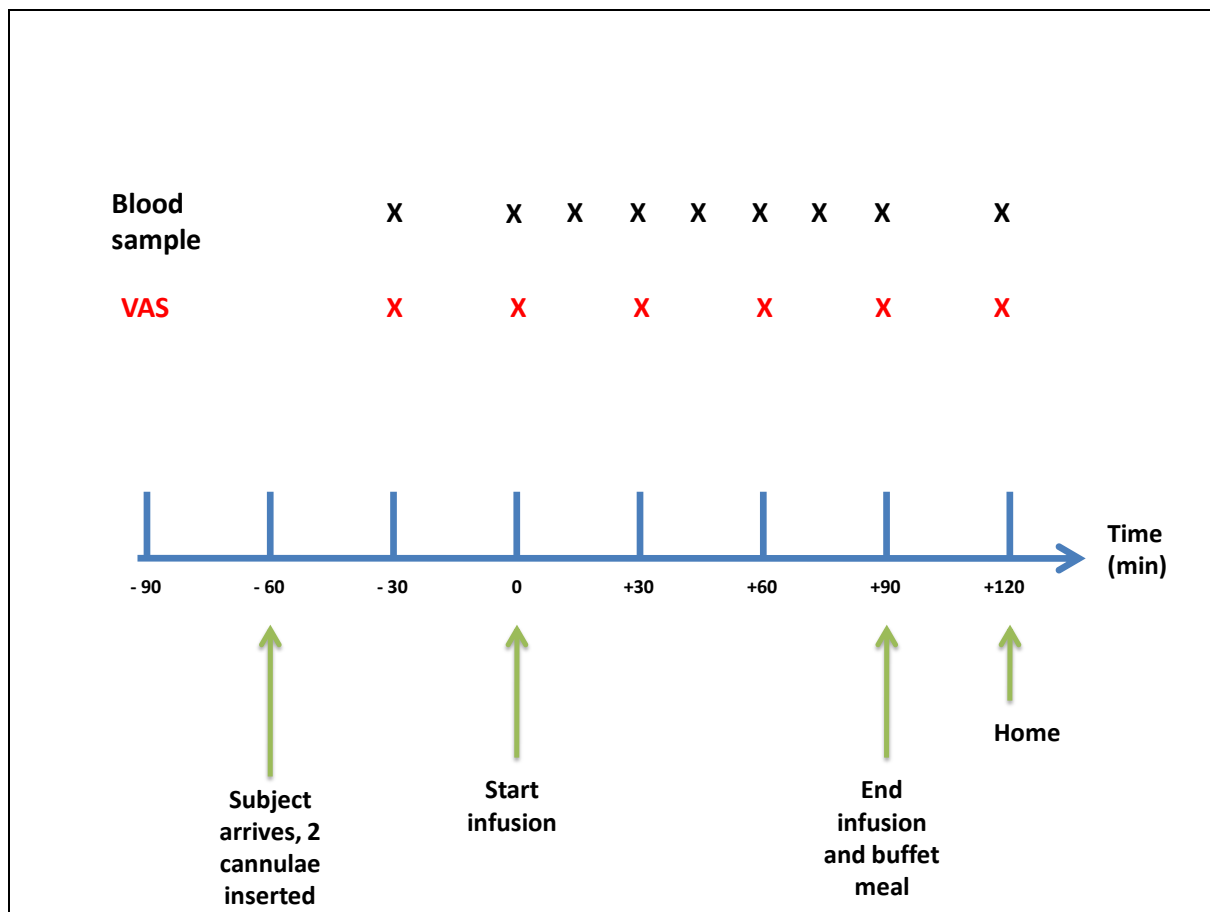


Figure 2.1: Protocol for Study 1. Healthy subjects underwent 90 minute infusions of saline, PYY 0.3 pmol/kg/min, GLP-1 0.8 pmol/kg/min, combined PYY 0.3 pmol/kg/min + GLP-1 0.8 pmol/kg/min following an overnight fast. An *ad libitum* buffet meal was served immediately after the infusion in order to measure energy intake. Blood sampling and VAS analysis was performed as indicated. Study visits were single blinded and in randomised order.

2.2.9. Measurement of total PYY

Total PYY immunoreactivity was measured with a specific and sensitive in-house radioimmunoassay (RIA), previously established (Adrian et al., 1985). Information regarding the principles of RIA can be found in Appendix 1. The antibody (Y21) was produced in rabbits against synthetic human PYY coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:50,000. The antibody cross reacted fully with both PYY₃₋₃₆ and PYY₁₋₃₆, but not with pancreatic polypeptide, neuropeptide Y, or other known gastrointestinal hormones. Radiolabelled ¹²⁵I-PYY was prepared by the iodogen method (Wood et al., 1981) and purified by HPLC. The specific activity of the ¹²⁵I-PYY label was 54 Bq/fmol. PYY standard was used at 1 pmol/ml and volumes ranging from 1 microlitre to 100 microlitres were added to generate the standard curve. The assay was performed in total volume of 0.7 ml of 0.06 M phosphate buffer (pH 7.3) containing 0.3% bovine serum albumin (BSA). The assay was incubated for three days at 4°C. Separation of the free and antibody bound radiolabel was by the addition of 0.1 ml sheep anti-rabbit antibody to each tube, along with 0.1 ml 10% polyethylene glycol (PEG) and 0.5 ml Triton X-100 in 0.05M phosphosaline, followed by centrifugation at 4°C for 20 minutes at 2500 rpm. The detection limit of the assay was 2 pmol/L, with an intra-assay coefficient of variation of 5.8%. The inter-assay coefficient of variation was 9.8%. All samples were assayed in duplicate and in one assay to avoid inter-assay variation.

2.2.10. Measurement of amidated GLP-1

Amidated GLP-1 like immunoreactivity was measured by a specific and sensitive in-house RIA, previously established (Kreymann et al., 1987). The antibody was produced in rabbits against human GLP-1 coupled to BSA and used at a final dilution of 1:50,000. The antibody cross reacted 100% with all amidated forms of GLP-1 but did not cross react with glycine

extended forms [GLP-1₁₋₃₇ and GLP-1₇₋₃₇] or any other known pancreatic or gastrointestinal peptide. Radiolabelled ¹²⁵I-GLP-1 was prepared by the iodogen method (Wood et al., 1981) and purified by HPLC. The specific activity of the ¹²⁵I-GLP-1 label was 48 Bq/fmol. GLP-1 standard was used at 0.5 pmol/ml and volumes ranging from 1 microlitre to 100 microlitres were added to generate the standard curve. 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 and 0.02 % Tween. The assay was incubated for three days at 4°C. Separation of the free and antibody bound radiolabel was by charcoal adsorption (charcoal and dextran were dissolved in 0.06 M phosphate buffer containing gelatine, and 0.3 ml of this solution was added to each tube). The limit of detection of the assay was 5 pmol/L with an intra-assay coefficient of variation of under 10%. The inter-assay coefficient of variation was also under 10%. All samples were assayed in duplicate and in one assay to avoid inter-assay variation.

2.2.11. RIA set up for measurement of total PYY and amidated GLP-1

Assay tubes were set up in duplicate according to the volumes described in table 2.1 below. All samples were added in duplicate. “Non-specific binding (NSB)” tubes, without primary antibody, were added at the beginning of the assay. Tubes containing half (1/2 X) and twice (2X) the standard volume of radiolabelled peptide were also included in order to confirm the quality of the radiolabel. Tubes with no sample (‘zero’ tubes) were placed at regular intervals throughout the assay, and standard (STD) curves (containing known quantities of unlabelled, pure peptide) were performed every 400 tubes, in order to calibrate the assay. Quality control (QC) samples, containing high, medium and low known concentrations of each peptide, were included at 3 points within each assay.

The known QC values for each assay (pmol/L) were:

PYY: High = 100 - 150 pmol/L, Medium = 50 - 100 pmol/L, Low = 10 - 40 pmol/L.

GLP-1: High = 120 - 180 pmol/L, Medium = 60 - 100 pmol/L, Low = 20 - 40 pmol/L.

The measured QC values for each hormone were within the above ranges. At the end of the assay, two tubes contained excess antibody (XS), to ensure that the volume of antibody added during the assay was indeed limiting.

TUBE IDENTITY	BUFFER VOLUME (μL)	LABEL VOLUME (μL)	ANTIBODY VOLUME (μL)	SAMPLE / STANDARD (μL)	TOTAL VOLUME (μL)
NSB	600	100	0	0	700
1/2 X	550	50	100	0	700
2 X	400	200	100	0	700
ZERO	500	100	100	0	700
STD 1-15	500	100	100	1-15	701-715
STD 20	480	100	100	20	700
STD 30	470	100	100	30	700
STD 50	450	100	100	50	700
STD 100	400	100	100	100	700
XS	0	100	600	0	700
Sample	400	100	100	100	700
QC	400	100	100	100	700

Table 2.1: Volumes of reactants for addition to PYY and GLP-1 in-house radioimmunoassay.

2.2.12. Statistical analysis of energy intake, VAS, haemodynamic parameters, blood glucose and gut hormone levels

Grouped data are represented as the mean $\pm$ Standard Error of Mean (SEM). Comparisons of energy intake and ratings of meal palatability were by repeated measures Analysis of Variance (ANOVA) with Tukey's multiple comparison post-test. VAS scores were adjusted for baseline and differences between $t = 0$ min and $t = 90$ min (the duration of the infusion) compared by repeated measures non-parametric Friedman's test with Dunn's multiple comparison post-test. Glucose levels were adjusted for baseline and differences between $t = 0$ min and $t = 90$ min compared by repeated measures ANOVA with Tukey's multiple comparison post-test. Comparisons of haemodynamic parameters between interventions were by one-way ANOVA with Tukey's multiple comparison post-test. For each subject, the area under the curve (AUC) of plasma gut hormone levels was calculated during the infusion period (0 - 90 min) and the mean values during each of the interventions were compared 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).

2.3. Results

2.3.1 Peptides

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 PYY₃₋₃₆ 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 showed no histological abnormalities (Appendix 2).

2.3.2. Energy intake

All subjects were fasted overnight before each study visit. For the 6 subjects in Study 1, 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 PYY₃₋₃₆ 0.3 pmol/kg/min, 3334 ± 961.3 kJ when they received the single infusion of GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and 2359 ± 825.7 kJ when they received the combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min. This is illustrated in figure 2.2 below. The difference in mean energy intake was statistically significant between the 4 visits ($p < 0.05$ for PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min vs. Fasted saline and GLP-1₇₋₃₆ amide 0.8 pmol/kg/min, $p < 0.01$ for PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min vs. PYY₃₋₃₆ 0.3 pmol/kg/min).

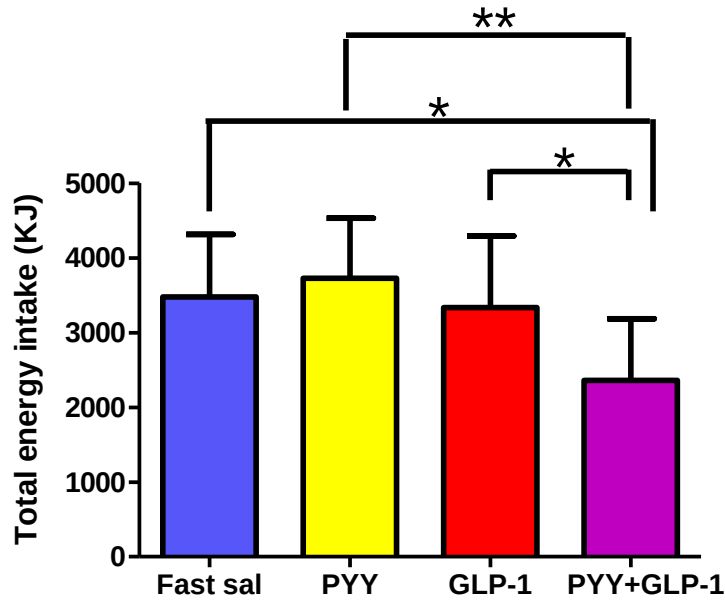


Figure 2.2: Mean *ad libitum* energy intake in Study 1. Six healthy subjects underwent 90 minute infusions of saline (Fast sal), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-35 amide} 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. An *ad libitum* buffet meal was served immediately after the infusion in order to measure energy intake. * denotes $p < 0.05$ for PYY+GLP-1 vs. Fast sal and GLP-1, **denotes $p < 0.01$ for PYY+GLP-1 vs. PYY.

2.3.3. Ratings of meal palatability

For the 6 subjects in Study 1, mean rating (at $t = 120$ min) of how tasty they found the buffet lunch on a 100 mm VAS scale was 61 ± 9.4 mm when they received saline, 65.2 ± 8.7 mm when they received the single infusion of PYY₃₋₃₆ 0.3 pmol/kg/min, 64.8 ± 7.8 mm when they received the single infusion of GLP-1_{7-36 amide} 0.8 pmol/kg/min and 56.7 ± 12.0 mm when they received the combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min. This is illustrated in figure 2.3 below. There was no significant difference between visits ($p = 0.48$).

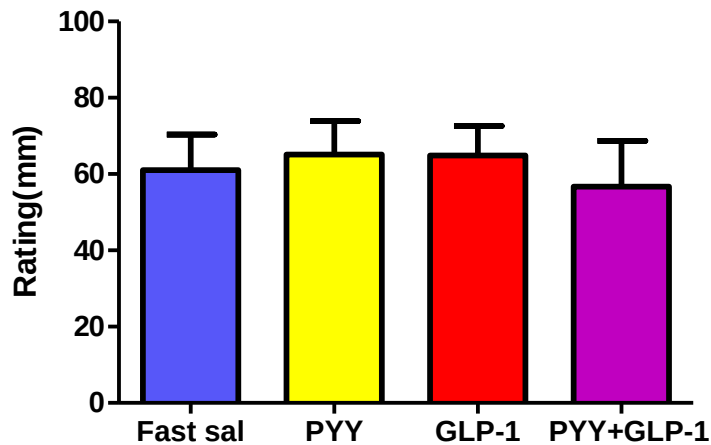


Figure 2.3: Meal rating following *ad libitum* meal in Study 1. Six healthy subjects underwent 90 minute infusions of saline (Fast sal), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. An *ad libitum* buffet meal was served immediately after the infusion and subjects rated this meal on a scale of 0 - 100.

2.3.4. Hunger, nausea and fullness scores

For the 6 subjects in Study 1, combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min resulted in a mean increase in nausea score by 22.5 mm from t = 0 min to t = 90 min (figure 2.4). 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) (figure 2.5). Analysis of the mean change in hunger and fullness scores between t = 0 min and t = 90 min revealed no significant difference between the control saline infusion, PYY₃₋₃₆ infusion, GLP-1_{7-36 amide} infusion or combined PYY₃₋₃₆ + GLP-1_{7-36 amide} infusion (p = 0.68) (figure 2.5).

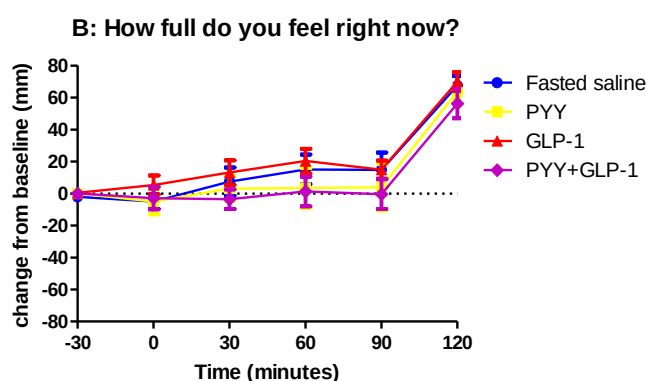
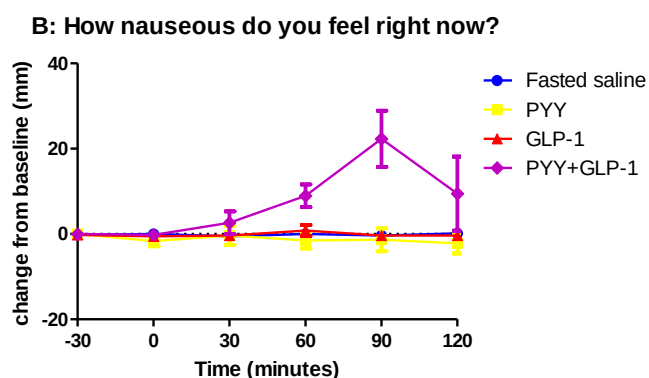
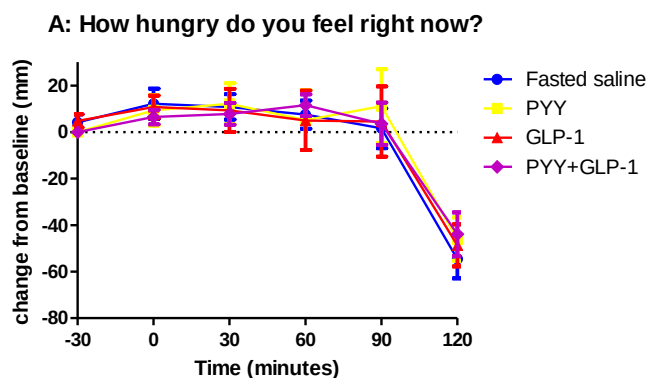


Figure 2.4: Hunger, nausea and fullness ratings during Study 1. Six healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On each day, the infusion was administered between t = 0 and t = 90 min. During the study, subjects completed 100 mm VAS questionnaires relating to hunger (A), nausea (B) and fullness (C)

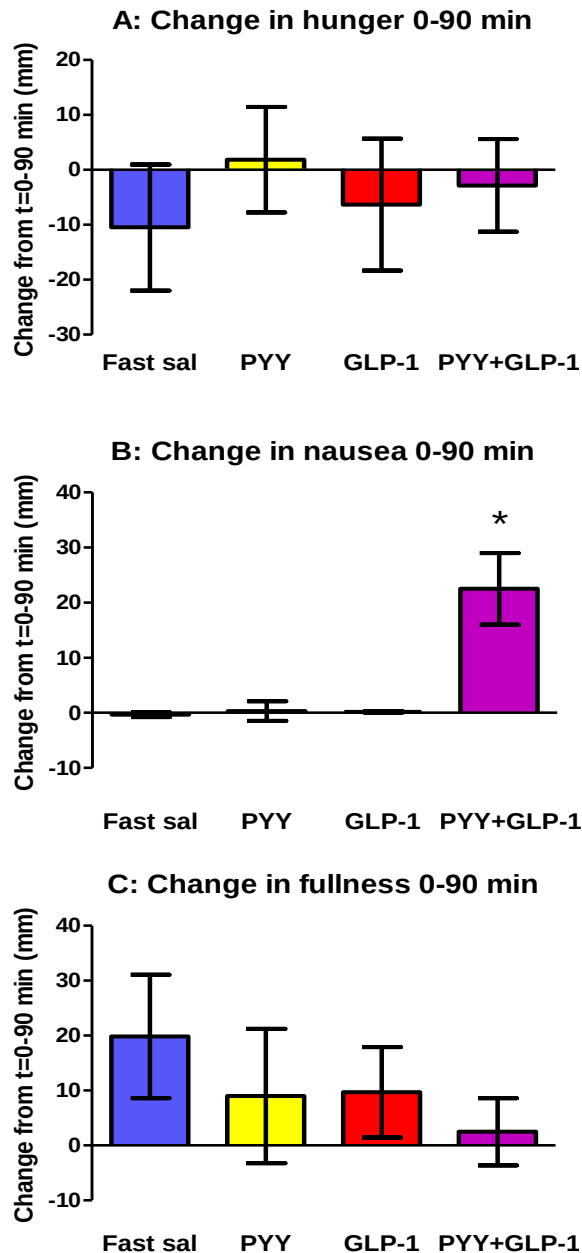


Figure 2.5: Change in hunger, nausea and fullness ratings during Study 1. Six healthy subjects underwent 90 minute infusions of saline (Fast sal), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On each day, the infusion was administered between t = 0 and t = 90 min. During the study, subjects completed 100 mm VAS questionnaires relating to hunger, nausea and fullness. The change in hunger (A), nausea (B) and fullness (C) from t = 0 to t = 90 min is shown.

* denotes $p < 0.05$ for PYY+GLP-1 vs. all other groups.

2.3.5. Haemodynamic parameters

For the 6 subjects in Study 1, blood pressure (systolic and diastolic BP) and pulse were not significantly different between infusions, as shown in figure 2.6 ($p = 0.23$ for systolic blood pressure, $p = 0.97$ for diastolic blood pressure and $p = 0.11$ for pulse)

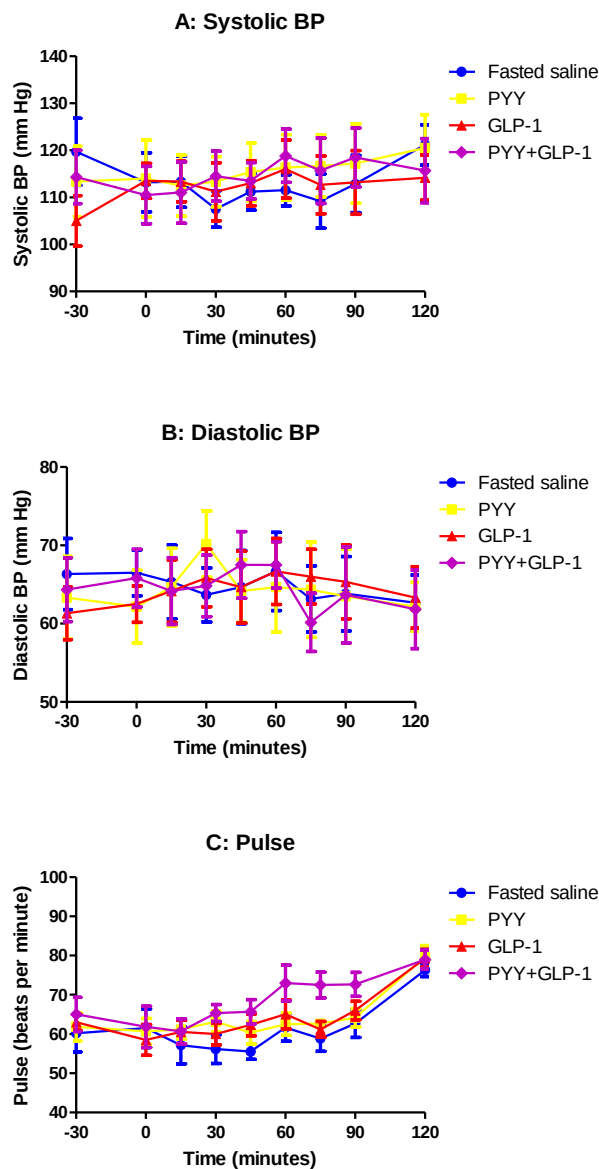


Figure 2.6: Haemodynamic parameters during Study 1. Six healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On each day, the infusion was administered between $t = 0$ and $t = 90$ min. During each study, systolic blood pressure (A), diastolic blood pressure (B) and pulse (C) were recorded.

2.3.6. Blood glucose

For the 6 subjects in Study 1, infusion of GLP-1₇₋₃₆ amide 0.8 pmol/kg/min, in addition to combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min, led to a greater fall in blood glucose between 0 and 90 min compared with infusion of saline or infusion of PYY₃₋₃₆ 0.3 pmol/kg/min alone (figure 2.7).

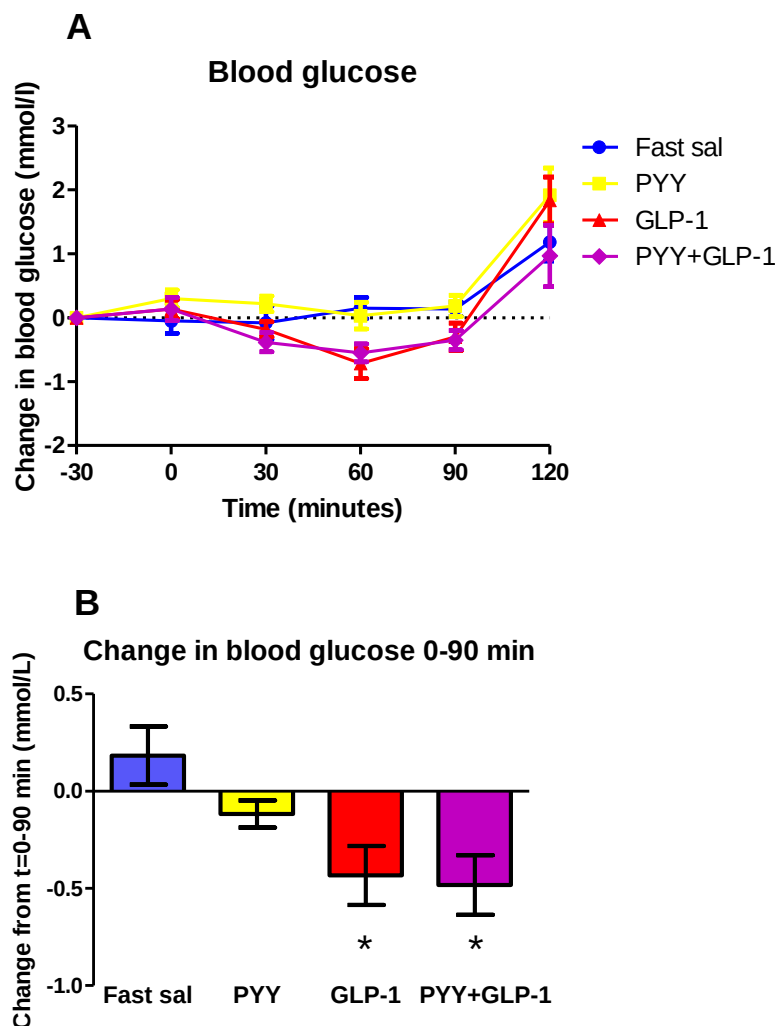


Figure 2.7: Blood glucose during Study 1. Six healthy subjects underwent 90 minute infusions of saline (Fast sal), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On each day, the infusion was administered between t = 0 and t = 90 min. During the study, the change in blood glucose from baseline was measured (figure 2.7 A). Figure 2.7 B shows the change in blood glucose from 0 - 90 min.

* denotes p<0.05 for GLP-1 and PYY+GLP-1 vs. Fast sal.

2.3.7. Plasma PYY levels in Study 1

Mean plasma concentration of total PYY (from time points $t = 15$ min to $t = 90$ min) was 27.5 ± 1.7 pmol/L when saline was infused, 66.8 ± 9.0 pmol/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 20.5 ± 0.4 pmol/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 87.3 ± 8.8 pmol/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused.

Peak mean plasma concentration of total PYY (during the entire study) was 33.3 ± 6.8 pmol/L during the saline study visit (occurring at $t = 30$ min), 91.0 ± 11.2 pmol/L during the PYY₃₋₃₆ 0.3 pmol/kg/min study visit (occurring at $t = 90$ min), 33.3 ± 3.7 pmol/L during the GLP-1₇₋₃₆ amide 0.8 pmol/kg/min study visit (occurring post-prandially at $t = 120$ min), and 106.5 ± 12.6 pmol/L during the PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min study visit (occurring at $t = 90$ min).

The mean AUC of total PYY levels (between 0 - 90 min) was 2553 ± 330 pmol min/L when saline was infused, 5464 ± 743 pmol min/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 1882 ± 224 pmol min/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 7267 ± 716 pmol min/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused. There was no significant difference between the mean AUC of total PYY levels between saline and GLP-1₇₋₃₆ amide 0.8 pmol/kg/min infusions. When PYY₃₋₃₆ was infused in combination with GLP-1₇₋₃₆ amide, the mean AUC of total PYY levels was higher than when PYY₃₋₃₆ was infused singly ($p < 0.05$).

Figure 2.8 shows the variation of plasma total PYY levels with time in Study 1.

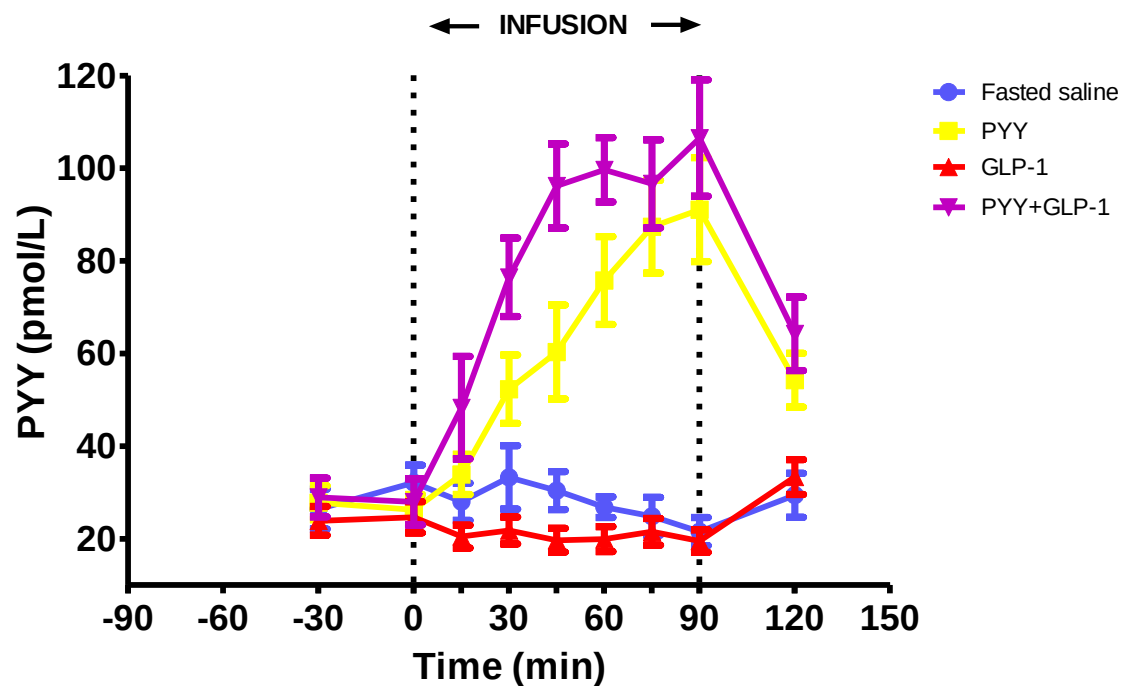


Figure 2.8: Variation of plasma total PYY levels with time during Study 1. Following an overnight fast, 6 healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{1-7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{1-7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1). On each study visit, the infusion was administered between t = 0 and t = 90 min. An *ad libitum* buffet lunch was served immediately after the infusion. Blood samples were taken for measurement of total PYY during each study at the time-points indicated.

2.3.8. Plasma GLP-1 levels in Study 1

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 PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 58.9 ± 6.0 pmol/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 59.4 ± 5.7 pmol/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused.

Peak mean plasma concentration of amidated GLP-1 (during the entire study) was 32.4 ± 4.7 pmol/L during the saline study visit (occurring post-prandially at $t = 120$ min), 26.3 ± 4.0 pmol/L during the PYY₃₋₃₆ 0.3 pmol/kg/min study visit (occurring post-prandially at $t = 120$ min), 73.3 ± 6.7 pmol/L during the GLP-1₇₋₃₆ amide 0.8 pmol/kg/min study visit (occurring at $t = 90$ min), and 73.0 ± 9.4 pmol/L during the PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min study visit (occurring at $t = 90$ min).

The mean AUC of amidated GLP-1 levels (between 0 - 90 min) was 1790 ± 248 pmol min/L when saline was infused, 1574 ± 268 pmol min/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 4897 ± 508 pmol min/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 4928 ± 585 pmol min/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused. There was no significant difference between the mean AUC of amidated GLP-1 levels between saline and PYY₃₋₃₆ 0.3 pmol/kg/min infusions. Furthermore, there was no significant difference between the mean AUC of amidated GLP-1 levels when GLP-1₇₋₃₆ amide was infused singly or in combination with PYY₃₋₃₆.

Figure 2.9 shows the variation of plasma amidated GLP-1 levels with time in Study 1.

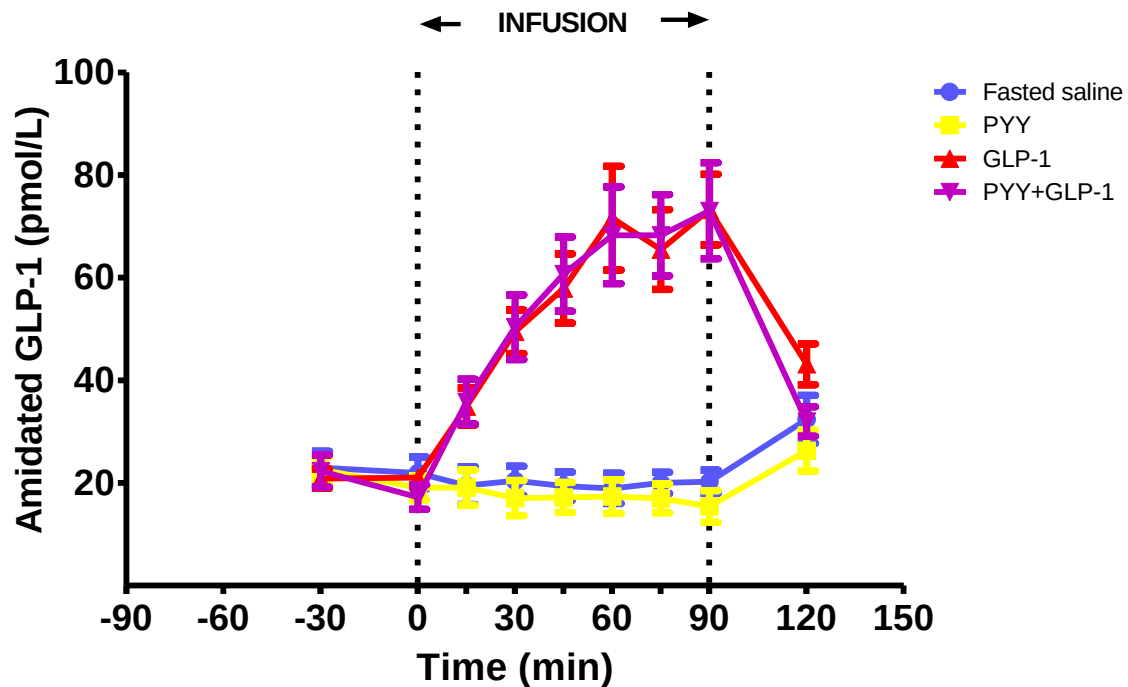


Figure 2.9: Variation of plasma amidated GLP-1 levels with time during Study 1. Following an overnight fast, 6 healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1). On each study visit, the infusion was administered between t = 0 and t = 90 min. An *ad libitum* buffet lunch was served immediately after the infusion. Blood samples were taken for measurement of amidated GLP-1 during each study at the time-points indicated.

2.4. Discussion

My dose verification study involved investigating the effect PYY₃₋₃₆ 0.3 pmol/kg/min and GLP-1_{7-36 amide} 0.8 pmol/kg/min both singly and in combination, infused intravenously over 90 minutes to a group of 6 subjects. The *ad libitum* test meal was served immediately after the termination of the infusion. Combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min led to a statistically significant reduction (32% reduction compared with the control saline visit) in *ad libitum* energy intake during the test meal, which is similar to the findings of Neary *et al.*, 2005. Single infusion of PYY₃₋₃₆ 0.3 pmol/kg/min led to non-significantly increased food intake (7% increase compared with the control saline visit), whereas single infusion of GLP-1_{7-36 amide} led to a small reduction in *ad libitum* energy intake during the test meal (4% reduction compared to the control saline visit). There was no significant modulation of meal rating in response to either single or combined infusions.

The combined infusion was associated with a degree of nausea (the mean increase in nausea rating from baseline to that at the peak of the infusion was 22.5 mm), although interestingly, there was no change in hunger or fullness ratings. Furthermore, although a degree of nausea outlined above was established through VAS questioning, at no stage did any subjects report to the investigators that they felt nauseated outside of the context of VAS analysis. Therefore, it could be argued that the degree of nausea induced by the combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min and GLP-1_{7-36 amide} 0.8 pmol/kg/min was tolerable.

Haemodynamic parameters were not significantly different between infusion of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1_{7-36 amide} 0.8 pmol/kg/min or combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min and GLP-1_{7-36 amide} 0.8 pmol/kg/min. The infusion of GLP-1_{7-36 amide}, as well as

the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide led to a small but statistically significant fall in blood glucose compared with infusion of saline. This would be expected, given the well established incretin effect of GLP-1.

A peak mean total PYY concentration of 106.5 pmol/L during the combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min infusion, and peak mean total PYY concentration of 91.07 pmol/L during the single PYY₃₋₃₆ 0.3 pmol/kg/min infusion, were similar to the circulating PYY concentrations achieved by Neary *et al.* (Neary *et al.*, 2005), when PYY₃₋₃₆ 0.4 pmol/kg/min + GLP-1₇₋₃₆ amide 0.4 pmol/kg/min were co-infused. Interestingly, Batterham *et al.* achieved a peak mean total circulating PYY concentration of 43.5 pmol/L when administering a single infusion of PYY₃₋₃₆ 0.8 pmol/kg/min (Batterham *et al.*, 2002). This may provide an explanation for why Batterham *et al.* did not report any nausea as a result of their PYY₃₋₃₆ infusion. Despite this modest elevation in circulating concentrations of PYY, 24 hour energy intake was reduced by 33% compared to saline in the study by Batterham *et al.* A degree of inter-assay variation in measurement may explain these differences. Variation between true and estimated peptide content of vials may also account for this mis-match. As outlined in the methods section, after the aliquotting process, I randomly selected representative vials of each peptide and sent them for amino acid analysis. These results were used to calculate the actual peptide content of the vials and therefore the correction factor when calculating dose. In the case of PYY₃₋₃₆, each representative 25 nmol vial was in fact found to contain 18.6 nmol, whereas in the case of GLP-1₇₋₃₆ amide, each 25 nmol vial contained a near exact quantity of peptide (25.4 nmol). Given that previous research may not have taken into account amino acid analysis, it is plausible that a dose of PYY₃₋₃₆ thought to be 0.8 pmol/kg/min by Batterham *et al.* may in fact have been a lower dose.

Analysis of circulating amidated GLP-1 levels in response to infusion in my study showed that compared with a previous study (Neary et al., 2005), peak circulating levels of amidated GLP-1 were similar in my study. Notably, I infused GLP-1_{7-36amide} at a higher dose than Neary's group. Inter-assay variation could have explained these differences.

Interestingly, when PYY₃₋₃₆ was infused in combination with GLP-1_{7-36amide}, circulating levels of PYY were higher than when the same dose of PYY₃₋₃₆ was infused singly. This may have influenced the results for energy intake and nausea. Reasons for this are unclear, but may be due to possible interactions between PYY₃₋₃₆ and GLP-1_{7-36amide} in the infusion system.

My dose verification study established doses of PYY₃₋₃₆ and GLP-1_{7-36amide} that were sub-anorectic when infused singly, but anorectic in combination. Additionally, these doses reduced food intake without causing intolerable nausea. This was of particular importance when planning the fMRI study, given that in the claustrophobic environment within the MRI scanner, nausea was likely to be accentuated. Furthermore, whilst nausea is relatively easy to tackle in a clinical investigation unit, it is more problematic if it occurs within the confines of an MRI scanner, with risk of aspiration pneumonia if the subject were to actually vomit when recumbent. Therefore, it was reassuring that my dose verification study identified doses of PYY₃₋₃₆ and GLP-1_{7-36amide} that when administered in combination, produced significant reduction in food intake without causing intolerable nausea. As a result, these doses (0.3 pmol/kg/min for PYY₃₋₃₆ and 0.8 pmol/kg/min for GLP-1_{7-36amide}) were taken forward into the fMRI study (Study 2).

CHAPTER 3

**Investigation of the effects of single and
combined administration of PYY₃₋₃₆ and
GLP-1₇₋₃₆ amide on appetite, energy intake and
neuronal activity in brain appetite centres
(Study 2)**

3.1. Introduction and rationale

BOLD fMRI has recently been used as a tool to investigate the changes in brain activity in response to nutritional status in humans. It has been demonstrated that brain activity within reward systems is increased in the fasted state compared to the fed state (La Bar et al., 2001, Goldstone et al., 2009, Fletcher et al., 2010). However, the use of BOLD fMRI to determine brain activity following peripheral administration of hormones affecting appetite in humans has been limited to a few studies (Batterham et al., 2007, Farooqi et al., 2007, Baicy et al., 2007, Rosenbaum et al., 2008, Malik et al., 2008). It has been shown that intravenous infusion of PYY₃₋₃₆ to human subjects results in reduced food intake and changes in brain activity in appetite regions (Batterham et al., 2007). However, there have been no human fMRI studies investigating the effect of administration of GLP-1 or co-administration of PYY and GLP-1 on brain activity in humans.

Following Roux-en-Y gastric bypass surgery (the most effective current treatment for obesity), patients demonstrate increased levels of the gut hormones PYY and GLP-1 (Borg et al., 2006, Le Roux et al., 2006). It is thought that elevated levels of these hormones act via the CNS to cause the reduced appetite and sustained weight loss observed following this intervention. It is therefore important to understand the CNS mechanisms by which the gut hormones PYY and GLP-1 mediate their combined anorectic effect in humans.

Together, these important unanswered questions formed the rationale for my investigation of the single and combined anorectic effects of peripherally administered PYY₃₋₃₆ and GLP-1_{7-36 amide} to healthy humans, using BOLD fMRI as a tool to assess the effect of these hormones on neuronal activation in brain regions implicated in appetite control.

3.2. Methods

3.2.1. Peptides

Freeze dried vials of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide used in this study were from the same batch of peptide vials as used in Study 1 (Section 2.2.1.). Based on the results of study 1, I decided to use PYY₃₋₃₆ at a dose of 0.3 pmol/kg/min and GLP-1₇₋₃₆ amide at a dose of 0.8 pmol/kg/min.

3.2.2. Subjects

Sixteen healthy right-handed subjects (11 male, 5 female) of mean age 29.5 years (range 21 - 36 years) and mean body mass index 22.1 kg/m² (range 18.3 - 25.1 kg/m²) were recruited by newspaper advertisement. 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 kJ, using a paired Student t test with a 0.05 two sided significance level. Ethical Approval, subject screening, consent, inclusion and exclusion criteria were as per Study 1 (Section 2.2.2.). Additionally, subjects who were claustrophobic, had any metal implants, or those who had undergone recent dental work were excluded, as this would have precluded the subject entering the MRI scanner. One male subject was excluded from the study after recruitment due to excessive head movement during the MRI scans, resulting in complete data from 15 subjects for analysis.

3.2.3. Design

Sixteen right-handed subjects (11 male, 5 female) each attended for 6 study visits, each at least 3 days apart. Overnight fasting from 22:00 h the night before and other preparation before each study visit was identical to Study 1 (Section 2.2.3.).

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 an identical protocol 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 this acclimatisation visit, the nature of the MRI scan experience was explained to the volunteers. The rest of this first visit was run in an identical fashion to the following visits with an additional structural brain scan (5 minutes duration) at the end of the scanning run to exclude organic disease. Results from the first saline visit are not included in the imaging analysis.

Following the first saline 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 PYY₃₋₃₆ infusion at 0.3 pmol/kg/min
4. Overnight fast followed by GLP-1_{7-36 amide} infusion at 0.8 pmol/kg/min
5. Overnight fast followed by combined PYY₃₋₃₆ and GLP-1_{7-36 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 inserted in both forearms at t = - 90 min, 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.

3.2.4. Peptide preparation and delivery

All syringes and lines used to deliver peptide were coated in gelofusin for 20 minutes prior to preparation, to minimise adsorption of peptide to the plastic walls. The vehicle for delivering peptide was a 10% solution of gelofusin in 0.9% saline, as per Study 1 (Section 2.2.4.). Vials of peptide were dissolved in 2.5 ml vehicle, and drawn up in a single syringe up to a total volume of 90 ml. The amount of peptide solution added to the delivery syringe varied according to the dose each subject received. The first 10 ml were slowly (over 5 minutes) purged through the delivery line before it was attached to a subject's infusion cannula. A MEDRAD MR Injector Spectris Solaris EP (Siemens UK, Camberley, Surrey) pump was used to deliver the infusions at a constant rate of 36 ml/hr. The intravenous infusion started in the MRI scanning room at 10:30 h ($t = 0$ min) and was delivered over 90 minutes.

3.2.5. Blood sampling and processing

Six ml blood samples were collected for gut hormone measurement at $t = -60, 0, 15, 30, 45, 60, 75, 90$ and 120 min and processed and stored as already described for Study 1 in Section 2.2.5. Three ml deadspace blood was collected and discarded at each sample point to account for the 100 cm long extension line.

3.2.6. Visual analogue scale scores

At $t = -60, 0, 90,$ and 120 min, subjects completed a series of VAS questionnaires. These comprised five food-related sensations (hunger, nausea, pleasantness to eat, how much one could eat, fullness) and additionally four non-food related sensations (warmth, irritability, anxiety, sleepiness). 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 (Flint et al., 2000).

3.2.7. Measurement of haemodynamic parameters and blood glucose

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. Measurement of these parameters was as per Study 1 (Section 2.2.7.).

3.2.8. Study meals

The *ad libitum* lunch buffet meal was as per Study 1 (Section 2.2.8.). On their fed visit, subjects received a set breakfast (2500 kJ), served in a private area between $09:30 - 09:50$ h ($t = -60$ to -40 min). The set breakfast served to subjects during one of the fMRI study visits (2500 kJ) consisted of 2 medium slices of wholemeal bread, 20 g strawberry jam, 20 g margarine, 2 slices of Leerdammer Swiss cheese, 40 g Bran flakes, 170 g semi-skimmed milk, and 220 g orange juice. Subjects were asked to consume the above breakfast within 20 minutes.

3.2.9. Functional MRI scan

At 10:30 h ($t = 0$ min), the subject was taken into the scanning room and the 90 minute infusion started. Twenty minutes after the start of the infusion (i.e. $t = 20$ min), the 60 minute fMRI scan began. T2*-weighted, dual echo, EPI images sensitive to BOLD contrast were acquired continuously on a 3 Tesla 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 $\sim 30^\circ$ coronally to the anterior-posterior commissural plane to minimize signal dropout in orbitofrontal and medial temporal regions (Repetition time (TR) = 2000 ms; dual echo times (TEs) = 13 and 31 ms; flip angle = 80° , slice thickness = 3.0 mm, matrix size = 64×64 , field of view = 225×225 mm, voxel size = $3.51 \times 3.51 \times 3.0$ mm). High-resolution T1-weighted anatomical scans (ADNI MPRAGE (Jack et al., 2008)) 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 = 9° , voxel size = $1 \times 1 \times 1$ mm).

During the fMRI scan, subjects were asked to watch and rate a series of colour images in a picture processing task. 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. During the fMRI picture processing task, 75 images were shown, divided into 50 images of foods and 25 non-food items (Beaver et al., 2006). There were two sets of 75 images, alternated between each scanning session. Images from each category were presented in counterbalanced order across participants and sessions. The images appeared for 5 seconds, interspersed with periods of rest (fixation on a blue octagon), with each block lasting 25 seconds.

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 private dining area where an *ad libitum* buffet meal (as previously described in Section 2.2.8.) was served. Subjects were allowed to go home at $t = 120$ min.

The protocol for Study 2 is summarised in figure 3.1 below.

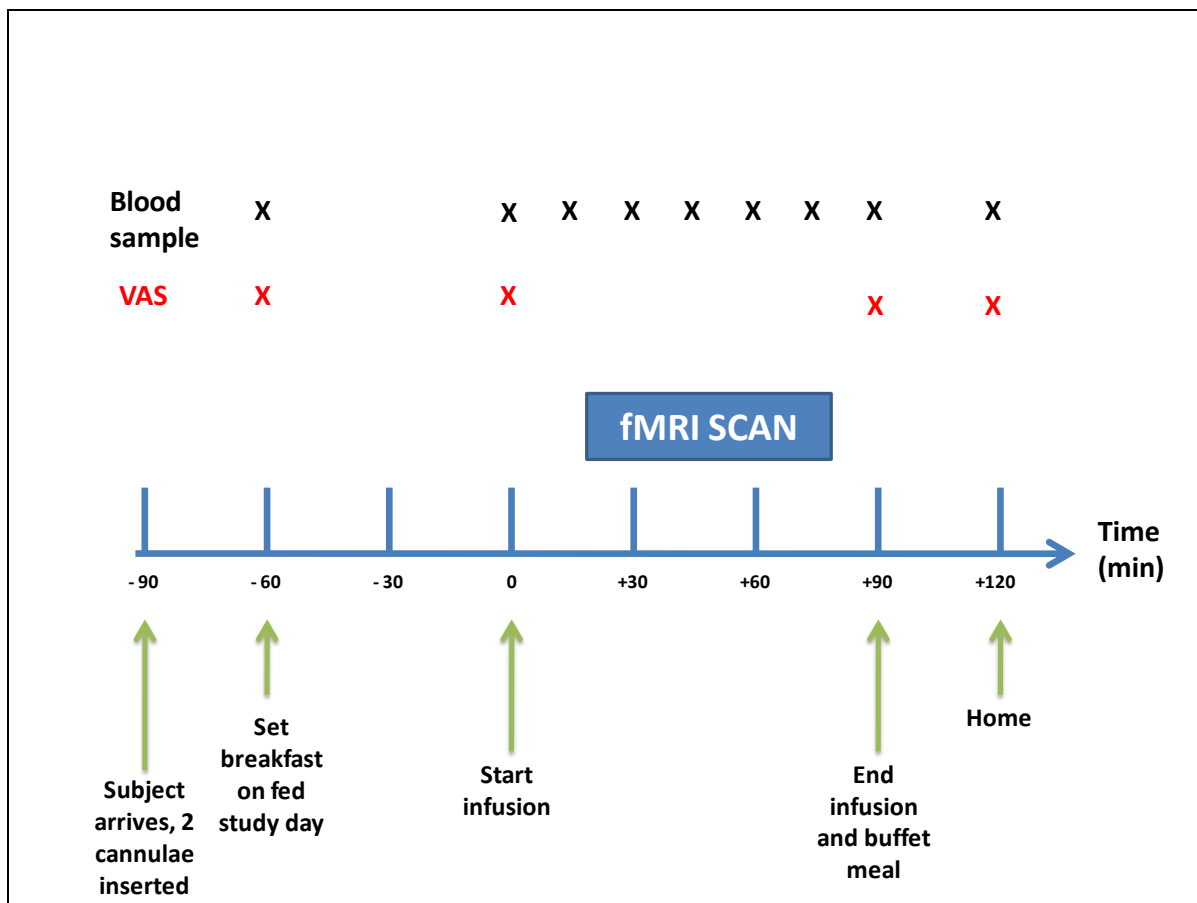


Figure 3.1: Protocol for Study 2. Following an overnight fast, sixteen healthy, normal-weight subjects each received the following interventions, in random order, over 5 separate study days in a single-blinded fashion:

1. 90 minute saline infusion (fasted saline, control visit)
2. Standard breakfast, then 90 minute saline infusion (fed saline)
3. 90 minute PYY₃₋₃₆ infusion at 0.3 pmol/kg/min
4. 90 minute GLP-1₇₋₃₆ infusion at 0.8 pmol/kg/min
5. 90 minute combined PYY₃₋₃₆ and GLP-1₇₋₃₆ 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 VAS after direct questioning) was performed.

3.2.10. Measurement of total PYY

Total PYY immunoreactivity was measured with a specific and sensitive in-house RIA, as previously described in Section 2.2.9. The RIA set up was as described in Section 2.2.11.

3.2.11. Measurement of PYY₃₋₃₆

Active PYY (PYY₃₋₃₆) immunoreactivity was measured with a specific and sensitive commercial RIA (Cat. # PYY-67HK, Millipore Corporation, MA, USA) (King *et al.*, 2011). The antibody was produced in Guinea Pigs against human PYY. The antibody cross reacted 100% with PYY₃₋₃₆, but 0% PYY₁₋₃₆ (for concentrations up to 247 pmol/L). PYY₃₋₃₆ standard was used at 2800 pg/ml (0.69 pmol/ml) and serially diluted for use in the standard curves, which were included every 200 tubes in the assay. The assay was performed in total volume of 0.4 ml of 0.08% sodium azide buffer (0.1 ml) with treated human serum matrix (0.1 ml). A sample volume of 0.1 ml and antibody volume of 0.1 ml were used. The assay tubes containing sample (or standard), antibody, buffer and matrix were incubated for 24 hours at 4°C, following which ¹²⁵I-PYY was added to each tube (0.1 ml) and the assay incubated for a further 24 hours. Separation of the free and antibody bound radiolabel was by the addition of 0.01 ml of Guinea Pig serum and 1 ml of precipitating reagent to each tube (containing goat anti-Guinea Pig IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M phosphosaline, 0.025M EDTA, 0.08% sodium azide) followed by centrifugation at 4°C for 20 minutes at 2500 rpm. The assay results in pg/ml were divided by 4049.52 (the molecular weight of 1 mole of PYY₃₋₃₆) and multiplied by 1000 in order to convert to pmol/L. The detection limit of the assay was 5 pmol/L, with an intra-assay coefficient of variation of 6.4% (for high levels of 54 pmol/L) and 11% (for low levels of 21 pmol/L). The inter-assay coefficient of variation was 7% (for high levels of 54 pmol/L) and 15% (for low levels of 21 pmol/L). All standard curve

tubes were assayed in duplicate, whereas all sample tubes were assayed in singlet. All samples were assayed in one assay to avoid inter-assay variation. The value of the QCs used were: high = 84 - 174 pmol/L; low = 24 - 50 pmol/L and measured QC values were within the above ranges.

Full details of the commercial RIA protocol for measurement of PYY₃₋₃₆ can be found at <http://www.millipore.com/catalogue/item/pyy-67hk>

3.2.12. Measurement of amidated GLP-1

Amidated GLP-1 like immunoreactivity was measured by a specific and sensitive in-house RIA, as previously described in Section 2.2.10. The RIA set up was as described in Section 2.2.11.

3.2.13. Measurement of active GLP-1 (GLP-1₇₋₃₆ amide & GLP-1₇₋₃₇)

Active GLP-1 immunoreactivity was measured with a specific and sensitive commercial enzyme-linked immunosorbent assay (ELISA) kit (Cat. # EGLP-35K, Millipore Corporation, MA, USA) (Adam *et al.*, 2005). Information regarding the principles of ELISA can be found in Appendix 1. Microtiter plates (96 well) were used to set up the assay, which had been pre-coated in anti-GLP-1 monoclonal antibody. The antibody cross reacted 100% with active forms of GLP-1 (GLP-1₇₋₃₆ amide & GLP-1₇₋₃₇) but undetectably with other forms of GLP-1 (GLP-1₁₋₃₆ amide, GLP-1₁₋₃₇). Volumes of 0.1 ml from GLP-1₇₋₃₆ ELISA standard vials ranging from 2 pmol/L to 100 pmol/L were used to generate the standard curves, which were placed throughout the assay at the beginning of each 96 well microtiter plate. The assay was performed in total volume of 0.2 ml. This comprised 0.1 ml of sample added to 0.1 ml of

phosphate buffered saline (pH 6.8), containing proprietary protease inhibitors, with Tween 20, 0.08% sodium azide and 1% BSA. Following addition of sample, plates were covered with a plate sealer and incubated for 24 hours at 4°C. Plates were washed to remove unbound materials, following which 0.2 ml detection conjugate (containing anti-GLP-1-alkaline phosphatase conjugate) was added to each well and the plates further incubated at room temperature for 2 hours. Plates were then washed again to remove unbound conjugate, following which 0.2 ml 4-methylumbelliferyl phosphate was added to each well. Plates were incubated at room temperature in the dark for at least 20 minutes, following which 0.05 ml stop solution was added to each well. The plates were read on a fluorescence plate reader with an excitation / emission wavelength of 355 nm / 460 nm. The detection limit of the assay was 2 pmol/L, with an intra-assay coefficient of variation of 9% (for high levels of 76 pmol/L) and 8% (for low levels of 4 pmol/L). The inter-assay coefficient of variation was <1% (for high levels of 76 pmol/L) and 13% (for low levels of 4 pmol/L). All samples were assayed in duplicate and in one assay to avoid inter-assay variation. The value of the QCs used were: high = 24 - 50 pmol/L; low = 4.4 - 9.2 pmol/L and measured QC values were within the above ranges.

Full details of the commercial ELISA protocol for measurement of active GLP-1 (GLP-1₇₋₃₆ amide & GLP-1₇₋₃₇) can be found at <http://www.millipore.com/catalogue/item/eglp-35k>

3.2.14. Statistical analysis of energy intake, VAS, haemodynamic parameters, blood glucose and gut hormone levels

Grouped data are represented as the mean $\pm$ SEM. Comparisons of energy intake, ratings of meal palatability, VAS scores, glucose levels, haemodynamic parameters and gut hormone levels during the infusion period were as per Study 1 (Section 2.2.12.). Comparison of

energy intake between the fasted acclimatisation saline visit (visit 1) and the control, fasted saline visit was by paired Student t-test. The effect of visit number on energy intake was analysed using two-way ANOVA, with the intervention as the column factor and visit number as the row factor. Linear regression analysis was performed to assess correlation between nausea and energy intake for the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. 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).

3.2.15. fMRI analysis: pre-processing of data

All fMRI data processing was carried out using FEAT (fMRI Expert Analysis Tool) version 5.98. fMRI data were preprocessed with FSL software (www.fmrib.ox.ac.uk/fsl/) in order to correct for motion, to register the echo planar functional images to the high-resolution anatomical (T1-weighted) scan of each individual, and to overlay images on a standardized Montreal Neurological Institute (MNI) atlas in order to allow for comparisons across individuals. Correction for slice timing was performed in view of the fact that each slice was acquired at a slightly different time within the TR; 36 slices were acquired within the TR of 2000 ms. Data were high-pass temporally filtered in order to remove low frequency drifts (e.g. drifts due to the cardiac and respiratory cycle) in image acquisition. Data were 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. Using the MCFLIRT tool within FSL (Jenkinson et al., 2002), individual subjects sessions with greater than 3.5 mm absolute head motion or severe stimulus-correlated motion (as exhibited by marked rim artefacts in intra-subject statistic maps) were excluded from further analysis. This led to a total of fifteen usable subjects with complete data from all infusion groups.

3.2.16. fMRI analysis: whole-brain activation with presentation of food-salient visual stimuli

For the fasted saline and fed saline visits, a whole-brain map of brain regions activated by food images (compared with non-food images) was created. Clusters were thresholded by means of parametric testing at the level of spatially contiguous clusters of voxels, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for multiple comparisons.

3.2.17. fMRI analysis: generation of whole brain maps of effect of feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration on brain activation by food images

Whole brain maps of the difference in BOLD signal between viewing images of food versus non-food were generated for each infusion (fasted saline, fed saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min, PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min). Comparison was made between the difference in BOLD signal between viewing images of food versus non-food for each of the above interventions, against the difference in BOLD signal between viewing images of food versus non-food during the fasted saline infusion. Clusters were thresholded by means of parametric testing at the level of $z > 1.6$, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for multiple comparisons.

3.2.18. fMRI ROI analysis: effect of feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration on brain activation by food images

During each infusion (fasted saline, fed saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min, PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min) the difference in regional mean BOLD signal intensity between periods of subject exposure to food images compared with non-food images was estimated at each of 6 pre-specified ROIs bilaterally-amygdala, caudate, insula, nucleus accumbens, OFC, and putamen. These were chosen based on previous studies (Batterham et al., 2007, Farooqi et al., 2007, Fletcher et al., 2010, LaBar et al., 2001, Malik et al., 2008) using an in-house MRI atlas (Tziortzi et al., 2011). All ROI signal change was 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 clusters of voxels, while controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for multiple comparisons) at the relevant atlas region. The accuracy of registration of ROIs on the BOLD functional data was confirmed by visual inspection of their overlay in relation to the functional images i.e. in subject space. Image acquisition for this ROI analysis was weighted towards the 2nd echo (TE = 31 ms).

These estimates of % BOLD signal change were then averaged over right and left homologous regions of amygdala, caudate, insula, nucleus accumbens, OFC, and putamen. For each study intervention (i.e. feeding or gut hormone infusion), I tested the null hypothesis that the within-subject difference between fasted saline, fed saline, PYY₃₋₃₆ infusion, GLP-1₇₋₃₆ amide infusion or combined PYY₃₋₃₆ + GLP-1₇₋₃₆ amide infusion in regional BOLD response to food images vs. non-food images was zero, i.e. [the gut hormone infusion or fed saline infusion BOLD response to food images] – [fasted saline BOLD response to food images] = $\Delta\text{BOLD} = 0$. The statistical model used to test this was a repeated measures

analysis of difference of means, with Dunnett's post-test between control (fasted saline) and each infusion session BOLD data. A separate analysis of left and right sides for each of the 6 ROIs was also performed. This was followed by an analysis of the effects of feeding, PYY₃₋₃₆ infusion, GLP-1₇₋₃₆ amide infusion and combined PYY₃₋₃₆ & GLP-1₇₋₃₆ amide infusion on changes in mean % BOLD signal change between viewing images of food vs. non-food in the total brain volume represented by the 6 *a priori* selected ROIs. Statistical analysis was by repeated measures ANOVA and Tukey's multiple comparison post-test.

For each subject undergoing the combined infusion of PYY₃₋₃₆ & GLP-1₇₋₃₆ amide, linear regression analysis of the change in nausea score from 0 - 90 min against the reduction in % BOLD signal change from the control saline infusion was performed for each ROI, averaged over right and left sides.

3.2.19. fMRI analysis: effect of feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration on hypothalamic and brainstem activation by food images

In a separate voxel-wise analysis, I examined the mean % change in BOLD signal when viewing food images versus non-food images in the hypothalamus and brainstem (midbrain) in subjects after feeding, PYY₃₋₃₆, GLP-1₇₋₃₆ amide or combined PYY₃₋₃₆ & GLP-1₇₋₃₆ amide infusion. I compared this with the mean % change in BOLD signal when viewing food images versus non-food images in the hypothalamus and brainstem (midbrain) when subjects were fasted and received saline (control). Voxels were thresholded by means of parametric testing at the level of $z > 1.6$, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for multiple comparisons. Image acquisition for this hypothalamic and brainstem response used a combined echo analysis, weighted towards the 1st echo (TE = 13 ms).

3.3. Results

3.3.1. Comparison of energy intake between acclimatisation visit and fasted saline visit

For the 15 subjects in Study 2, energy intake during the *ad libitum* buffet lunch during the fasted saline acclimatisation study visit (visit 1) and the control, fasted saline visit (randomly distributed across the remaining 5 study visits) was analysed (figure 3.2). This revealed there to be significantly lower energy intake during the *ad libitum* buffet lunch during the acclimatisation study visits compared with the control saline study visits (energy intake at *ad libitum* buffet lunch (kJ): fasted acclimatisation saline, 3715 ± 419.4 vs fasted (control) saline, 4039 ± 399 , $p = 0.049$) (Figure 3.2).

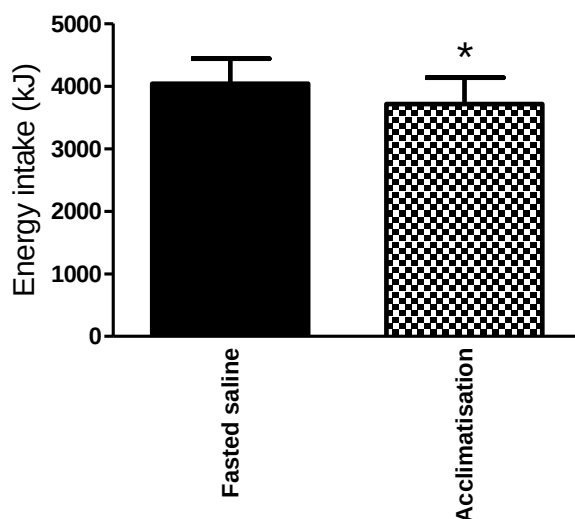


Figure 3.2: Mean *ad libitum* energy intake during acclimatisation visit and control saline visit in Study 2. Fifteen healthy subjects underwent an initial acclimatisation visit where they received a 90 minute infusion of saline following an overnight fast (acclimatisation), followed by 5 further randomised visits, one of which comprised a 90 minute infusions of saline following an overnight fast. An *ad libitum* buffet meal was served immediately after each infusion in order to measure energy intake. Energy intake during the acclimatisation visit and control fasted saline visit is shown. * denotes $p < 0.05$ for acclimatisation vs fasted saline.

3.3.2. Analysis of visit order effects on energy intake

For the 15 subjects in Study 2, following the acclimatisation visit, where subjects received saline only, the remaining 5 study visits were randomised in terms of the infusion administered (fasted saline, fed saline, PYY₃₋₃₆, GLP-1₇₋₃₆ amide, PYY₃₋₃₆ + GLP-1₇₋₃₆ amide). Analysis of the mean energy intake during the *ad libitum* lunch across the 5 randomised visits revealed that there were no order effects across study visits and that subjects did not eat progressively less during each subsequent visit as a result of habituation to the study meal: mean energy intake at *ad libitum* buffet lunch (kJ): visit 2 3798 ± 498; visit 3 3229 ± 334; visit 4 3531 ± 330; visit 5 3354 ± 411; visit 6 3116 ± 323. The effect of the interventions on energy intake was not significantly different between visits ($p = 0.16$). Furthermore, energy intake did not change significantly with visit number ($p = 0.74$). This is illustrated in figure 3.3.

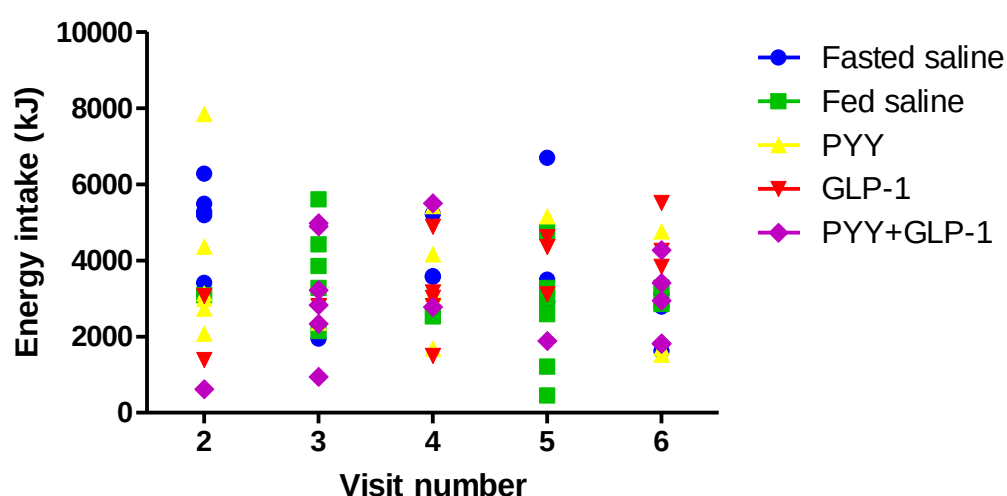


Figure 3.3: Visit order effects on energy intake in Study 2. Following an acclimatisation visit, 15 healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. The interventions were administered in randomised order. On each study visit, each subject received the same *ad libitum* buffet meal immediately after the infusion in order to measure energy intake. There were no effects on energy intake of subjects by visit number ($p = 0.74$).

3.3.3. Effects of feeding, PYY₃₋₃₆ and GLP-1_{7-36 amide} administration on subsequent energy intake

Following saline, PYY or GLP-1 (either as single or combined administration) in 15 fasted healthy human subjects, *ad libitum* energy intake was measured during a buffet lunch. The effects of PYY and GLP-1 were compared to those seen naturally following a breakfast meal. Consumption of a standard breakfast by subjects before saline infusion led to a $23.5\% \pm 8.3\%$ reduction in energy intake during the subsequent *ad libitum* buffet lunch, compared to when subjects received only saline infusion (energy intake at *ad libitum* buffet lunch (kJ): fasted (control), 4039 ± 399 vs. fed, 3090 ± 335 , $p < 0.01$) (Figure 3.4 A). Infusion of PYY₃₋₃₆, GLP-1_{7-36 amide} or combined PYY₃₋₃₆ and GLP-1_{7-36 amide} to fasted subjects resulted in $12.3\% \pm 11.2\%$, $15.7\% \pm 7.5\%$ and $27.0\% \pm 9.4\%$ reductions in energy intake, respectively, at the subsequent *ad libitum* buffet lunch compared to when subjects received saline infusion (energy intake at *ad libitum* buffet lunch (kJ): fasted saline 4039 ± 399 ; PYY₃₋₃₆ 3542 ± 452 ; GLP-1_{7-36 amide} 3407 ± 302 ; PYY₃₋₃₆ + 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 3.4 A).

The additive effects of single infusions of PYY₃₋₃₆ and GLP-1_{7-36 amide} were compared with combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} on energy intake at the *ad libitum* lunch. The summed reduction in energy intake by each of the single peptide infusions was comparable with the reduction after the combined infusion ($p = 0.86$) (Figure 3.4 B).

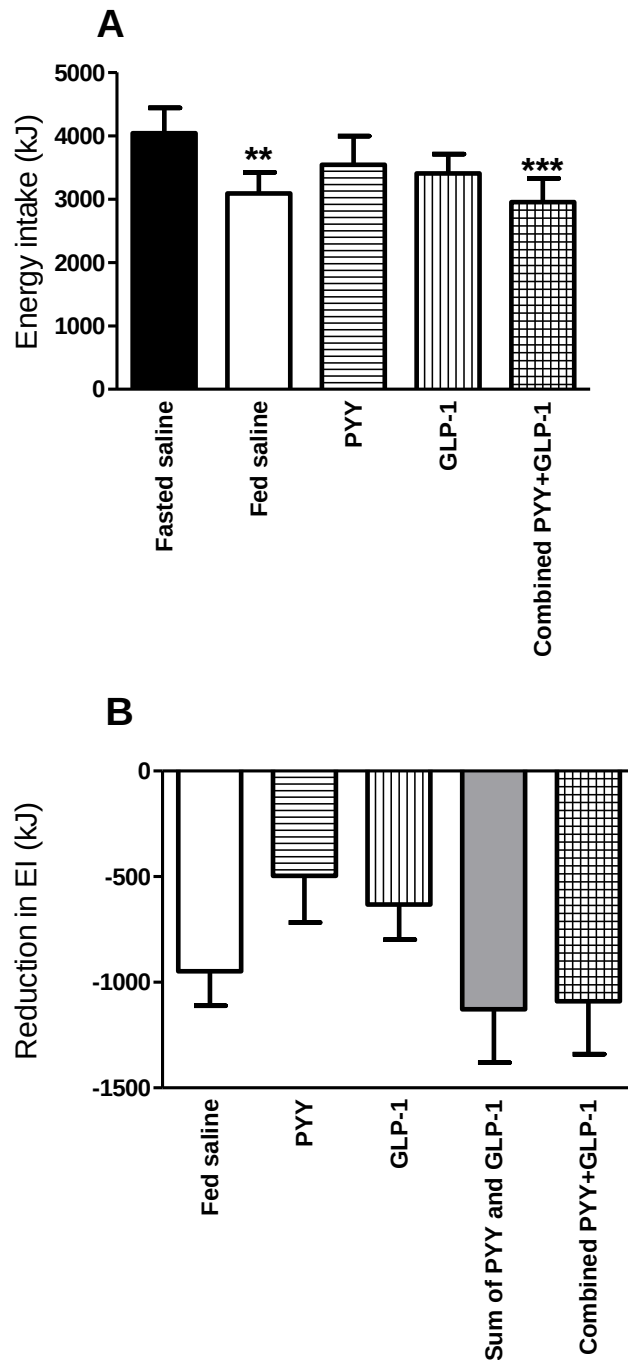


Figure 3.4: Mean *ad libitum* energy intake in Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (Combined PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion (Fed saline). An *ad libitum* buffet meal was served immediately after the infusion in order to measure energy intake (figure 3.4 A). Figure 3.4 B shows the reduction in energy intake during the buffet meal for each infusion versus the fasted saline infusion and also the sum of the individual effects of PYY and GLP-1 in reducing energy intake (sum of PYY and GLP-1). ** denotes $p < 0.01$ vs. fasted saline. *** denotes $p = 0.0001$ vs. fasted saline.

3.3.4. Ratings of meal palatability

For the 15 subjects in Study 2, mean rating (at $t = 120$ min) of how tasty they found the buffet lunch on a 100 mm VAS scale was 71.6 ± 4.1 mm when they received saline, 66.3 ± 3.4 mm when they received saline after a set breakfast, 68.7 ± 3.9 mm when they received the single infusion of PYY₃₋₃₆ 0.3 pmol/kg/min, 67.8 ± 3.5 mm when they received the single infusion of GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and 64.4 ± 4.1 mm when they received the combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min. This is illustrated in figure 3.5 below. There was no significant difference between visits ($p = 0.11$).

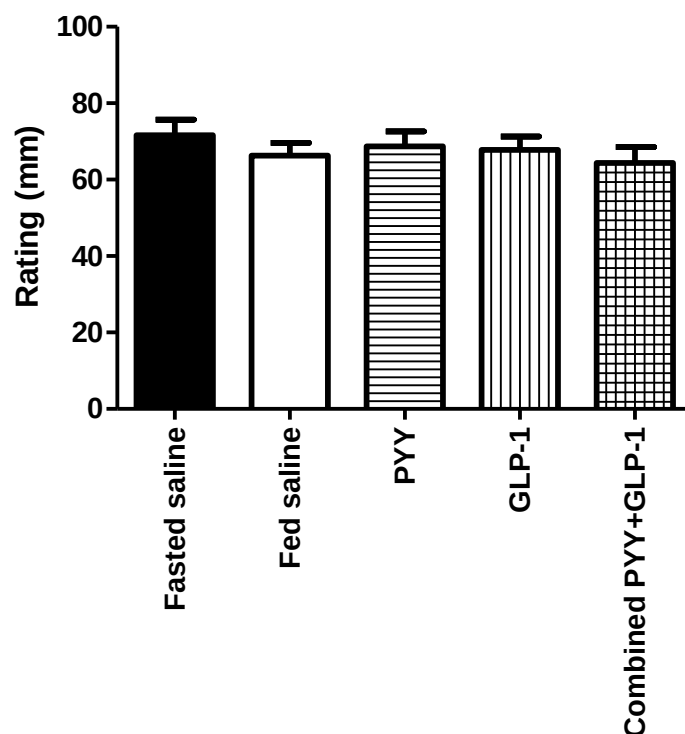


Figure 3.5: Meal rating following *ad libitum* meal in Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (Combined PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion (Fed saline). An *ad libitum* buffet meal was served immediately after the infusion and subjects rated this meal on a scale of 0 - 100.

3.3.5. Analysis of the effect of feeding and gut hormone infusion on food related VAS parameters

For the 15 subjects in Study 2, during the course of each study day, food related VAS parameters varied as shown in figure 3.6.

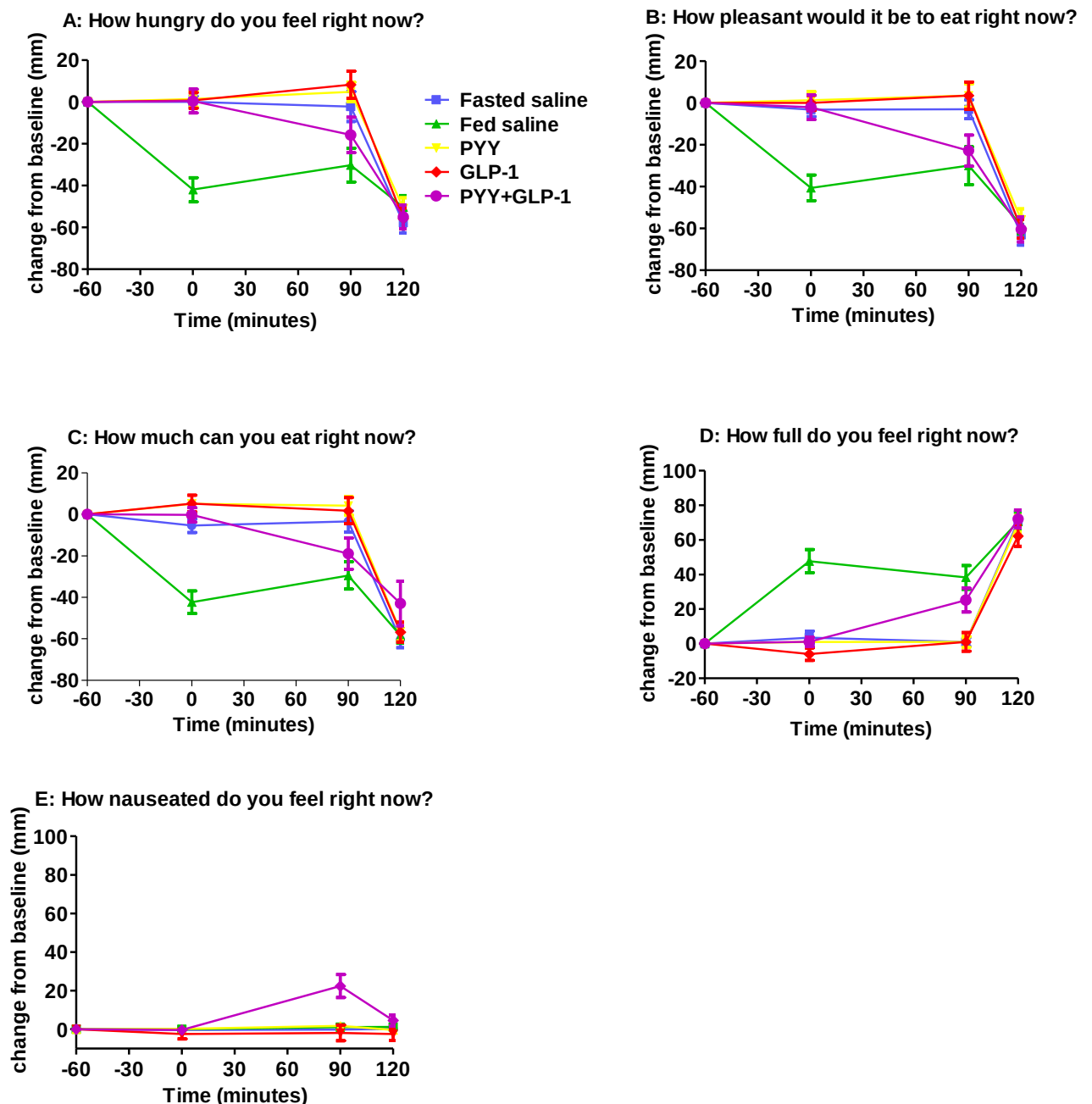


Figure 3.6: Food related VAS parameters during Study 2. During each study, 15 subjects completed 100 mm VAS questionnaires relating to hunger (A), pleasantness to eat (B), anticipated food consumption (C), fullness (D) and nausea (E).

VAS scores of appetite by subjects confirmed that consumption of the standard breakfast reduced hunger, reduced perceived pleasantness of eating more food and reduced anticipated further consumption, as well as increasing a sense of “fullness” (figure 19). Similarly, in fasted subjects, combined infusion of PYY₃₋₃₆ 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 3.6). By contrast, the single infusions of PYY₃₋₃₆ or GLP-1_{7-36 amide} were not associated with significant changes in subjective feelings of hunger, perceived pleasantness of eating, anticipated food consumption, “fullness” or nausea (figure 3.6). The mean change in each VAS parameter during the course of each infusion is shown in table 3.1.

Change in VAS (mm)	Fasted saline	Fed saline	PYY	GLP-1	PYY+GLP-1
Hunger	-2.27 ± 6.4	13.43 ± 4.9	3.40 ± 5.6	7.47 ± 5.8	-16.20 ± 9.1 ^a
Pleasantness to eat	0.13 ± 5.7	10.07 ± 6.1	2.27 ± 6.1	3.53 ± 5.4	-20.73 ± 9.1 ^b
How much one could eat	2.00 ± 4.9	12.36 ± 3.4	-0.93 ± 4.5	-3.47 ± 5.0	-18.67 ± 8.7 ^c
Fullness	-2.47 ± 4.9	-9.21 ± 4.7	-0.07 ± 3.6	7.07 ± 2.7	24.07 ± 7.5 ^d
Nausea	0.20 ± 0.2	1.14 ± 0.7	1.53 ± 1.5	0.60 ± 1.9	22.73 ± 5.9 ^e

Table 3.1: Analysis of food related VAS parameters from t = 0 to t = 90 min during each infusion in Study 2.

Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion (Fed saline). On each day, the infusion was administered between t= 0 and t= 90 min. During the study, subjects completed 100 mm VAS questionnaires relating to hunger, nausea, pleasantness to eat, how much they could prospectively eat and fullness. The change in VAS scores from baseline (t=0 min) to the end of the infusion (t= 90 min) are shown. ^a denotes p = 0.03 for PYY+GLP-1 vs Fed saline. ^b denotes p = 0.02 for PYY+GLP-1 vs Fed saline. ^c denotes p = 0.007 for PYY+GLP-1 vs Fed saline. ^d denotes p = 0.0002 for PYY+GLP-1 vs Fed saline and p<0.01 for PYY+GLP-1 vs Fasted saline and PYY. ^e denotes p<0.0001 for PYY+GLP-1vs all other groups. p = ns for Fasted saline vs. Fed saline.

3.3.6. Correlation of nausea with energy intake

For the 15 subjects in Study 2, for the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, there was no significant correlation between nausea and energy intake during the *ad libitum* buffet lunch ($p = 0.19$, $r^2 = 0.13$, figure 3.7).

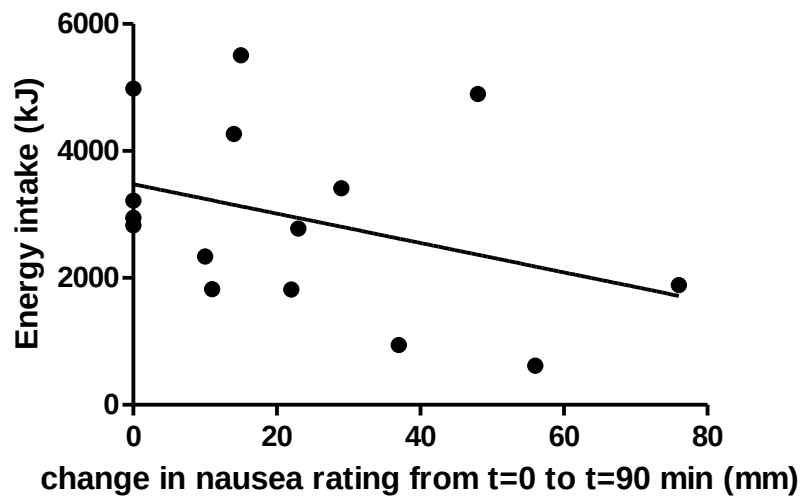


Figure 3.7: Correlation of nausea with energy intake during combined PYY₃₋₃₆ and GLP-1₇₋₃₆ amide infusion during Study 2. For each subject, the change in VAS nausea scores from baseline ($t = 0$ min) to the end of the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide ($t = 90$ min) are shown, correlated against *ad libitum* energy intake during the buffet lunch. $p = 0.19$, $r^2 = 0.13$.

3.3.7. Analysis of the effect of feeding and gut hormone infusion on non-food related VAS parameters

For the 15 subjects in Study 2, alteration of non-food related VAS parameters (warmth, irritability, anxiety and sleepiness) during the course of the studies is shown in figure 3.8.

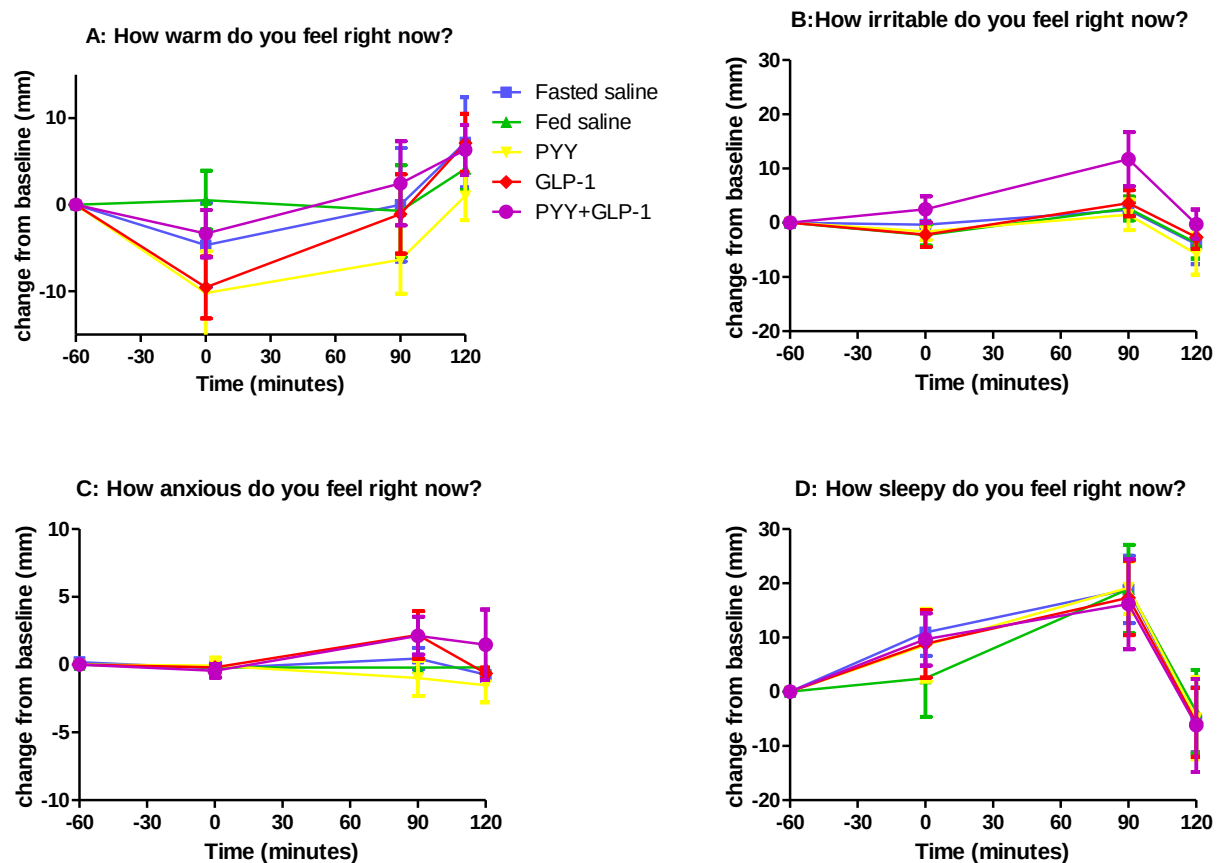


Figure 3.8: Non-food related VAS parameters during Study 2. During each study, 15 subjects completed 100 mm VAS questionnaires relating to warmth (A), irritability (B), anxiety (C) and sleepiness (D).

The change in non-food VAS ratings during the period of infusion (0 - 90 min) were not significantly different between infusion of saline, PYY₃₋₃₆, GLP-1₇₋₃₆ amide, combined infusion of PYY₃₋₃₆ + GLP-1₇₋₃₆ amide, or infusion of saline following a standard breakfast ($p = 0.64$ for warmth, $p = 0.67$ for irritability, $p = 0.31$ for anxiety, $p = 0.90$ for sleepiness, figure 3.9)

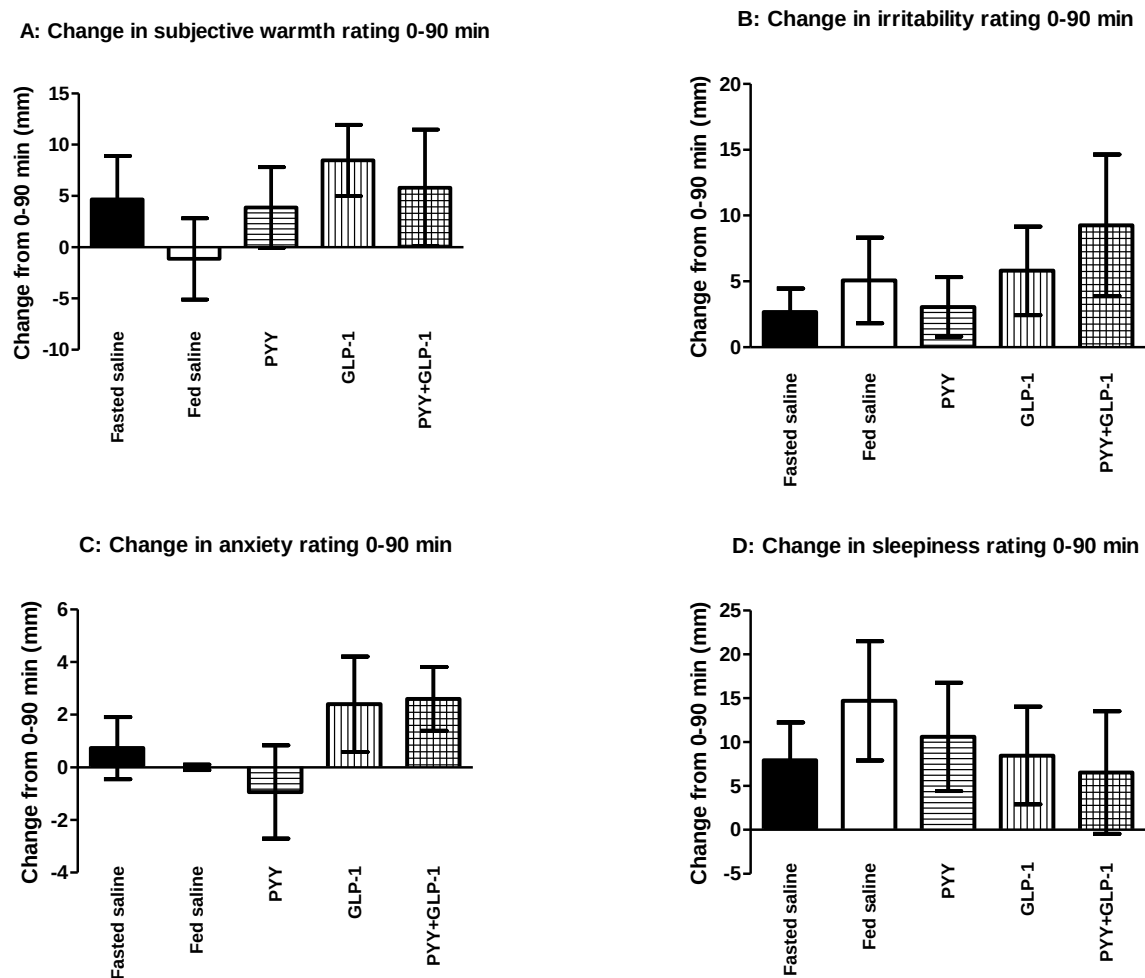


Figure 3.9: Change in non-food related VAS parameters during Study 2. During each study, 15 subjects completed 100 mm visual analogue scale questionnaires relating to warmth (A), irritability (B), anxiety (C) and sleepiness (D). The change in VAS scores from baseline ($t = 0$ min) to the end of each infusion ($t = 90$ min) are shown.

3.3.8. Haemodynamic parameters

For the 15 subjects in Study 2, blood pressure (systolic and diastolic BP) and pulse were not significantly different between infusions, as shown in figure 3.10 ($p=0.66$ for systolic blood pressure, $p=0.56$ for diastolic blood pressure and $p=0.40$ for pulse).

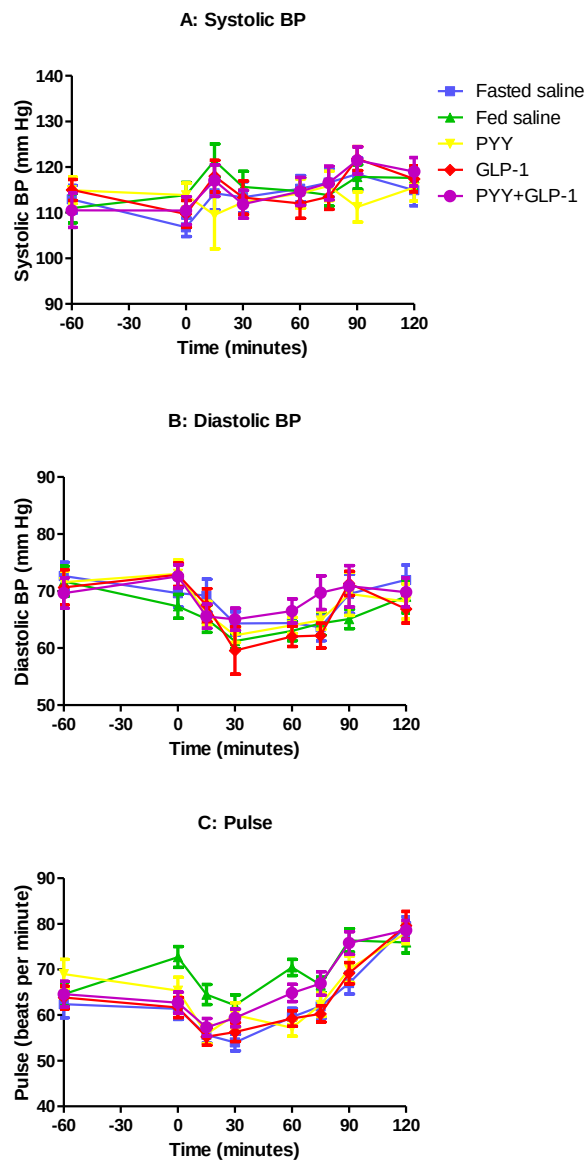


Figure 3.10: Haemodynamic parameters during Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion (Fed saline). On each day, the infusion was administered between $t = 0$ and $t = 90$ min. During each study, systolic blood pressure (A), diastolic blood pressure (B) and pulse (C) were recorded.

3.3.9. Blood glucose

For the 15 subjects in Study 2, infusion of GLP-1₇₋₃₆ amide 0.8 pmol/kg/min or combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min did not lead to a significant change in blood glucose between 0 and 90 min compared with infusion of saline or PYY₃₋₃₆ 0.3 pmol/kg/min alone (figure 3.11). Feeding subjects breakfast at t = - 60 min led to an initial rise in blood glucose by t = 0 min, which returned to baseline during the course of the 90 min saline infusion (figure 3.11)

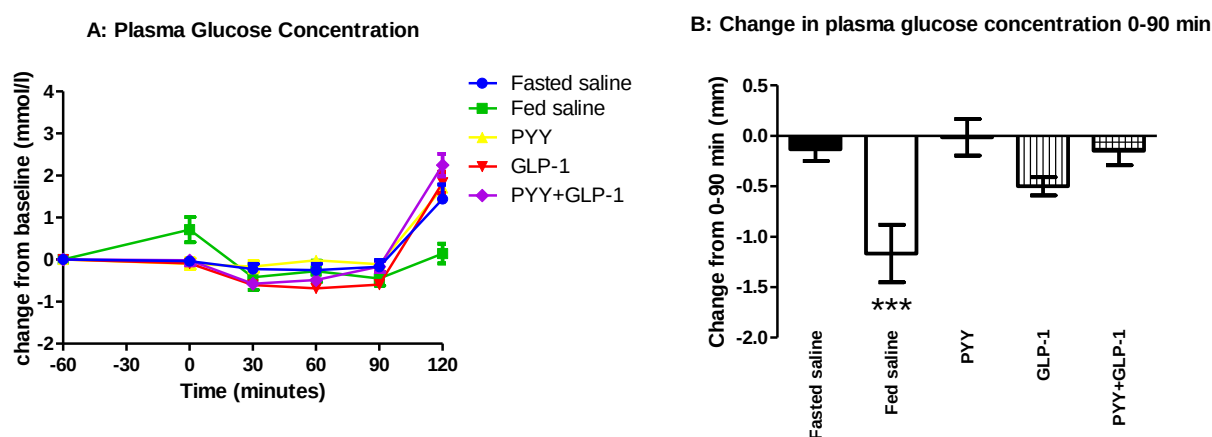


Figure 3.11: Blood glucose during Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted sal), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast at t = -60 min followed by a 90 minute saline infusion (Fed saline). On each day, the infusion was administered between t = 0 and t = 90 min. During the study, the change in blood glucose from baseline was measured (figure 3.11 A). Figure 3.11 B shows the change in blood glucose from 0 - 90 min.

*** denotes $p < 0.001$ for Fed saline vs Fasted saline and PYY.

3.3.10. Total PYY levels in Study 2

Mean plasma concentration of total PYY (from time points $t = 15$ min to $t = 90$ min) was 31.5 ± 0.9 pmol/L during the fasted saline infusion, 33.2 ± 0.7 pmol/L during the fed saline infusion, 65.0 ± 4.8 pmol/L during the PYY₃₋₃₆ infusion, 30.0 ± 1.5 pmol/L during the GLP-1₇₋₃₆ amide infusion, and 69.8 ± 7.5 pmol/L during the combination infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide (figure 3.12).

Peak mean plasma concentration of total PYY (during the entire study) was 42.3 ± 6.2 pmol/L during the fasted saline study visit (occurring post-prandially at $t = 120$ min), 42.5 ± 3.9 pmol/L during the fed saline study visit (occurring post-prandially at $t = 120$ min), 78.8 ± 6.7 pmol/L during the PYY₃₋₃₆ study visit (occurring at $t = 90$ min), 37.5 ± 5.6 pmol/L during the GLP-1₇₋₃₆ amide study visit (occurring post-prandially at $t = 120$ min), and 93.5 ± 8.4 pmol/L during the combination PYY₃₋₃₆ and GLP-1₇₋₃₆ amide study visit (occurring at $t = 90$ min).

The mean AUC of total PYY levels (between 0 - 90 min) was 2892 ± 525 pmol min/L when saline was infused in the fasted state, 3010 ± 346 pmol min/L when saline was infused after the set breakfast, 5519 ± 402 pmol min/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 2713 ± 533 pmol min/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 5821 ± 482 pmol min/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused. There was no significant difference between the mean AUC of total PYY levels between fasted saline, fed saline and GLP-1₇₋₃₆ amide 0.8 pmol/kg/min infusions. Furthermore, there was no significant difference between the mean AUC of total PYY levels when PYY₃₋₃₆ was infused singly or in combination with GLP-1₇₋₃₆ amide.

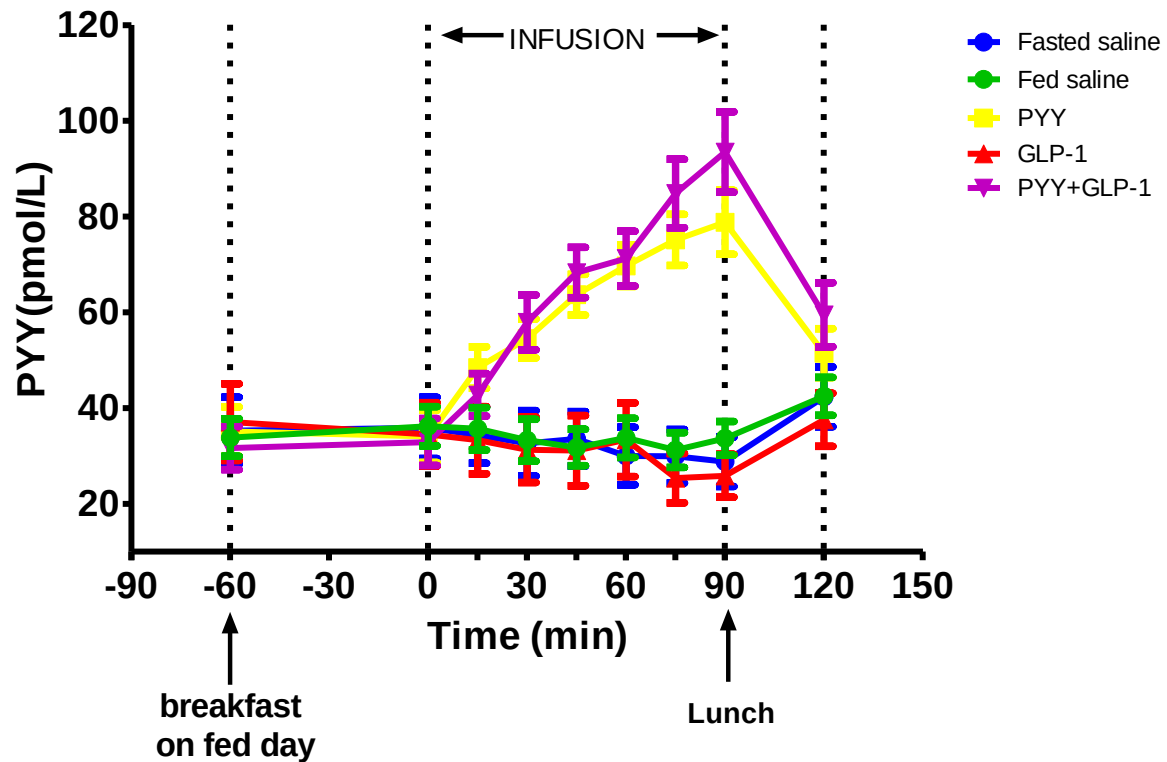


Figure 3.12: Variation of plasma total PYY levels with time during Study 2. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1). On one visit, after an overnight fast, they received a set breakfast at t = -60 min followed by a 90 minute saline infusion (Fed saline). On each study visit, the infusion was administered between t = 0 and t = 90 min. An *ad libitum* buffet lunch was served immediately after the infusion. Blood samples were taken for measurement of total PYY during each study at the time-points indicated.

3.3.11. PYY₃₋₃₆ levels in Study 2

Mean plasma concentration of PYY₃₋₃₆ (from time points t = 15 min to t = 90 min) was 18.3 ± 0.6 pmol/L during the fasted saline infusion, 20.6 ± 0.5 pmol/L during the fed saline infusion, 49.2 ± 4.2 pmol/L during the PYY₃₋₃₆ infusion, 16.6 ± 0.6 pmol/L during the GLP-1₇₋₃₆ amide infusion, and 51.2 ± 4.8 pmol/L during the combination infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide (figure 3.13).

Peak mean plasma concentration of PYY₃₋₃₆ (during the entire study) was 22.7 ± 2.5 pmol/L during the fasted saline study visit (occurring post-prandially at t = 120 min), 27.3 ± 3.1 pmol/L during the fed saline study visit (occurring post-prandially at t = 0 min), 59.8 ± 3.7 pmol/L during the PYY₃₋₃₆ study visit (occurring at t = 90 min), 23.1 ± 3.0 pmol/L during the GLP-1₇₋₃₆ amide study visit (occurring post-prandially at t = 0 min), and 63.6 ± 2.7 pmol/L during the combination PYY₃₋₃₆ and GLP-1₇₋₃₆ amide study visit (occurring at t = 90 min).

The mean AUC of PYY₃₋₃₆ levels (between 0 - 90 min) was 1668 ± 194 pmol min/L when saline was infused in the fasted state, 1914 ± 127 pmol min/L when saline was infused after the set breakfast, 4132 ± 201 pmol min/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 1558 ± 193 pmol min/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 4257 ± 127 pmol min/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused. There was no significant difference between the mean AUC of PYY₃₋₃₆ levels between fasted saline, fed saline and GLP-1₇₋₃₆ amide 0.8 pmol/kg/min infusions. Furthermore, there was no significant difference between the mean AUC of PYY₃₋₃₆ levels when PYY₃₋₃₆ was infused singly or in combination with GLP-1₇₋₃₆ amide.

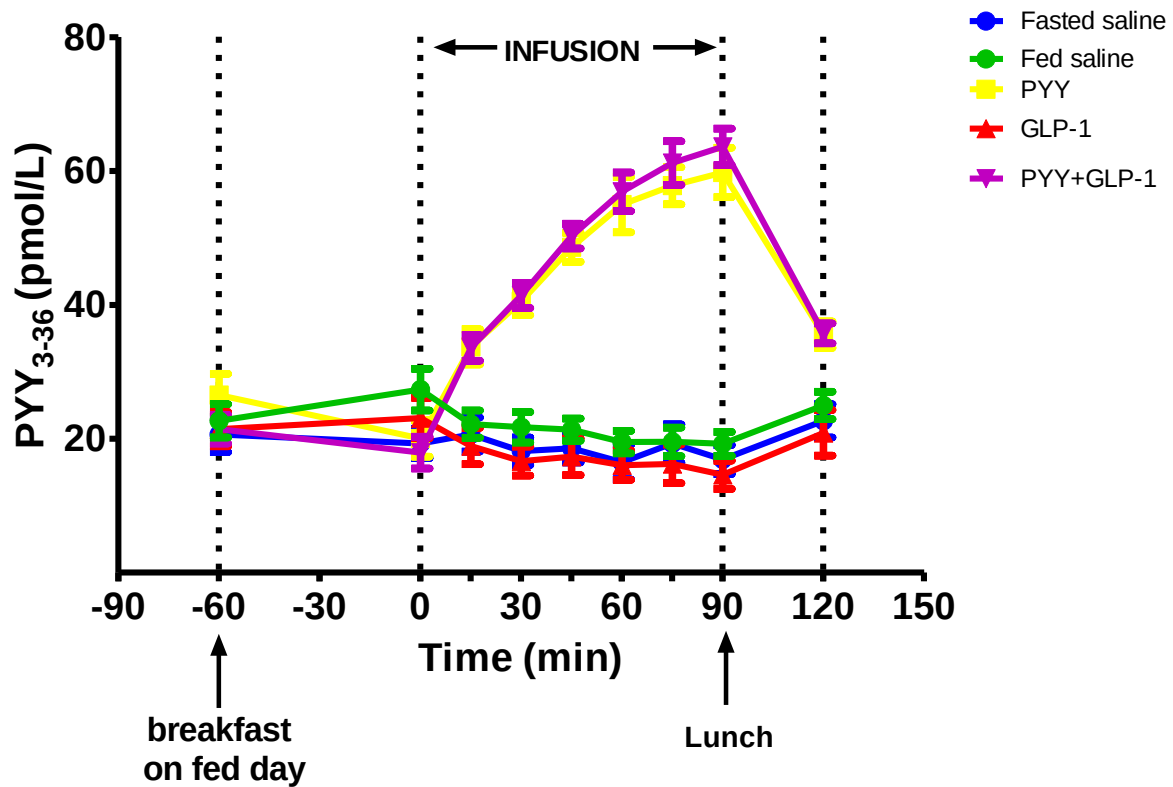


Figure 3.13: Variation of plasma PYY₃₋₃₆ levels with time during Study 2. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1). On one visit, after an overnight fast, they received a set breakfast at t = -60 min followed by a 90 minute saline infusion (Fed saline). On each study visit, the infusion was administered between t = 0 and t = 90 min. An *ad libitum* buffet lunch was served immediately after the infusion. Blood samples were taken for measurement of PYY₃₋₃₆ during each study at the time-points indicated.

3.3.12. Amidated GLP-1 levels in Study 2

Mean plasma concentration of amidated GLP-1 (from time points $t = 15$ min to $t = 90$ min) was 18.3 ± 0.2 pmol/L during the fasted saline infusion, 22.4 ± 0.4 pmol/L during the fed saline infusion, 18.2 ± 0.5 pmol/L during the PYY₃₋₃₆ infusion, 40.6 ± 3.2 pmol/L during the GLP-1_{7-36 amide} infusion, and 42.8 ± 2.8 pmol/L during the combination infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} (figure 3.14).

Peak mean plasma concentration of amidated GLP-1 (during the entire study) was 33.0 ± 5.8 pmol/L during the fasted saline study visit (occurring post-prandially at $t = 120$ min), 42.9 ± 7.4 pmol/L during the fed saline study visit (occurring post-prandially at $t = 120$ min), 35.9 ± 7.4 pmol/L during the PYY₃₋₃₆ study visit (occurring post-prandially at $t = 120$ min), 47.2 ± 5.2 pmol/L during the GLP-1_{7-36 amide} study visit (occurring at $t = 75$ min), and 51.2 ± 5.0 pmol/L during the combination PYY₃₋₃₆ and GLP-1_{7-36 amide} study visit (occurring at $t = 90$ min).

The mean AUC of amidated GLP-1 levels (between 0 - 90 min) was 1667 ± 334 pmol min/L when saline was infused in the fasted state, 2059 ± 388 pmol min/L when saline was infused after the set breakfast, 1655 ± 307 pmol min/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 3457 ± 440 pmol min/L when GLP-1_{7-36 amide} 0.8 pmol/kg/min was infused, and 3622 ± 387 pmol min/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min was infused. There was no significant difference between the mean AUC of amidated GLP-1 levels between fasted saline, fed saline and PYY₃₋₃₆ 0.3 pmol/kg/min infusions. Furthermore, there was no significant difference between the mean AUC of amidated GLP-1 levels when GLP-1_{7-36 amide} was infused singly or in combination with PYY₃₋

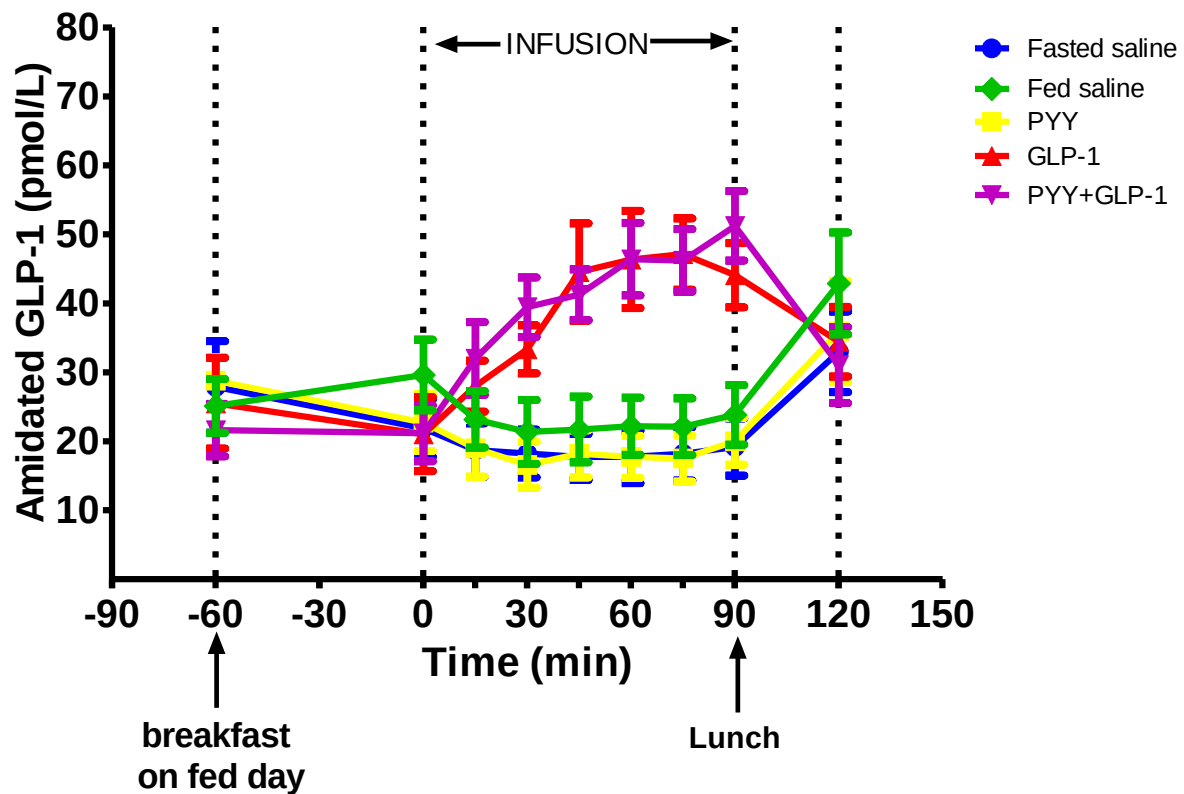


Figure 3.14: Variation of plasma amidated GLP-1 levels with time during Study 2. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1). On one visit, after an overnight fast, they received a set breakfast at t = -60 min followed by a 90 minute saline infusion (Fed saline). On each study visit, the infusion was administered between t = 0 and t = 90 min. An *ad libitum* buffet lunch was served immediately after the infusion. Blood samples were taken for measurement of amidated GLP-1 during each study at the time-points indicated.

3.3.13. Active GLP-1 levels (GLP-1₇₋₃₆ amide & GLP-1₇₋₃₇) in Study 2

Mean plasma concentration of active GLP-1 (from time points $t = 15$ min to $t = 90$ min) was 3.9 ± 0.5 pmol/L during the fasted saline infusion, 3.9 ± 0.4 pmol/L during the fed saline infusion, 2.9 ± 0.2 pmol/L during the PYY₃₋₃₆ infusion, 9.7 ± 1.0 pmol/L during the GLP-1₇₋₃₆ amide infusion, and 11.8 ± 1.1 pmol/L during the combination infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide (figure 3.15).

Peak mean plasma concentration of active GLP-1 (during the entire study) was 5.3 ± 1.7 pmol/L during the fasted saline study visit (occurring at $t = 15$ min), 8.7 ± 3.4 pmol/L during the fed saline study visit (occurring post-prandially at $t = 120$ min), 6.5 ± 2.3 pmol/L during the PYY₃₋₃₆ study visit (occurring post-prandially at $t = 120$ min), 13.2 ± 2.6 pmol/L during the GLP-1₇₋₃₆ amide study visit (occurring at $t = 75$ min), and 14.5 ± 2.0 pmol/L during the combination PYY₃₋₃₆ and GLP-1₇₋₃₆ amide study visit (occurring at $t = 75$ min).

The mean AUC of active GLP-1 levels (between 0 - 90 min) was 341 ± 115 pmol min/L when saline was infused in the fasted state, 341 ± 97 pmol min/L when saline was infused after the set breakfast, 264 ± 77 pmol min/L when PYY₃₋₃₆ 0.3 pmol/kg/min was infused, 819 ± 194 pmol min/L when GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused, and 986 ± 164 pmol min/L when the combination of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min was infused. There was no significant difference between the mean AUC of active GLP-1 levels between fasted saline, fed saline and PYY₃₋₃₆ 0.3 pmol/kg/min infusions. Furthermore, there was no significant difference between the mean AUC of active GLP-1 levels when GLP-1₇₋₃₆ amide was infused singly or in combination with PYY₃₋₃₆.

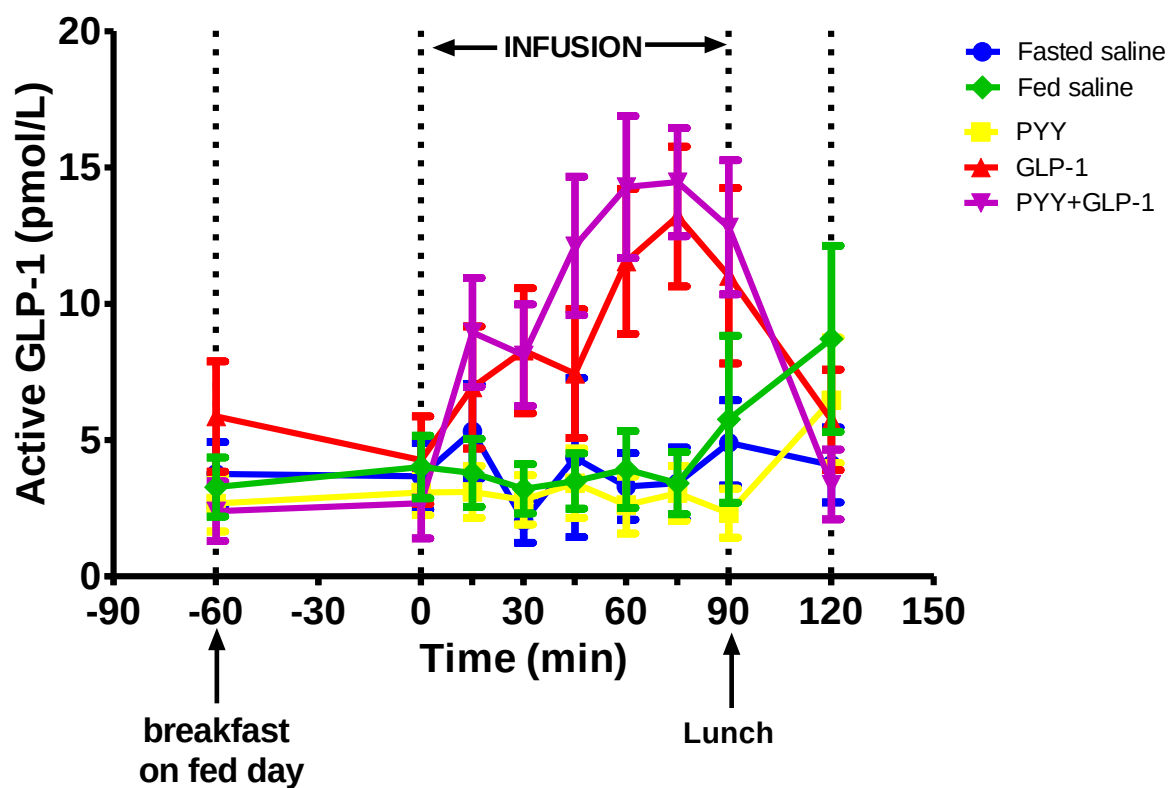


Figure 3.15: Variation of plasma active GLP-1 levels with time during Study 2. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{1-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{1-36 amide} 0.8 pmol/kg/min (PYY+GLP-1). On one visit, after an overnight fast, they received a set breakfast at t = -60 min followed by a 90 minute saline infusion (Fed saline). On each study visit, the infusion was administered between t = 0 and t = 90 min. An *ad libitum* buffet lunch was served immediately after the infusion. Blood samples were taken for measurement of active GLP-1 (GLP-1_{7-36 amide} and GLP-1₇₋₃₇) during each study at the time-points indicated.

3.3.14. Brain activation with presentation of food-salient visual stimuli

Brain activation with presentation of food-salient images was assessed using BOLD fMRI. For this, a whole-brain cluster wise analysis was used. I found that when subjects infused with saline (in either the fasted or fed state) viewed images of food, there was greater BOLD signal in several brain regions compared with when non-food images were viewed (Figure 3.16 and table 3.2).

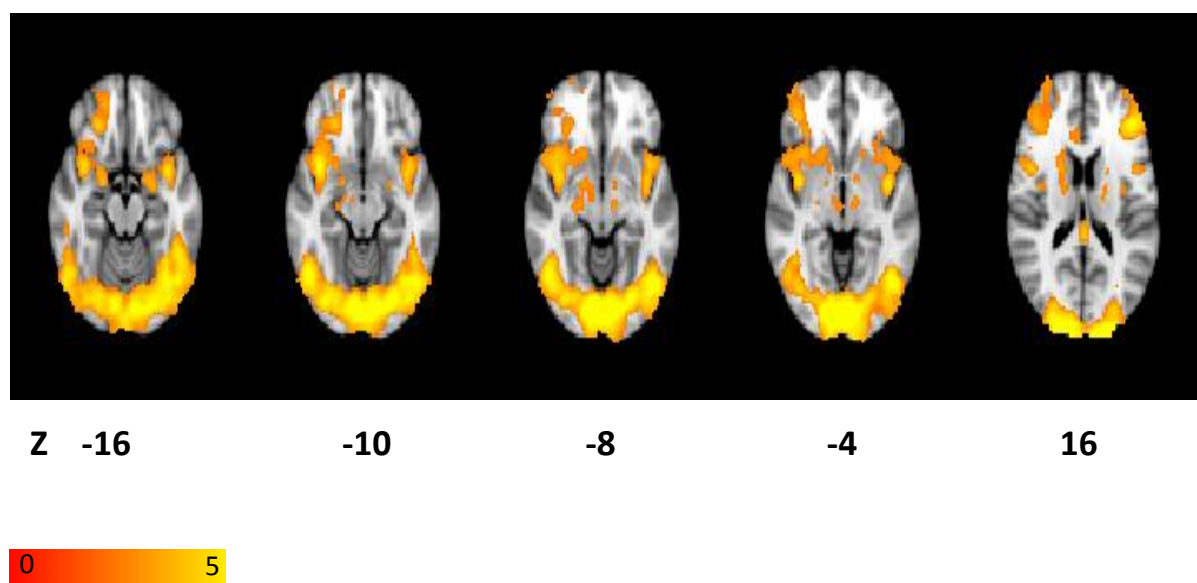


Figure 3.16: Brain regions where Δ BOLD (when subjects viewed images of food compared with when they viewed images of non-food) was significantly greater than zero in Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. 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) by food images (compared with non-food images) is shown. Z indicates distance (mm) superior or inferior to the inter-commissural plane in standard stereotactic space. Clusters were thresholded by means of parametric testing at the level of spatially contiguous clusters, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for the number of comparisons.

I then *a priori* chose to test 6 ROIs (amygdala, caudate, insula, nucleus accumbens, OFC and putamen) based on previous research in the field (LaBar et al., 2001, Batterham et al., 2007, Farooqi et al., 2007, Malik et al., 2008, Fletcher et al., 2010). The whole-brain cluster-wise analysis supported the selection of these ROIs (Table 3.2).

Brain region	MNI co-ordinates of local maxima (mm)			Max z
	X	Y	Z	
R Occipital	14	-96	10	6.41
R Occipital	6	-86	-8	6.2
L Occipital	-12	-100	8	6.19
L Occipital	-8	-86	-8	6.13
L Occipital	-46	-68	-8	5.93
R Occipital	16	-98	20	5.78
L Inferior frontal gyrus/ frontal pole	-38	34	10	5.63
L Inferior frontal gyrus/ frontal pole	-44	38	12	5.42
L Insula	-38	-6	4	5.09
R Insula	40	8	-12	5.07
L Insula	-40	-4	0	5.02
R Insula	40	-2	-2	4.88
Posterior cingulate	0	-32	20	4.75
Posterior cingulate	0	-34	26	4.51
Paracingulate gyrus	8	12	42	4
Paracingulate gyrus	-8	18	40	3.87
L Superior frontal gyrus	-18	2	54	3.79
Posterior cingulate	-4	-14	24	3.78
R Lateral occipital	32	-62	36	5.16
R Lateral occipital	32	-66	34	5.11
R Posterior supramarginal gyrus	44	-40	44	3.98

R Angular gyrus	36	-54	38	3.95
R Anterior supramarginal gyrus	52	-30	42	3.92
R Angular gyrus	32	-48	32	3.91
R Postcentral gyrus	50	8	28	4.41
R Inferior frontal gyrus	48	8	14	4.05
R Inferior frontal gyrus	56	10	12	3.91
R Postcentral gyrus	56	8	40	2.9
R Middle frontal gyrus	48	14	42	2.38
L Amygdala	-24	-4	-18	3.73
R Amygdala	22	0	-18	3.76
L Caudate	-18	14	10	2.53
R Caudate	18	4	20	3.33
L Orbitofrontal cortex	-34	34	2	3.8
R Orbitofrontal cortex	26	36	-14	4.12
L Insula	-38	-6	4	5.09
R Insula	40	8	-12	5.07
L Putamen	-20	4	8	3.01
R Putamen	18	16	-6	3.42
L Nucleus accumbens	-14	16	-6	2.42
R Nucleus accumbens	16	16	-8	3

Table 3.2: 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 in Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. During each infusion, a BOLD fMRI scan was performed, incorporating a picture processing task where images of food and non-food were shown and the difference in brain activation compared. Imaging measures (cluster peaks meeting a criterion of $p < 0.05$ for food images > non-food images, $z > 1.6$) for saline infusion (in the fed and fasted state) are shown. The response of each of the *a priori* ROIs to the picture processing task for saline infusion (in the fed and fasted state) is highlighted in bold in the second half of the table.

3.3.15. Effect of feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration on brain activation by food images: whole brain analysis.

The difference in BOLD signal between viewing images of food versus non-food was reduced in the insula, nucleus accumbens, inferior frontal gyrus and post-central gyrus when subjects were fed breakfast (figure 3.17).

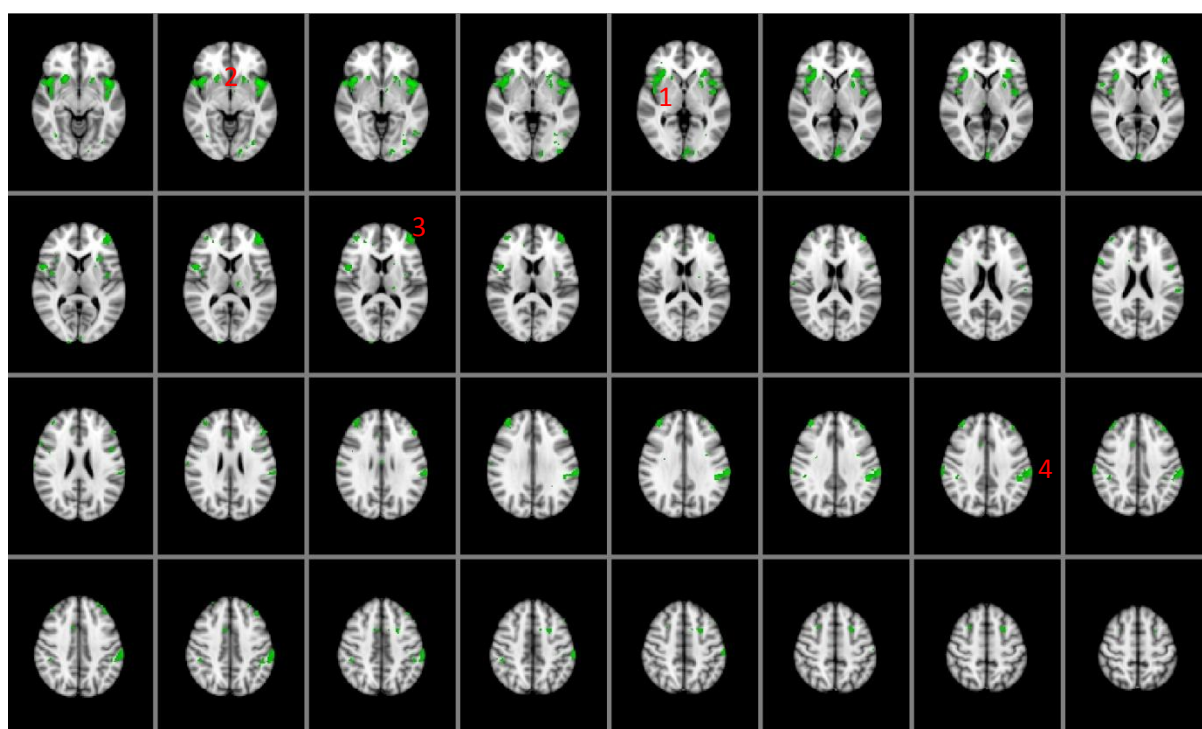


Figure 3.17: Whole brain map showing attenuation of BOLD fMRI signal with feeding. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ 0.8 pmol/kg/min. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. A BOLD fMRI scan was performed during each infusion, which comprised a picture processing task, where images of food and non-food were shown. During the fasted saline infusion, when subjects viewed images of food, there was greater activation in several brain regions compared with non-food images were viewed. This difference was reduced on the day when subjects were fed a set breakfast prior to the saline infusion. Green areas highlight brain regions where eating breakfast led to a reduction in the mean BOLD fMRI signal difference between viewing images of food versus non-food compared with the fasted saline infusion. 1= Insula. 2 = Nucleus accumbens. 3 = Inferior frontal gyrus. 4 = Post-central gyrus. Clusters were thresholded by means of parametric testing at the level of $z > 1.6$, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for the number of comparisons.

The difference in BOLD signal between viewing images of food versus non-food was reduced in the caudate, insula, nucleus accumbens, OFC, putamen and post-central gyrus when subjects were infused with PYY₃₋₃₆ (figure 3.18),

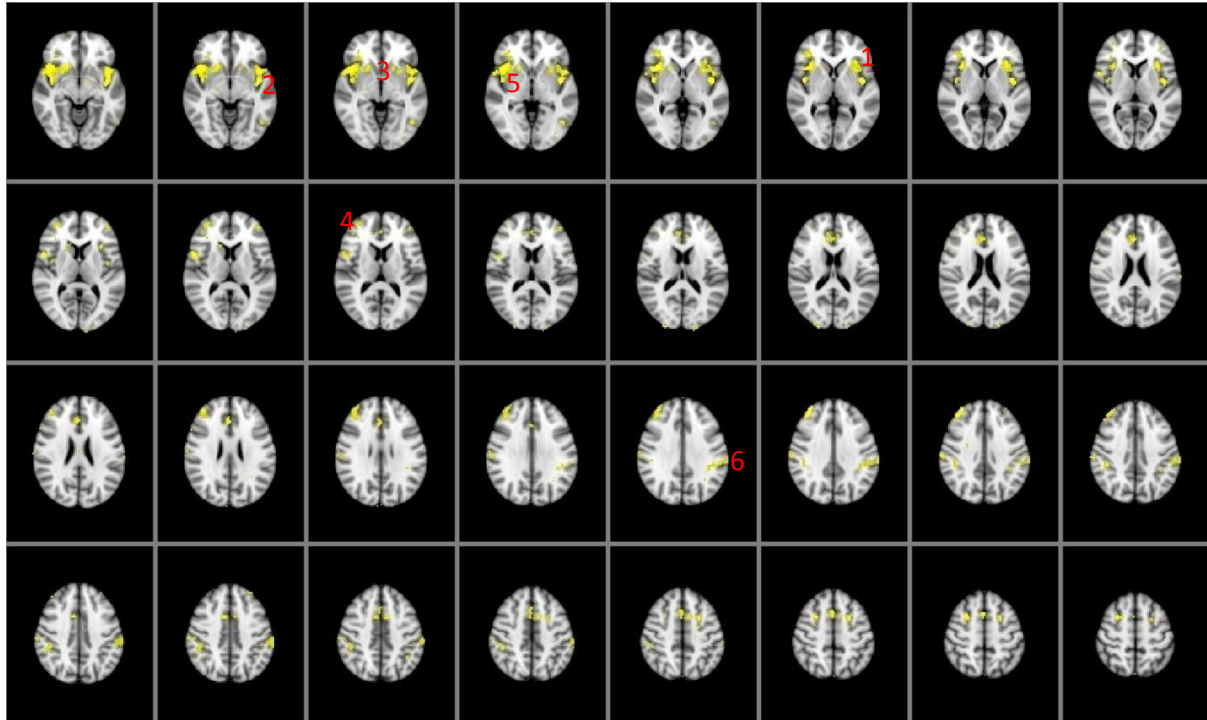


Figure 3.18: Whole brain map showing attenuation of BOLD fMRI signal with infusion of PYY₃₋₃₆. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ 0.8 pmol/kg/min. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. A BOLD fMRI scan was performed during each infusion, which comprised a picture processing task, where images of food and non-food were shown. During the fasted saline infusion, when subjects viewed images of food, there was greater activation in several brain regions compared with non-food images were viewed. This difference was reduced on the day when subjects were administered a 90 minute infusion of PYY₃₋₃₆ 0.3 pmol/kg/min. Yellow areas highlight brain regions where infusion of PYY₃₋₃₆ led to a reduction in the mean BOLD fMRI signal difference between viewing images of food versus non-food compared with the fasted saline infusion. 1 = Caudate. 2 = Insula. 3 = Nucleus Accumbens. 4 = OFC. 5 = Putamen. 6 = Post-central gyrus. Clusters were thresholded by means of parametric testing at the level of $z > 1.6$, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for the number of comparisons.

The difference in BOLD signal between viewing images of food versus non-food was reduced in the insula when subjects were infused with GLP-1₇₋₃₆ amide (figure 3.19).

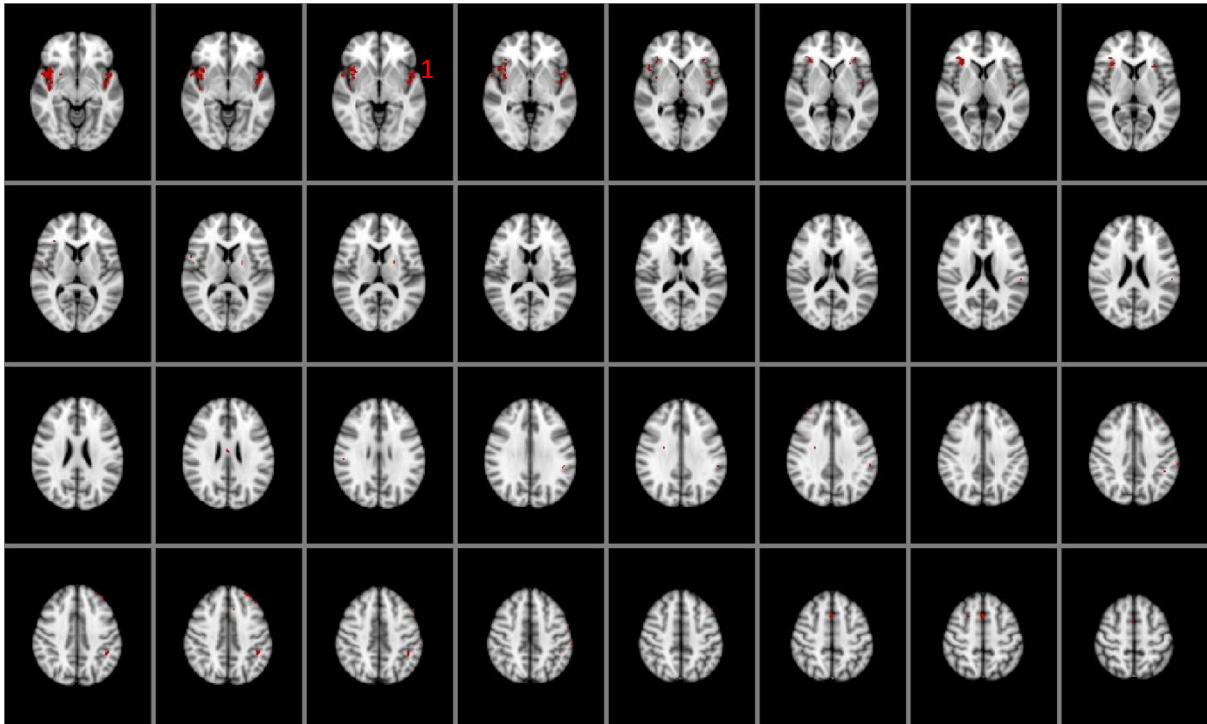


Figure 3.19: Whole brain map showing attenuation of BOLD fMRI signal with infusion of GLP-1₇₋₃₆ amide. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ 0.8 pmol/kg/min. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. A BOLD fMRI scan was performed during each infusion, which comprised a picture processing task, where images of food and non-food were shown. During the fasted saline infusion, when subjects viewed images of food, there was greater activation in several brain regions compared with non-food images were viewed. This difference was reduced on the day when subjects were administered a 90 minute infusion of GLP-1₇₋₃₆ amide 0.8 pmol/kg/min. Red areas highlight brain regions where infusion of GLP-1₇₋₃₆ amide led to a reduction in the mean BOLD fMRI signal difference between viewing images of food versus non-food compared with the fasted saline infusion. 1 = Insula. Clusters were thresholded by means of parametric testing at the level of $z > 1.6$, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for the number of comparisons.

The difference in BOLD signal between viewing images of food versus non-food was reduced in the amygdala, insula, inferior frontal gyrus, OFC, post-central gyrus and occipital cortex when subjects were infused with a combination of PYY₃₋₃₆ & GLP-1₇₋₃₆ amide (figure 3.20).

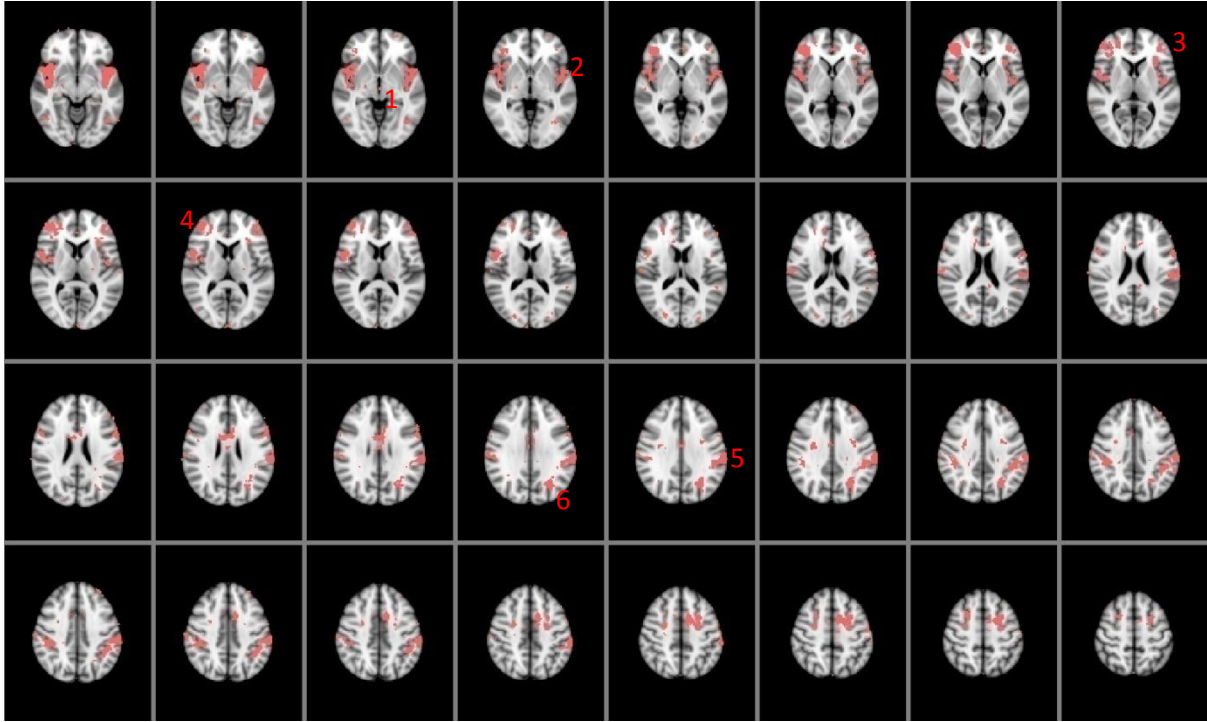


Figure 3.20: Whole brain map showing attenuation of BOLD fMRI signal with combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. Following an overnight fast, 15 healthy subjects underwent 90 minute infusions of saline, PYY₃₋₃₆ 0.3 pmol/kg/min, GLP-1₇₋₃₆ amide 0.8 pmol/kg/min and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ 0.8 pmol/kg/min. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion. A BOLD fMRI scan was performed during each infusion, which comprised a picture processing task, where images of food and non-food were shown. During the fasted saline infusion, when subjects viewed images of food, there was greater activation in several brain regions compared with non-food images were viewed. This difference was reduced on the day when subjects were administered a 90 minute infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min. Pink areas highlight brain regions where infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide led to a reduction in the mean BOLD fMRI signal difference between viewing images of food versus non-food compared with the fasted saline infusion. 1 = Amygdala. 2 = Insula. 3 = Inferior frontal gyrus. 4 = OFC. 5 = Post-central gyrus. 6 = Occipital cortex. Clusters were thresholded by means of parametric testing at the level of $z > 1.6$, simultaneously controlling the family-wise probability of type 1 error at $p < 0.05$, corrected for the number of comparisons.

3.3.16. Effect of feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration on brain activation by food images: ROI analysis

I examined each of the individual *a priori* selected ROIs, noting the 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, PYY₃₋₃₆, GLP-1₇₋₃₆ amide or combined PYY₃₋₃₆ & GLP-1₇₋₃₆ amide infusion, all compared with fasted saline infusion. This is illustrated for the amygdala (table 3.3 A), caudate (table 3.3 B), insula (table 3.3 C), nucleus accumbens (table 3.3 D), OFC (table 3.3 E) and putamen (table 3.3 F).

AMYGDALA (FOOD MINUS NON-FOOD)	CLUSTER PEAK	MNI CO-ORDINATES OF CLUSTER PEAK (mm)			
COMPARISON	Voxels (n)	X	Y	Z	Max z
PYY+GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
PYY+GLP-1<Fasted saline	94	-12	-2	-20	2.48
Fed saline>Fasted saline	6	14	0	-18	2.28
Fed saline<Fasted saline	0	n/a	n/a	n/a	n/a
GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
GLP-1<Fasted saline	2	-20	-7	-16	1.81
PYY>Fasted saline	0	n/a	n/a	n/a	n/a
PYY<Fasted saline	5	-21	0	-18	1.92

3.3 A

CAUDATE (FOOD MINUS NON-FOOD)	CLUSTER PEAK	MNI CO-ORDINATES OF CLUSTER PEAK (mm)			
COMPARISON	Voxels (n)	X	Y	Z	Max z
PYY+GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
PYY+GLP-1<Fasted saline	11	-1	-5	19	2.08
Fed saline>Fasted saline	0	n/a	n/a	n/a	n/a
Fed saline<Fasted saline	9	2	19	4	1.96
GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
GLP-1<Fasted saline	13	5	0	16	2.23
PYY>Fasted saline	7	-16	-11	22	1.77
PYY<Fasted saline	46	15	20	4	2.45

3.3 B

INSULA (FOOD MINUS NON-FOOD)	CLUSTER PEAK	MNI CO-ORDINATES OF CLUSTER PEAK (mm)			
COMPARISON	Voxels (n)	X	Y	Z	Max z
PYY+GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
PYY+GLP-1<Fasted saline	796	2	4	-5	3.13
Fed saline>Fasted saline	0	n/a	n/a	n/a	n/a
Fed saline<Fasted saline	734	0	9	-2	3.07
GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
GLP-1<Fasted saline	265	16	7	-6	2.43
PYY>Fasted saline	4	-17	-13	14	1.86
PYY<Fasted saline	1198	4	7	4	3.15

3.3 C

NUCLEUS ACCUMBENS (FOOD MINUS NON-FOOD)	CLUSTER PEAK	MNI CO-ORDINATES OF CLUSTER PEAK (mm)			
COMPARISON	Voxels (n)	X	Y	Z	Max z
PYY+GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
PYY+GLP-1<Fasted saline	5	-11	12	-11	1.88
Fed saline>Fasted saline	0	n/a	n/a	n/a	n/a
Fed saline<Fasted saline	26	-4	12	-10	3.02
GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
GLP-1<Fasted saline	0	n/a	n/a	n/a	n/a
PYY>Fasted saline	0	n/a	n/a	n/a	n/a
PYY<Fasted saline	53	-7	14	-9	2.91

3.3 D

ORBITOFRONTAL CORTEX (FOOD MINUS NON-FOOD)	CLUSTER PEAK	MNI CO-ORDINATES OF CLUSTER PEAK (mm)			
COMPARISON	Voxels (n)	X	Y	Z	Max z
PYY+GLP-1>Fasted saline	22	-7	63	-12	1.91
PYY+GLP-1<Fasted saline	628	-12	30	-18	2.83
Fed saline>Fasted saline	305	-12	48	-16	2.38
Fed saline<Fasted saline	207	-10	21	-12	2.35
GLP-1>Fasted saline	133	-21	53	-12	2.31
GLP-1<Fasted saline	90	23	25	-7	2.56
PYY>Fasted saline	24	-32	29	-18	2.06
PYY<Fasted saline	594	-2	26	-13	2.72

3.3 E

PUTAMEN (FOOD MINUS NON-FOOD)	CLUSTER PEAK	MNI CO-ORDINATES OF CLUSTER PEAK (mm)			
COMPARISON	Voxels (n)	X	Y	Z	Max z
PYY+GLP-1>Fasted saline	0	n/a	n/a	n/a	n/a
PYY+GLP-1<Fasted saline	3	10	9	-11	1.87
Fed saline>Fasted saline	5	8	-15	2	1.76
Fed saline<Fasted saline	200	-11	11	-4	2.83
GLP-1>Fasted saline	5	32	-8	2	1.75
GLP-1<Fasted saline	5	6	3	1	1.79
PYY>Fasted saline	10	30	-13	4	1.94
PYY<Fasted saline	269	4	12	-5	2.76

3.3 F

Table 3.3: Cluster peaks within the 6 *a priori* ROIs where Δ BOLD (when subjects viewed images of food compared with when they viewed images of non-food) was significantly greater than zero in Study 2. Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1_{7-36 amide} 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion (Fed saline). During each infusion, a BOLD fMRI scan was performed, incorporating a picture processing task where images of food and non-food were shown. Cluster peaks (meeting a criterion of $p < 0.05$, $z > 1.6$ corrected) for treatment contrasts versus Fasted saline are shown within the amygdala (3.3 A), caudate (3.3 B), insula (3.3 C), nucleus accumbens (3.3 D), OFC (3.3 E) and putamen (3.3 F).

Following this, I examined the mean % change in BOLD signal in subjects after feeding, PYY₃₋₃₆, GLP-1_{7-36 amide} or combined PYY₃₋₃₆ & GLP-1_{7-36 amide} infusion across the individual *a priori* selected ROIs and compared this with the mean % change in BOLD signal when subjects were fasted and received saline (control). There were similar reductions in mean % change in BOLD signal for all of the ROIs studied following feeding or gut hormone administration, compared to when subjects were fasted and received saline (figure 3.21).

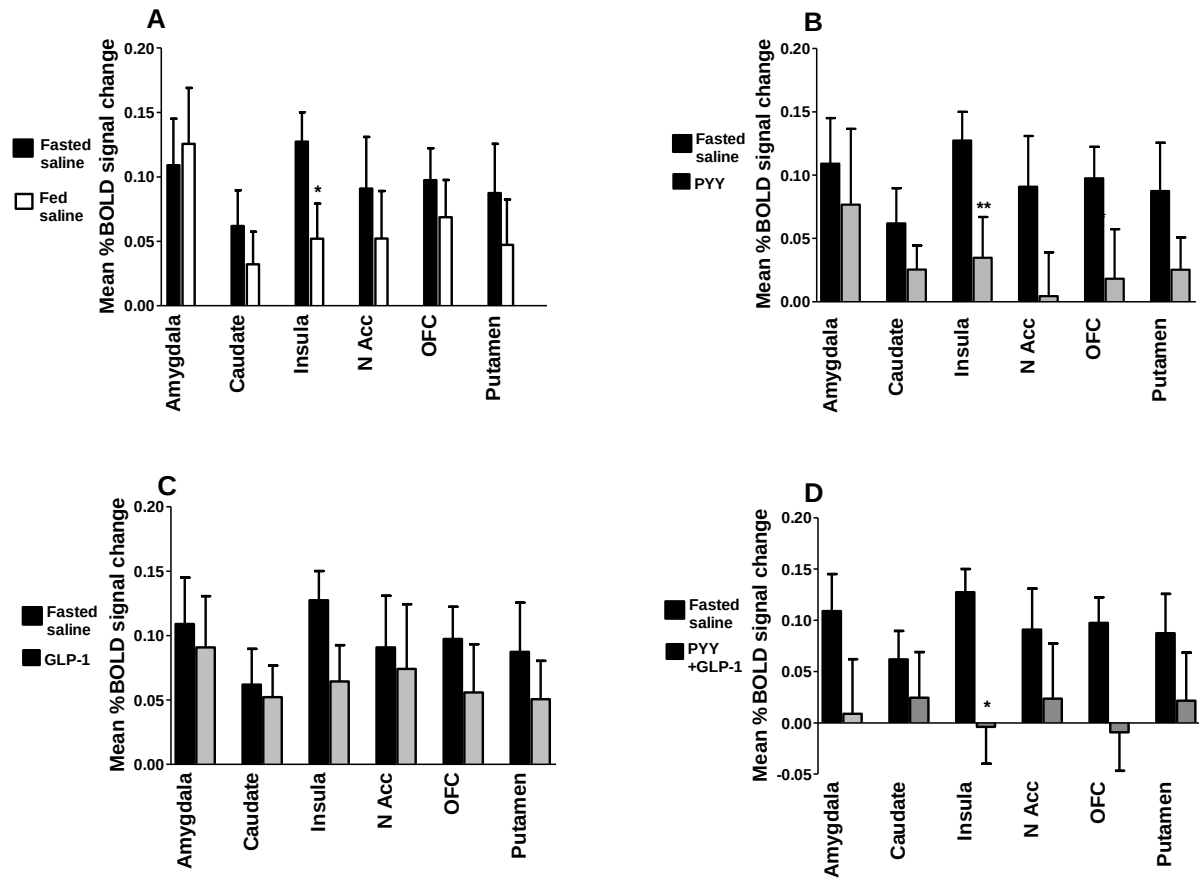


Figure 3.21: Modulation of BOLD signal across brain ROIs by feeding, or either individual or combined gut hormone infusions.

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 3.21 A), PYY₃₋₃₆ after an overnight fast (figure 3.21 B), GLP-1_{7-36 amide} after an overnight fast (figure 3.21 C) and combined PYY₃₋₃₆ + GLP-1_{7-36 amide} after an overnight fast (figure 3.21 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. Data are shown for individual ROIs (amygdala, insula, caudate, nucleus accumbens (N Acc), OFC and putamen), combined for left and right hemispheres and grouped for all 15 subjects. PYY denotes PYY₃₋₃₆, GLP-1 denotes GLP-1_{7-36 amide}.

* 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.

Analysis of left and right hemisphere ROIs individually (figure 3.22) showed that reductions in mean % BOLD signal change (when subjects viewed images of food compared to when they viewed images of non-food) were significant for the following comparisons in the following ROIs:

Fed saline vs. fasted saline in:

left insula ($p = 0.016$)

right insula ($p = 0.034$)

PYY₃₋₃₆ vs. fasted saline in:

left insula ($p = 0.007$)

right insula ($p = 0.005$)

left nucleus accumbens ($p = 0.013$)

left OFC ($p = 0.019$)

GLP-1_{7-36 amide} vs. fasted saline in:

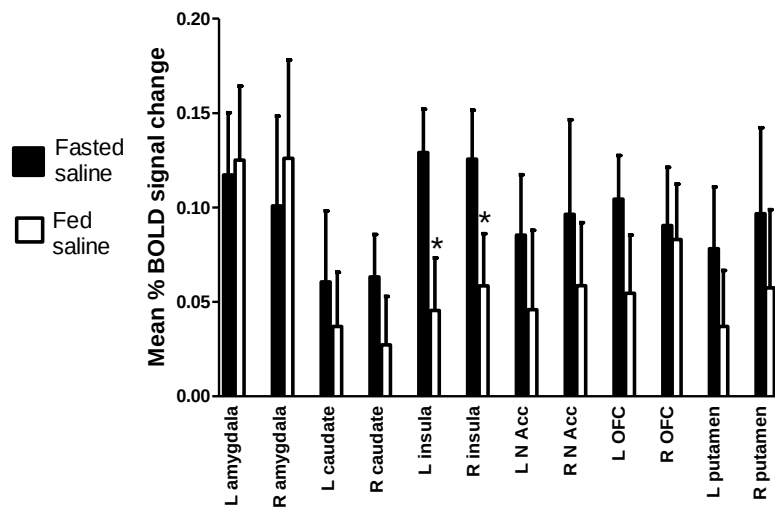
right insula ($p = 0.049$)

Combined PYY₃₋₃₆ & GLP-1_{7-36 amide} vs. fasted saline in:

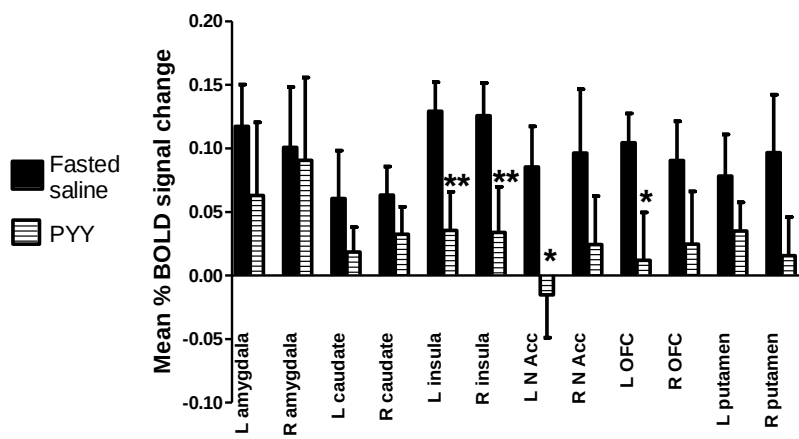
left insula ($p = 0.014$)

right insula ($p = 0.014$)

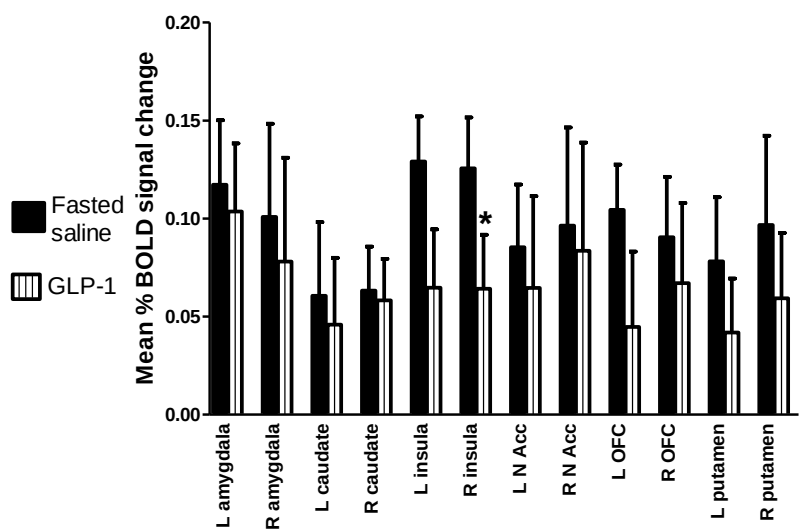
3.22 A



3.22 B



3.22 C



3.22 D

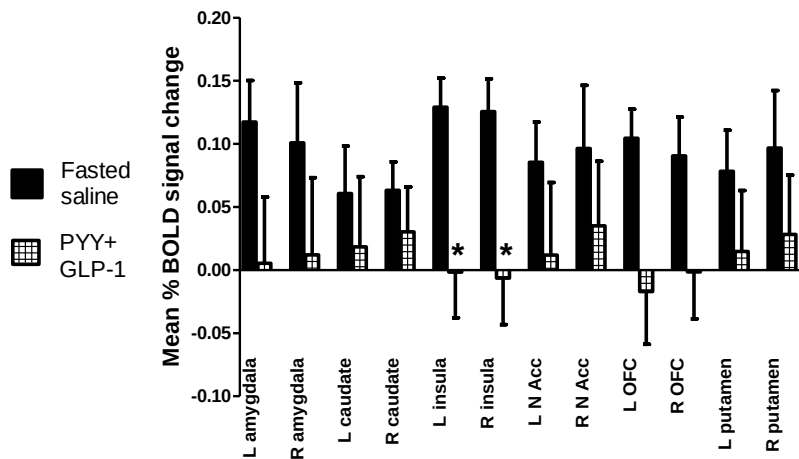


Figure 3.22: Modulation of BOLD signal across brain ROIs by feeding, or either individual or combined gut hormone infusions- left and right sided analysis.

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 3.22 A), PYY₃₋₃₆ after an overnight fast (figure 3.22 B), GLP-1_{7-36 amide} after an overnight fast (figure 3.22 C) and combined PYY₃₋₃₆ + GLP-1_{7-36 amide} after an overnight fast (figure 3.22 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. Data are shown for individual ROIs and for left and right sides (amygdala, insula, caudate, nucleus accumbens (N Acc), OFC and putamen), grouped for all 15 subjects. PYY denotes PYY₃₋₃₆, GLP-1 denotes GLP-1_{7-36 amide}.

* denotes p < 0.05 vs. fasted saline. ** denotes p < 0.01 vs. fasted saline.

The reduction in mean % BOLD signal change with the combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} was similar to the summed reduction in mean % BOLD signal changes after individual administrations of the two hormones (figure 3.23).

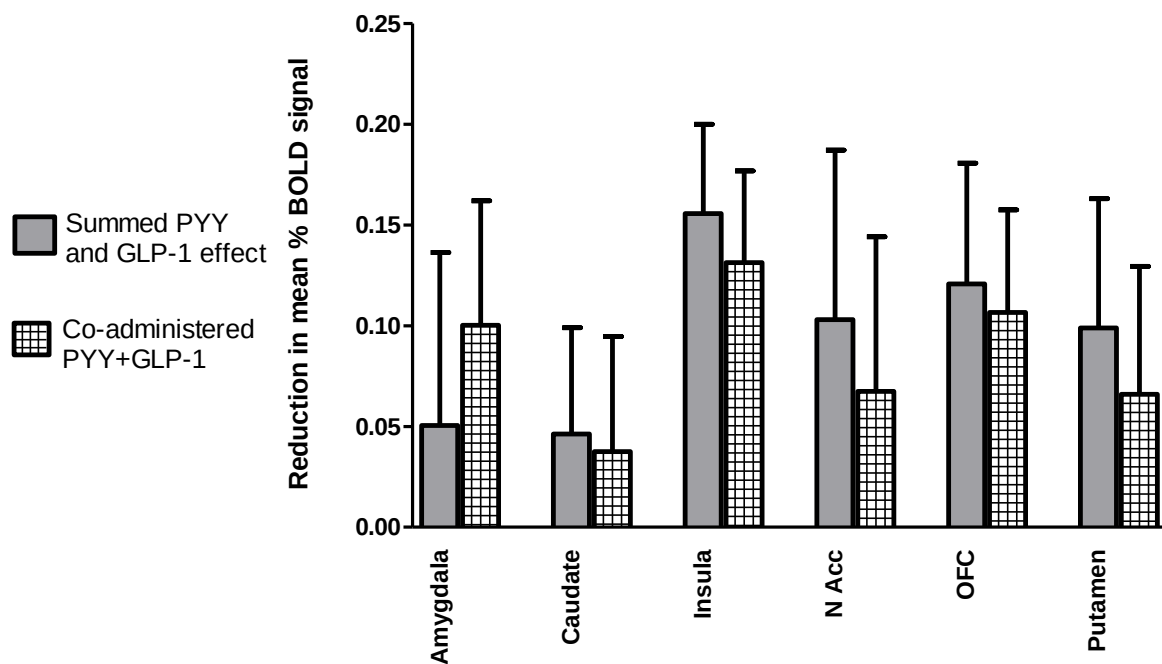


Figure 3.23: Summed reduction in mean % BOLD signal change in ROIs with individual administration of PYY₃₋₃₆ and GLP-1_{7-36 amide}, compared with the reduction in mean % BOLD signal change after combined administration of PYY₃₋₃₆ and GLP-1_{7-36 amide}.

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 individual effect of PYY₃₋₃₆ and GLP-1_{7-36 amide} (grey bars). This is compared with 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 for the combination infusion of PYY₃₋₃₆ & GLP-1_{7-36 amide} (hatched bars). Data are shown for individual ROIs (amygdala, insula, caudate, nucleus accumbens (N Acc), OFC and putamen), combined for left and right hemispheres and are grouped for all 15 subjects. PYY denotes PYY₃₋₃₆, GLP-1 denotes GLP-1_{7-36 amide}. $p = ns$ for all ROIs.

I then tested the effects of feeding, PYY₃₋₃₆ infusion, GLP-1_{7-36 amide} infusion and combined PYY₃₋₃₆ & GLP-1_{7-36 amide} infusion on changes in mean % BOLD signal in the total brain volume represented by the 6 *a priori* selected ROIs (Figure 3.24). When fasted subjects viewed images of food compared with when they viewed images of non-food, there was a significant reduction ($p < 0.001$) in the mean % BOLD fMRI signal change after administration of PYY₃₋₃₆ or after co-administration of PYY₃₋₃₆ and GLP-1_{7-36 amide}, compared to when subjects were fasted and received saline infusion (control). There was a non-significant reduction in the mean % BOLD signal change in the selected ROIs following feeding or after GLP-1_{7-36 amide} administration compared to when subjects were fasted and received saline infusion.

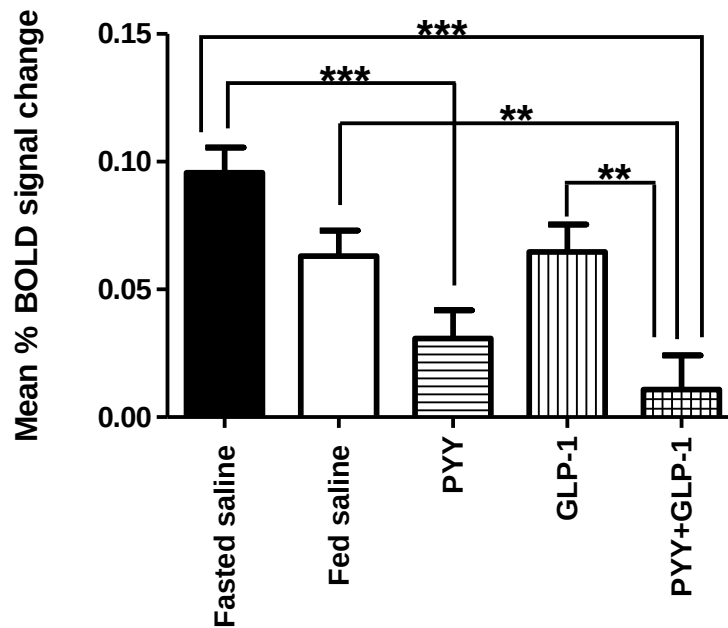


Figure 3.24: Modulation of BOLD signal across collective brain ROIs by feeding, or either individual or combined gut hormone infusions. Fifteen healthy subjects underwent 90 minute infusions of saline (Fasted saline), PYY₃₋₃₆ 0.3 pmol/kg/min (PYY), GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (GLP-1) and combined PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1₇₋₃₆ amide 0.8 pmol/kg/min (PYY+GLP-1) following an overnight fast. On one visit, after an overnight fast, they received a set breakfast followed by a 90 minute saline infusion (Fed saline). 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 (food images minus non-food images) across all 6 ROIs studied (amygdala, insula, caudate, nucleus accumbens, OFC and putamen), is shown for each of the interventions.

** p<0.01, *** p<0.001.

To investigate the possible effect of nausea on the MRI findings, I analysed whether there is any significant correlation between change in nausea score from 0 - 90 min (the time of the infusion) and the reduction in % BOLD signal change from the control saline infusion for all subjects in each of the 6 ROIs (averaged over left and right hemispheres) during the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. There was significant correlation between the change in nausea score and reduction in % BOLD signal change in the amygdala ($r^2 = 0.35$, $p = 0.02$), but not in the caudate ($r^2 = 0.02$, $p = 0.62$), insula ($r^2 = 0.05$, $p = 0.44$), nucleus accumbens ($r^2 = 0.06$, $p = 0.36$), OFC ($r^2 = 0.05$, $p = 0.45$) or putamen ($r^2 = 0.09$, $p = 0.28$). This is shown in figure 3.25.

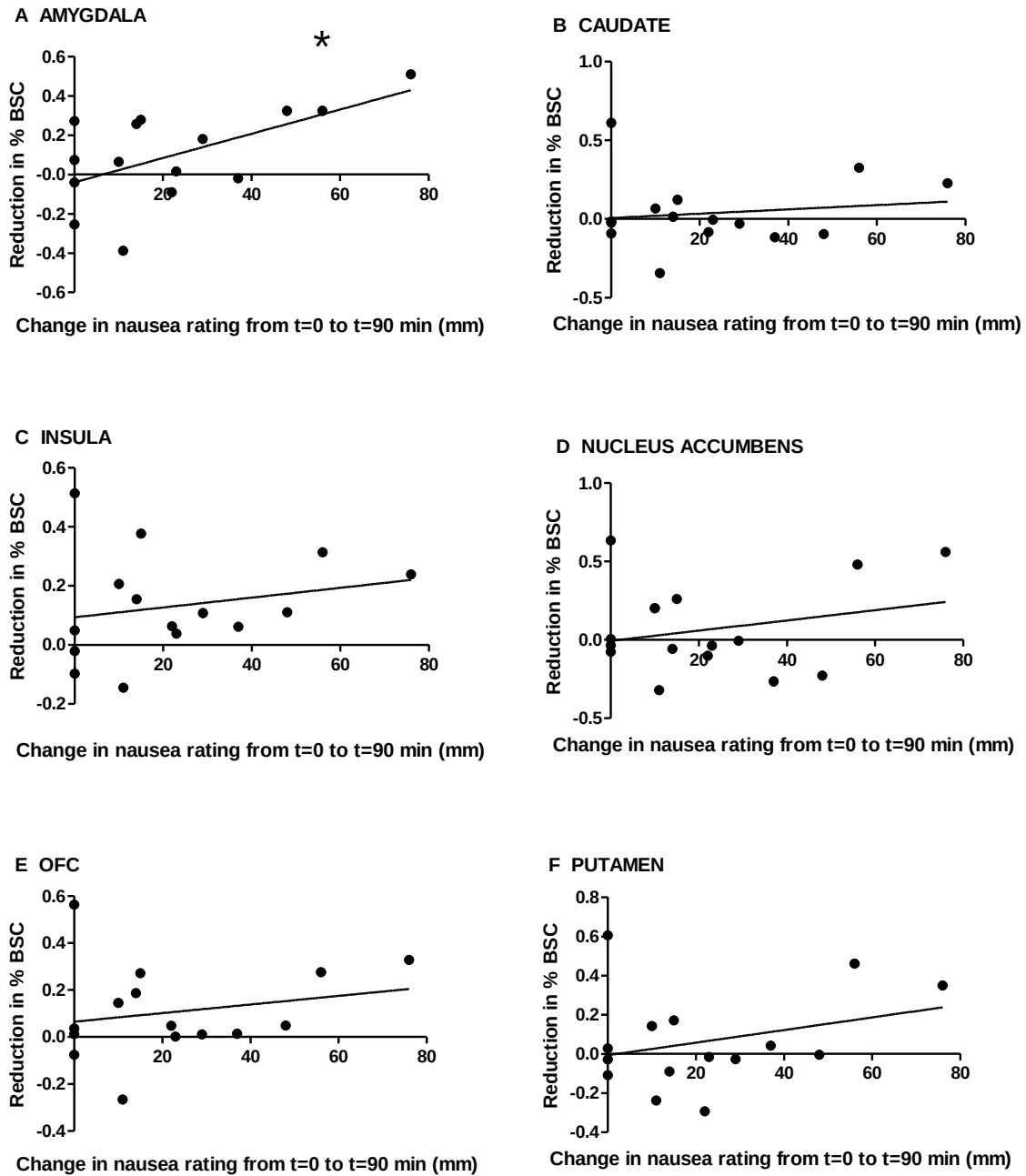


Figure 3.25: Correlation of nausea with reduction in % BOLD signal change during combined PYY₃₋₃₆ and GLP-1₇₋₃₆ amide infusion for each ROI. For each subject, the change in VAS nausea scores from baseline (t = 0 min) to the end of the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide (t = 90 min) are shown, correlated against reduction in % BOLD signal change (% BSC) from the control fasted saline infusion. This was done separately for each of the 6 ROIs (amygdala (A), caudate (B), insula (C), nucleus accumbens (D), OFC (E) and putamen (F), with % BSC data combined for left and right hemispheres. * denotes p<0.05

3.3.17. Effect of feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration on brain activation by food images: analysis of hypothalamic and brainstem responses

The whole brain cluster-wise analysis of brain regions responsive to the picture processing BOLD fMRI task (table 3.2) revealed that for the fasted saline and fed saline visits, the hypothalamus and brainstem (midbrain) BOLD signal was not significantly greater when subjects viewed images of food compared with non-food.

Following this, in a voxel-wise analysis, I examined the mean % change in BOLD signal in subjects after feeding, PYY₃₋₃₆, GLP-1₇₋₃₆ amide or combined PYY₃₋₃₆ & GLP-1₇₋₃₆ amide infusion in the hypothalamus and brainstem (midbrain) and compared this with the mean % change in BOLD signal when subjects were fasted and received saline (control). This revealed that in the hypothalamus and brainstem, there was no effect of feeding or gut hormone administration on mean % change in BOLD signal (between viewing images of food compared with non-food) compared with the fasted saline infusion (figure 3.26).

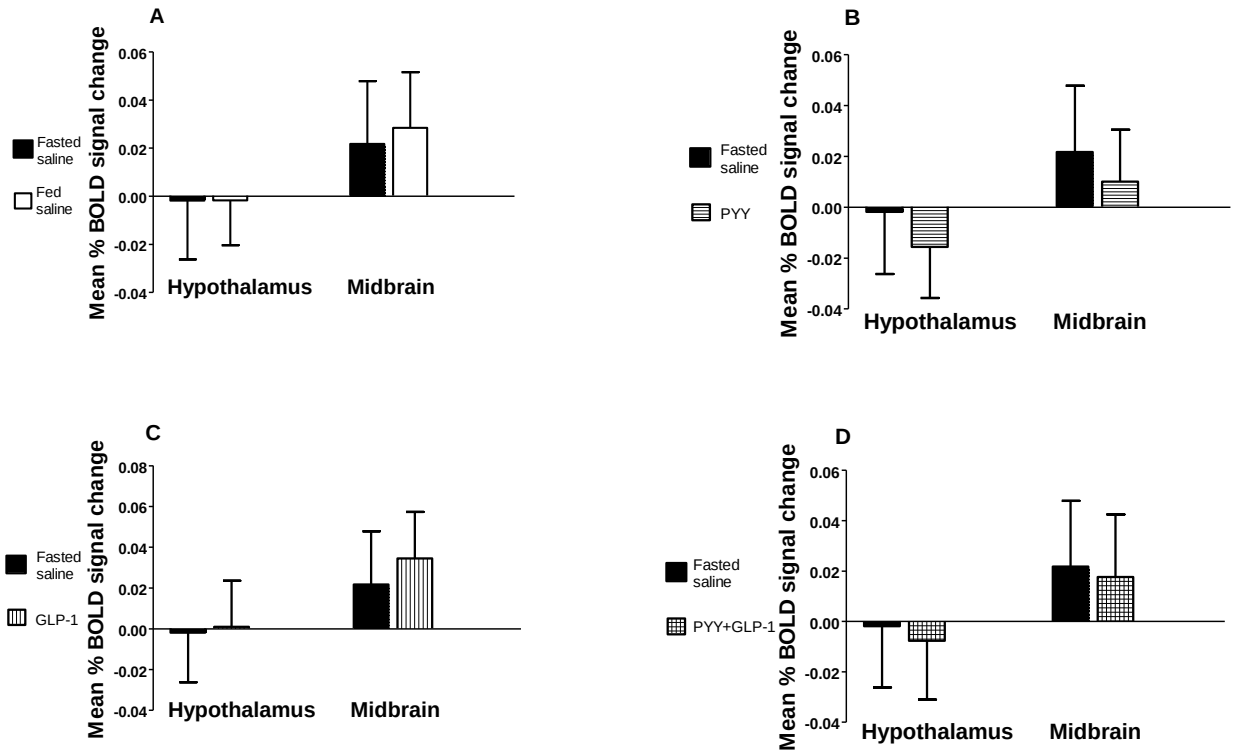


Figure 3.26: Modulation of BOLD signal in the hypothalamus and brainstem (midbrain) by feeding, or either individual or combined gut hormone infusions.

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 (A), PYY₃₋₃₆ after an overnight fast (B), GLP-1_{7-36 amide} after an overnight fast (C) and combined PYY₃₋₃₆ + GLP-1_{7-36 amide} after an overnight fast (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. Data are shown for the midbrain and hypothalamus and grouped for all 15 subjects. PYY denotes PYY₃₋₃₆, GLP-1 denotes GLP-1_{7-36 amide}. $p = ns$ for all interventions compared with fasted saline.

3.4. Discussion

3.4.1. Discussion of the effects of single and combined administration of PYY₃₋₃₆ and GLP-1_{7-36 amide} on appetite, energy intake and neuronal activity in brain appetite centres.

By means of this study (Study 2), I examined the independent and combined effects of PYY₃₋₃₆ and GLP-1_{7-36 amide} on appetite, subsequent food intake and neuronal activity in regions of the brain contributing to appetitive processing and behaviour. Fifteen healthy, lean subjects underwent BOLD fMRI scanning on five separate occasions at least 3 days apart, during which they received each of the following 90 minute infusions in random order: a saline infusion following an overnight fast (the fasted, control visit); a saline infusion following a large breakfast (the fed visit); an infusion of PYY₃₋₃₆ at 0.3 pmol/kg/min; an infusion of GLP-1_{7-36 amide} at 0.8 pmol/kg/min; a combined infusion of PYY₃₋₃₆ 0.3 pmol/kg/min + GLP-1_{7-36 amide} 0.8 pmol/kg/min (all the gut hormone infusions were performed after an overnight fast). The subjects were then served a free intake buffet lunch at the end of each infusion, in order to assess effects on food intake.

Prior to the 5 randomised visits detailed above, each subject underwent an initial acclimatisation visit, where they received a 90 minute infusion of saline following an overnight fast, underwent a BOLD fMRI scan and were then served an *ad libitum* buffet lunch. The protocol was almost identical to that of the subsequent randomized visits (the only difference being that during the acclimatisation visit, an extra 5 minute structural MRI scan was performed at the end of the BOLD fMRI picture processing task). The purpose of the acclimatization visit was to ensure that subjects were comfortable with the research environment and study protocol prior to the randomized study visits. The value of this visit is

illustrated by the fact that subjects consumed 8% fewer calories at lunch during the acclimatization visit compared to the control, fasted saline visit later on.

Analysis of the mean energy intake during the *ad libitum* lunch during the 5 randomised study visits revealed that when subjects consumed breakfast, they consumed 24% fewer calories at lunch than if they had continued fasting through the morning. Administering either of the gut hormones PYY₃₋₃₆ or GLP-1_{7-36 amide} to fasted subjects also reduced the amount of food they consumed at lunch, although to a smaller extent (administration of PYY₃₋₃₆ reduced *ad libitum* food consumption at lunch by 12% compared to saline, whereas administration of GLP-1_{7-36 amide} reduced *ad libitum* food consumption at lunch by 16% compared to saline). Notably, when PYY₃₋₃₆ and GLP-1_{7-36 amide} were co-administered to fasted subjects, the reduction in food consumption at lunch (27%) was comparable to that observed in subjects who had eaten breakfast and received saline (24%). Whilst my experimental procedure was not designed to draw conclusions about dose-response relationships for the effects of these hormones, the effects of combined gut hormone infusion appeared to be additive, with no loss of potency when the hormones were administered in combination. I postulate that administration of these anorectic gut hormones in combination induces the satiety similar or identical to that of a meal, but without the associated caloric load. This is consistent with expectations based on endogenous physiological responses observed post-prandially, whereby several anorectic gut hormones are co-secreted and are thought to act together to limit further food intake.

The same *ad libitum* study lunch was served during all visits. To investigate the possibility of subjects being progressively less enticed by the meal, resulting in lower energy intake at each subsequent visit, I performed analysis of the mean energy intake (of the 15 subjects) during the *ad libitum* lunch across the 5 randomised visits. This analysis provided

reassurance that subjects did not eat progressively less during each subsequent visit as a result of habituation to the study meal.

Haemodynamic parameters and non-food related VAS parameters were not significantly different between infusion of saline, PYY₃₋₃₆, GLP-1₇₋₃₆ amide, combined infusion of PYY₃₋₃₆ & GLP-1₇₋₃₆ amide or infusion of saline following consumption of breakfast. The effect of eating breakfast was as expected, to cause a rise in blood glucose levels, which then subsequently fell back towards baseline during the course of the saline infusion that followed. Interestingly, in contrast to Study 1, the infusion of GLP-1₇₋₃₆ amide or the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide did not lead to a significant reduction in blood glucose compared with infusion of saline after an overnight fast. In Study 1, the blood glucose during the control saline infusion rose slightly, whereas the blood glucose during the GLP-1₇₋₃₆ amide infusion and the combined PYY₃₋₃₆ & GLP-1₇₋₃₆ amide infusion fell, perhaps creating a statistical artefact.

During the course of each of the infusions described above, using BOLD fMRI, I tested for differences in relative activation in several pre-selected brain ROIs when subjects viewed images of food compared with images of non-food items. The *a priori* selected ROIs comprised the amygdala, caudate, insula, nucleus accumbens, OFC and putamen. These regions were selected based on the basis of previous studies of satiety and appetitive hormone responses, and have been hypothesized to contribute to the brain network for control of appetite (Batterham et al., 2007, Farooqi et al., 2007, Fletcher et al., 2010, LaBar et al., 2001, Malik et al., 2008). As predicted, my data demonstrated that in each of the 6 brain reward ROIs, viewing images of food was associated with a greater BOLD fMRI signal than viewing non-food images in the fasted state. Consumption of a standard breakfast reduced this brain activation, extending earlier observations where a similar protocol was used (LaBar et al., 2001). Notably, I did not observe a fed-fasted difference within the

amygdala, in contrast to the earlier study by La Bar *et al.* Activation of the insula was most sensitive to modulation by consumption of a meal, with similar trends in the caudate and putamen (dorsal striatum), nucleus accumbens (ventral striatum) and OFC. My findings are also largely consistent with recent studies demonstrating that fasting selectively increases BOLD fMRI activation to pictures of high-calorie food compared with pictures of low calorie food in similar ROIs and that this activation is attenuated in the fed state (Goldstone *et al.*, 2009, Fletcher *et al.*, 2010)

I then assessed the change in BOLD signal following infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, either singly or in combination, in fasted subjects. When PYY₃₋₃₆ 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 prominent in the insula, left nucleus accumbens and the left OFC.

My work formed the first published study to describe effects of GLP-1₇₋₃₆ amide infusion on brain activation in humans. Infusion of GLP-1₇₋₃₆ amide alone led to consistent reductions in mean % BOLD signal change compared with fasted saline across all of the selected ROIs, with the greatest change in the right insula.

I also characterized the combined effects of infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on brain activity for the first time. I found consistent reductions in mean % BOLD signal change relative to infusion of saline across all of the selected ROIs, similar to those observed following a meal. As with GLP-1₇₋₃₆ amide alone, the insula appeared particularly sensitive to hormonal modulation. Consistent with effects on *ad libitum* energy intake, summation of the

effect of each single hormone in reducing mean % BOLD signal change was comparable with the reduction in mean % BOLD signal change after the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. This provides novel evidence in humans that the actions of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on brain responses to food-salient stimuli are additive, explaining the way in which gut hormones co-secreted physiologically after meals may work in concert to limit further food intake and cause satiety.

This insight into the neuroendocrinology of appetite highlights numerous new questions for future studies. The functional anatomical localizations of brain activations after hormone administration suggest that ascending connections from vagal nuclei modulate the dopaminergic reward system: it was in the insular cortex (which is situated at an interface of homeostatic and cognitive systems with activity related to establishing salience of external stimuli for appropriate behavioural reactions (Menon and Uddin, 2010) that I observed brain activity to be most significantly modulated by both feeding and gut hormone infusion. Another region where I observed changes was in the nucleus accumbens (forming the major part of the ventral striatum); this area is thought to play an important role in the mediation of hedonic drive and action (e.g., food-seeking behaviours) (Baldo and Kelley, 2007).

Recent evidence has identified a role for the OFC in linking food memory with food reward. Connectivities of the OFC with the hippocampus, amygdala and insula (Kringelbach, 2005) may facilitate a central role in coordinating appetitive behaviour. Changes in OFC activation were also reported in an earlier investigation of PYY₃₋₃₆ effects (Batterham et al., 2007). In that study, subsequent food intake was predicted by the modulation of OFC signal with PYY₃₋₃₆ infusion, but not with saline infusion after fasting, when subsequent food intake was better correlated with changes in hypothalamic signal. It was postulated that the presence of PYY₃₋₃₆ switches regulation of food intake from a homeostatic brain region (hypothalamus) to

a hedonic region (OFC). However, differences in methodology used (my study used task-activated BOLD fMRI data, whereas Batterham *et al.* tested for changes in resting state BOLD fMRI) confounds direct comparisons between the studies.

Whereas single infusion of PYY₃₋₃₆ to fasted subjects led to greatest modulation of BOLD signal in the left and right insula, left OFC and left nucleus accumbens, single infusion of GLP-1_{7-36 amide} infusion led to significant modulation of signal only in the right insula. This may in part be due to fundamental differences in the way in which these anorectic gut hormones act to mediate post-prandial satiety. However, notably, combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} led to significant modulation of BOLD signal in the insula alone, suggesting that the study was somewhat underpowered to detect differences in BOLD signal due to considerable inter-subject variance of this parameter. Furthermore, despite the fact that GLP-1_{7-36 amide} infusion led to relatively modest changes in BOLD signal compared with infusion of PYY₃₋₃₆, the reduction in energy intake during the *ad libitum* meal was greater for GLP-1_{7-36 amide}. This may be due to additional effects of GLP-1_{7-36 amide} in mediating fullness by inhibiting gastric emptying.

Although there was no modulation of subjective ratings of meal palatability by either single or combined administration of the gut hormones, the combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} was associated with a mild increase in VAS nausea and fullness scores, along with a corresponding reduction in hunger, pleasantness of prospective food consumption and planned prospective food consumption. Significantly, at no stage did any subjects report to the investigators that they felt nauseated outside of the context of VAS analysis. Therefore, the degree of nausea induced by the combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} was tolerable. Furthermore, the absence of significant correlation between nausea and *ad libitum* energy intake at lunch during the combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide} provides

reassurance that PYY₃₋₃₆ and GLP-1_{7-36 amide} do not inhibit food intake merely by inducing nausea. Analysis of the effect of nausea on BOLD signal change revealed that there was a significant correlation between the change in nausea score during the combined PYY₃₋₃₆ and GLP-1_{7-36 amide} infusion and the reduction in % BOLD signal change compared to the control saline infusion only in the amygdala, and not in any of the other ROIs. This may reflect the general role of the amygdala in processing of emotion. Notably, there was no significant correlation between nausea and reduction in % BOLD signal change in the insula, which was the brain region that showed greatest modulation with the combined infusion of PYY₃₋₃₆ and GLP-1_{7-36 amide}.

3.4.2. Comparison of the effects of PYY₃₋₃₆ and GLP-1_{7-36 amide} on neuronal activity related to appetite with those of other appetite-modulating hormones.

It is of interest to interpret my results in the context of similar human fMRI studies investigating neuronal effects of other appetite-modulating hormones. Consistent trends can be found in reports of modulation of the BOLD fMRI signal when viewing food-related images in the presence or absence of these hormones, with the directionality of neuronal effects seemingly dependent on whether the hormone under scrutiny is anorectic or orexigenic.

Leptin, a hormone secreted by adipocytes, is thought to form part of the feedback loop for maintaining long term energy homeostasis; when fat stores are replete, increased levels of leptin signal to the brain to reduce subsequent food intake in order to maintain weight neutrality. However, whilst this is an oversimplification, given the well known phenomenon of leptin resistance in obesity, in global terms, we may consider leptin to be an anorectic

hormone. In an fMRI study testing for brain activation contrasts between viewing high and low calorie food images (i.e. slight methodological differences to my study, as I used the signal contrast between all food and non-food images), subcutaneous leptin replacement in congenitally leptin deficient individuals resulted in reduced BOLD activation in the insula, temporal and parietal cortex (Baicy et al., 2007). Therefore, my results for PYY₃₋₃₆ and GLP-1₇₋₃₆ amide administration, showing attenuation of signal in the insula, caudate, putamen and nucleus accumbens, are consistent with this previous study of leptin. In another fMRI study that used a protocol similar to mine, when leptin was replaced subcutaneously in individuals with congenital leptin deficiency, BOLD activation contrast between viewing food and non-food images was reduced in the caudate, putamen, globus pallidus and nucleus accumbens (Farooqi et al., 2007). I found similar activation changes following infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. However, in my study the most significant reduction in mean % BOLD signal change following infusion of PYY₃₋₃₆ and / or GLP-1₇₋₃₆ amide was in the insula, left nucleus accumbens and the left OFC. This implies that leptin, compared with PYY₃₋₃₆ or GLP-1₇₋₃₆ amide, modulates distinct neural networks. I speculate that this may be related to the longer-term anorectic signaling by the adipokine leptin, relative to the more acute anorectic effects of post-prandially released gut hormones.

It is also interesting to consider my results in relation to the effects of the hormone insulin on cue-induced brain activation. Although exogenous insulin therapy often leads to weight gain when administered to patients with diabetes (which may be consequential to its overall peripheral anabolic effects and also due to the stimulation of appetite secondary to a fall in blood glucose), endogenous insulin release is postprandial and central anorectic effects limit further food intake. In a study where insulin was administered intranasally whilst maintaining euglycaemia, BOLD fMRI activation (in response to food versus non-food images) was reduced in the fusiform gyri, right hippocampus, right temporal superior cortex and right frontal middle cortex (Guthoff et al., 2010). The attenuation of BOLD signal in higher cortical

centres by insulin is consistent with my data. I speculate that subtle differences in brain responses between insulin and the anorectic gut hormones PYY₃₋₃₆ and GLP-1₇₋₃₆ amide may reflect the different roles of these hormones in energy homeostasis: anorectic gut hormones are thought to mediate short term signalling, whereas insulin may be implicated both in short and longer term signalling of energy homeostasis.

My results are also interesting in relation to a previous study investigating 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 BOLD activation when subjects viewed images of food compared to when they viewed images of non-food in the amygdala, OFC, insula, visual areas and striatum. By contrast, in my study, the gut hormones PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, which acutely inhibit food intake, resulted in a reduction in mean % BOLD signal change in these ROIs. These results suggest that these ROIs form CNS networks, which when activated by ghrelin, mediate hunger and when inhibited by the anorectic gut hormones PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, mediate satiety.

To investigate the effects of hypoglycaemia (another orexigenic stimulus), a recent study reported increased BOLD activation in the insula and striatum, along with increased hunger after mild hypoglycaemia was induced (Page et al., 2011). Thus my work, along with other fMRI studies, suggests that whilst anorectic hormones attenuate activity in brain regions supporting salience and reward, orexigenic stimuli enhance activation in these same areas.

3.4.3. Comparison of the effects of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on neuronal activity related to appetite with those observed after gastric bypass surgery.

Here, I have reported both total and active concentrations of PYY, in addition to amidated and active concentrations of GLP-1. In contrast to study 1, there was no significant difference in plasma levels of PYY achieved between single infusion of PYY₃₋₃₆ compared with combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. Following the exogenous infusion of PYY₃₋₃₆, the profile of total and active hormone levels in plasma was similar. Furthermore, following the exogenous infusion of GLP-1₇₋₃₆ amide, the profile of amidated and active hormone levels in plasma was similar. This suggests that the standard practice of measuring total PYY concentrations and amidated GLP-1 concentrations is valid. Total PYY concentrations and amidated GLP-1 concentrations are more commonly reported in the literature; review of the corresponding hormone levels in my study thus allows for meaningful comparison with existing relevant data. My results revealed that during the combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, the mean total PYY concentration achieved was 70 pM and the mean amidated GLP-1 concentration was 43 pM. Interestingly, the mean circulating levels of total PYY and amidated GLP-1 following breakfast were 33 pM and 22 pM, respectively. Although levels of total and active hormone levels were slightly higher after breakfast than when subjects continued to fast through the morning, this did not reach statistical significance. This is surprising, given that we would expect increased levels of both hormones post-prandially. One explanation for this may have been that the set breakfast was served between $t = -60$ and $t = -40$ min and the next plasma sample was not taken until $t = 0$ min, thereby missing the early peak of post-prandial PYY and GLP-1.

Although my infused circulating total PYY and amidated GLP-1 levels were slightly higher than those observed physiologically after breakfast, there was a comparable reduction in *ad*

libitum energy intake at lunch following combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide and following infusion of saline after breakfast. I hypothesize that this may reflect the combined effects of several other gut hormones and satiety factors in addition to PYY₃₋₃₆ and GLP-1₇₋₃₆ amide that are thought to play a role in mediating satiety as part of the physiological response to feeding.

The plasma concentrations of total PYY and amidated GLP-1 that I have explored are pertinent when considering the levels observed after Roux-en-Y gastric bypass, the most effective current treatment for obesity. Gastric bypass patients demonstrate exaggerated post-prandial levels of total PYY and amidated GLP-1 (Borg et al., 2006, le Roux et al., 2006). In the study by Le Roux *et al.*, a mean 90 min post-prandial total PYY level of 40 pM was observed following Roux-en-Y gastric bypass. In the same study, a mean 30 min post-prandial amidated GLP-1 level of 47 pM was observed. These levels are comparable to plasma levels of total PYY and amidated GLP-1 that were observed in my study. Therefore, although circulating levels achieved in my study may be regarded as somewhat supraphysiological within the normal population, they are of direct functional relevance for understanding the effects of PYY and GLP-1 in patients who have undergone gastric bypass surgery. PYY and GLP-1 appear to be the most important gut hormones implicated in mediating reduced appetite and promoting weight loss following gastric bypass (le Roux et al., 2007) although there are likely to be changes in other gut hormones that are not routinely measured.

It is therefore of interest to compare my imaging results in healthy, lean individuals to those found in a fMRI studies of obese individuals pre- and post- gastric bypass surgery. In a recent study, reductions in striatal neural response, along with reductions in wanting and liking for high- relative to low-calorie food cues, were observed one month post- Roux en-Y

gastric bypass surgery compared with pre-surgery (Ochner et al., 2012). In a previous study by the same group (Ochner et al., 2011) post-surgery (compared with pre-surgery), there was attenuation of high calorie food cue associated fMRI BOLD signal in the VTA, ventral striatum, putamen, lentiform nucleus, posterior cingulate and dorsomedial prefrontal cortex. Hunger also was reduced after surgery (Ochner et al., 2011). These results are striking, given that I also found similar attenuation of BOLD signal in reward areas when I administered PYY₃₋₃₆ and GLP-1₇₋₃₆ amide. Although circulating gut hormone levels were not measured in the gastric bypass study (Ochner et al, 2011), the authors postulated post-surgical reductions in ghrelin levels as an explanation for their imaging observations. Alternatively, I speculate that elevated levels of PYY and GLP-1 post-bypass would explain their findings. Thus, whilst fMRI is at present used only as a research tool in endocrinology, these studies imply translation for the pre-operative work-up of obese patients; it may help highlight those patients who are more susceptible to the anorectic effects of gut hormones and who may therefore benefit most from surgery. Although there are as yet no published studies as such, a recent study of fMRI response to viewing high-calorie food versus control pictures in obese individuals before and after a 12 week psychosocial weight loss program revealed that obese individuals who were least successful in losing weight during the treatment displayed greater pre-treatment BOLD activation to high calorie food vs. control pictures in the nucleus accumbens, ACC, and insula. Furthermore, greater post-treatment BOLD activation in the insula, VTA, putamen, and fusiform gyrus predicted less successful weight maintenance at 9 months follow up (Murdaugh et al., 2012).

3.4.4. Limitations of my study

In addition to the brain reward regions discussed thus far, the hypothalamus and certain brainstem nuclei are also known to be important in the homeostatic control of food intake. Direct information on hypothalamic and brainstem responses was not available in my study. In a separate voxel-wise analysis, I did not observe significant responses in these regions using the picture processing BOLD fMRI task. Previous similar studies failed to observe changes in hypothalamic activation (Malik et al., 2008) or provided no comment on it (Farooqi et al., 2007), although other BOLD fMRI investigations have quantified hypothalamic signal (Batterham et al., 2007, Fletcher et al., 2010). Approaches that control for effects of motion of the hypothalamus and brainstem during the cardiorespiratory cycle could enable the study of activation responses in future work (Pattinson et al., 2009). The small size of the hypothalamus (approximately 5 mm in diameter) limits its resolution using fMRI acquisition parameters applied in my study. Additionally, magnetic susceptibility signal loss due to the air-tissue interface of the adjacent sinuses limits assessment of any hypothalamic signal changes. Thus, the ascending pathways mediating actions of circulating modulators of appetite remain incompletely understood.

In my BOLD fMRI analysis, although there was consistent modulation of % BOLD signal change by feeding, PYY₃₋₃₆ and GLP-1₇₋₃₆ amide infusion across all ROIs studied, statistical significance of results was largely limited to the insula. This is most likely explained by the large inter-individual variation in BOLD fMRI signal across subjects, thus creating a large standard error at the group level, which is a limiting factor where statistical power is concerned. The study was powered based on the outcome of reduction in food intake during the *ad libitum* lunch following feeding or gut hormone infusion; a sample size of 16 was required in order to have 90% power to detect a difference in mean food intake of 950 kJ,

assuming a standard deviation of differences of 1140 kJ, using a paired t test with a 0.05 two sided significance level. A much larger subject group may well have allowed further statistically significant imaging results to emerge at the group level, notwithstanding constraints on scanner time and cost.

A further limitation of my work stems from the concept of *a priori* choosing ROIs to assess the action of feeding or gut hormone infusion. Although I chose ROIs based on the results of existing data in the field and also my hypotheses as to their role in food reward, it should be noted that an ROI-based analysis, although statistically providing more sound results, possesses the inherent problem of potentially missing other regions of the brain which are modulated by gut hormones. Notably however, my initial analysis of the effect of the picture processing task, examining the brain regions where BOLD signal contrast between viewing images of food and non-food was greatest, used data from the fasted saline infusion scans and 'fed saline' infusion scans. This verified that my choice of ROIs was appropriate and that other brain regions significantly responsive to the picture processing task were not being missed. Furthermore, the similarity in brain response to feeding as when PYY₃₋₃₆ and GLP-1_{7-36 amide} were infused provides reassurance regarding my choice of ROIs for assessing the action of PYY₃₋₃₆ and GLP-1_{7-36 amide}.

Here, I have presented all BOLD fMRI data as the contrast in BOLD signal during the picture processing task when subjects viewed images of food versus non-food, and the effect that feeding or gut hormone infusion had on modulating this signal contrast. Presenting data as a contrast of BOLD signal is standard practice in the fMRI literature; I chose to compare the signal contrast between viewing images of food and non-food, as the image processing task that I used was already set up in this way (Beaver et al., 2006). Contrasting the BOLD signal in response to viewing images of appetizing food versus bland food, or alternatively

appetizing food versus non-food would have been alternative approaches that I could have used that are also widely reported in the literature. Regardless of the type of contrast used to present the data, an inherent limitation of fMRI analysis stems from this; namely that results are not presented directly as 'BOLD signal', but instead as 'BOLD signal change'.

Furthermore, it is important to appreciate that BOLD fMRI signal is not a direct marker of neuronal activity; increased neuronal activity in given brain region elicits a local haemodynamic response, which causes an increase in blood flow above and beyond that necessary for tissue demands. Provided that tissue oxygen extraction remains the same, this results in a locally reduced ratio of deoxyhaemoglobin to oxyhaemoglobin concentration. The ensuing differences in local magnetic field inhomogeneities can be translated into imaging outcomes, whereby the locally reduced ratio of deoxyhaemoglobin to oxyhaemoglobin leads to a longer $T2^*$, producing an increased image intensity. If tissue oxygen extraction increased as well, then the ratio of deoxyhaemoglobin to oxyhaemoglobin concentration would be higher, thus in fact paradoxically leading to reduced signal in more active tissue. It is therefore important to note that although BOLD fMRI focuses on the initial haemodynamic response observed in metabolically active tissue, the BOLD response is also influenced by local changes in oxygen extraction, which may confound results.

CHAPTER 4

General Discussion

4.1. Summary

I have characterized the effects of single and combined administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on brain BOLD fMRI activations in humans. My data shows for the first time that combined infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide leads to an anorectic effect which is similar to that observed following a meal. In keeping with this, combined administration modulates brain activations implicated in appetite control to an extent similar to that observed physiologically after a meal. By administering PYY₃₋₃₆ and GLP-1₇₋₃₆ amide to fasted individuals, it is therefore possible to 'trick' the brain into feeling 'satiated' in ways that appear indistinguishable from the effects of a meal. These findings provide direct evidence that the combined action of gut hormones including PYY₃₋₃₆ and GLP-1₇₋₃₆ amide in the brain could explain post-prandial satiety.

There is additional exciting data to suggest that following gastric bypass, where it is known that appetite is reduced and that levels of PYY and GLP-1 are elevated, patterns of brain activation are similar to those seen in my study after administration of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide in healthy volunteers (Ochner et al., 2011, Ochner et al., 2012). I have speculated on the significance of some of the brain areas engaged in appetitive processing and suggest that a continued and deeper understanding of the neurobiology of appetite will pave the way to the development of gut hormones as effective anti-obesity therapies. Given the consistency of the imaging data so far, it 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.

4.2. Further work: imaging the hypothalamus

The use of arterial spin labelling (ASL) MRI promises to be a novel technique for the investigation of appetitive pathways, potentially allowing for better functional assessment of the hypothalamus. By magnetically tagging a column of blood outside the brain, when this tagged blood enters a brain region of interest, distortions of the local magnetic field in the given region of interest can be assessed. ASL more directly reflects the changes in local brain perfusion and has favourable noise properties over BOLD for intersession comparisons (Wang et al., 2011)

It is also possible to envisage that using a combination of BOLD and ASL analysis for reward regions of interest would allow a better dissection of changes in local blood flow versus changes in tissue oxygen extraction; theoretically, as the BOLD signal is based on the overall outcome of blood flow and oxygen extraction, whereas the ASL signal focuses on blood flow alone, subtraction of ASL from BOLD may provide a marker of local oxygen extraction in metabolically active tissues.

Other approaches to provide better BOLD fMRI signal in the hypothalamus could also be considered in future work; early studies that have concentrated on hypothalamic signal change have involved conventional T2*-weighted sequences to functionally scan a sagittal plane through the hypothalamus continuously, acquiring images pre- and post- a given stimulus. By concentrating all image processing ability on this thin, central slice, greater attention can be devoted to a ROI corresponding to the hypothalamus, although clearly at the expense of imaging the rest of the brain. In this manner, Matsuda *et al.* demonstrated that oral glucose ingestion produced a profound transient reduction in hypothalamic BOLD fMRI signal in lean subjects (Matsuda et al., 1999). Interestingly, from a clinical perspective,

this observation has been extended and it has been subsequently demonstrated that the reduction in hypothalamic BOLD signal following glucose ingestion was absent in patients with Type 2 diabetes, theorising that inappropriate hypothalamic processing of nutrient availability may be involved in the aetiology of the disease (Vidarsdottir et al., 2007). This group has also alluded to the effects of gut hormones on hypothalamic activity following glucose ingestion. In healthy men, despite causing a threefold lower rise in blood glucose concentration than intravenous glucose administration, oral glucose ingestion led to a much more robust reduction hypothalamic BOLD signal (Smeets et al., 2007). As well as the effect of taste, it was postulated that hormones such as GLP-1, released in response to nutrients entering the gut, accounted for this greater hypothalamic response to oral rather than intravenous glucose.

In 2000, Liu *et al.* described a new technique called time clustering analysis (TCA), whereby the number of voxels reaching maximum signal intensity as a function of time was examined (Liu et al., 2000). In this way, TCA records the time windows in which maximal brain responses occur and from which ROI analysis can be appropriately extracted. By scanning a 1cm midsagittal slice, they were able to further concentrate resolution on the hypothalamus and found that significant hypothalamic deactivation occurred 10 minutes following glucose ingestion. They also showed that the degree to which the hypothalamic signal reduced post-glucose ingestion negatively correlated with fasting plasma insulin levels.

However, in contrast to the above findings, a recent study by Purnell *et al.* in 2011 did not detect any signal change in the hypothalamus in response to either glucose or fructose infusion in 9 healthy, normal-weight subjects (Purnell et al., 2011). The authors postulated that differential activation of excitatory and inhibitory nuclei within the hypothalamus may have cancelled out any overall signal change. This illustrates the ongoing limitations when attempting to visualise the human hypothalamus in fMRI studies. Clearly, in order to

maximally capture hypothalamic signal, dedicated imaging paradigms are required, which may limit the ability to simultaneously image brain reward regions.

4.3. Further work: fMRI responses in obesity

My work in this thesis has focused on the effect of normal feeding and the anorectic gut hormones PYY₃₋₃₆ and GLP-1₇₋₃₆ amide on the BOLD fMRI response in individuals of normal weight. Translation of these findings into obese individuals are supported by the recent fMRI studies of patients pre- and post- bariatric surgery; the similar activation patterns in brain reward regions in my work to those observed post- bariatric surgery, where levels of PYY and GLP-1 would presumably be elevated, are supportive (Ochner et al., 2011, Ochner et al., 2012). However, a limitation of this body of work lies in the fact that fMRI studies of bariatric surgery have not directly reported levels of measured anorectic gut hormone levels.

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 one such study, increasing BMI positively predicted BOLD activation (in relation to viewing high calorie food images versus non-food images) in the dorsal striatum, anterior insula and lateral OFC (Rothmund et al., 2007). A further study of obese women demonstrated that pictures of high calorie foods (versus non-food) produced greater activation in an obese group compared with controls in several brain regions, including the ventral and dorsal striatum, insula, ACC, amygdala and OFC (Stoeckel et al., 2008). Functional connectivity analysis indicated that obese women displayed a relative deficiency in the amygdala's modulation of the OFC and ventral striatum, along with excessive modulation of the ventral striatum by the OFC (Stoeckel et al., 2009). Collectively, results from the above studies suggest that a

hyper-reactive reward system to high calorie food cues is involved in the pathophysiology of obesity.

Several studies have shown that obese individuals also respond differently to satiation compared with normal weight individuals, hypothesizing that in obesity, there is a degree of dysregulation in CNS appetite regions, which may contribute to overeating and hence weight gain. One such study measured BOLD fMRI 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 (Martin et al., 2010), thus concurring with the previous observations (Stoeckel et al., 2008). During the post-meal condition, obese participants also showed greater BOLD activation in the medial prefrontal cortex compared with healthy weight controls. A further 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 (Dimitropoulos et al., 2012). 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 ACC (Dimitropoulos et al., 2012).

The CNS response to the hormones discussed in this thesis may also be different in overweight or obese individuals. As discussed above, in 1999, Matsuda *et al.* studied the hypothalamic response before and after oral glucose ingestion. Although they showed that oral glucose ingestion produced a profound transient reduction in hypothalamic signal in 10

lean subjects, this response was both significantly delayed and attenuated in 10 obese individuals. More tellingly, it was shown that following glucose ingestion, the time taken to reach the maximum hypothalamic inhibitory response was closely correlated to fasting plasma glucose and insulin levels (which, as expected, were significantly higher in the obese group) rather than BMI per se, suggesting hypothalamic dysfunction as either the cause or consequence of insulin resistance (Matsuda et al., 1999).

To extend the above findings to the study of reward regions, a later study incorporating visual food and non-food cue methodology revealed that BOLD fMRI activation in the right hippocampus (with respect to high calorie food images versus other images) correlated positively with fasting insulin levels and waist circumference (but not BMI) in 12 normal weight and 12 obese adolescents who had all been fed a standard breakfast a few hours prior to scanning (Wallner-Liebmann et al., 2010). On the other hand, in that study, there was significant negative correlation between activation of the medial right superior frontal gyrus and the left thalamus (again, with respect to the contrast between high calorie food images versus other images) with fasting insulin levels. The authors postulated that their results reflected a permissive role of insulin in the control of eating behaviour by the hippocampus. I further speculate that in those with a larger waist circumference and high circulating levels of insulin (i.e. peripherally insulin resistant individuals), a degree of central insulin resistance in the hippocampus may explain the greater activation in this region, despite higher insulin levels. Indeed, a magnetoencephalography study showed that food-image related brain activity in the fusiform gyrus was increased by local intranasal administration of insulin, but that this was reduced in overweight subjects, implying a degree of cerebral insulin resistance (Guthoff et al., 2011).

Some studies have reported that obese individuals have lower basal fasting levels of PYY and have a smaller rise in postprandial levels (Batterham et al., 2003). Obesity does not appear to be associated with resistance to PYY₃₋₃₆, as in obese subjects, there was a 30% in a reduction in *ad libitum* food intake at a buffet meal served 2 hours after completion of a 90 minute infusion of PYY₃₋₃₆ (Batterham et al., 2003). This compared with a 31% reduction in food intake in a group of lean subjects who also received a peripheral infusion of PYY₃₋₃₆. Additionally, there was also a comparable reduction in 24 hour energy intake in both lean and obese subjects following PYY₃₋₃₆ infusion. Furthermore, in the case of GLP-1₇₋₃₆ amide, the reduction in *ad libitum* energy intake for overweight / obese subjects (10.5%) was comparable with that of lean subjects (13.2%) (Verdich et al., 2001). Further fMRI studies of BOLD signal in response to infusion of PYY₃₋₃₆ and GLP-1₇₋₃₆ amide in overweight / obese subjects, would nevertheless be interesting to validate the findings from early infusion studies.

4.4. Conclusion

The use of fMRI to study appetite control in humans is a rapidly evolving field. There appears to be a degree of consistency between studies, suggesting that in the fasted state, greater BOLD activation is seen in several brain regions implicated in reward processing when food cues (and in particular high calorie food cues) are presented to subjects. Obese subjects seem to show heightened response to such food cues, which may fundamentally contribute to increased wanting of food (and thus increased subsequent food intake) in obese individuals. The fMRI data also seems to be in agreement that the consumption of a meal (or other surrogate markers of feeding, such as a rise in glucose) tends to deactivate key appetite regions in normal weight individuals, and furthermore, that this satiety response may also be abnormal in those with obesity. Further studies should lead to consolidation of existing concepts on the role of individual brain regions and their interconnections in physiological and pathological satiety responses.

Building on this fundamental understanding of the change in BOLD signal between the fasted and fed states, recent studies have suggested that in general, orexigenic signals, which are in abundance in the fasted state, increase the BOLD response in brain reward regions, whereas anorectic signals, which predominate in the fed state, correspondingly deactivate the same regions. Subtle differences in brain responses between these studies may reflect the slightly different roles of the different hormonal signals in short term versus longer term energy homeostasis, and also may reflect differential action on various appetite pathways. Nevertheless, studies in obese patients, assessing the effect of gastric bypass surgery on the BOLD fMRI response, have further concurred with the results of studies investigating the BOLD response following the exogenous administration of orexigenic and anorectic hormones, thus consolidating the overall body of work in the area.

Although fMRI is at present used only as a research tool to study appetite, the overall goal in the future will be to translate this research into the clinical arena. An obvious role would be to help develop pharmacological treatments for obesity, by means of assessing the fMRI response to administration of novel agents in obese individuals. Alternatively, we may envisage the use of fMRI as a tool to help predict response to gastric bypass surgery; it would be interesting to investigate whether particular patterns of fMRI brain activation pre-operatively predict response to surgery. To aid effective comparison of results, it would be enormously useful to strive towards greater consistency between studies, particularly with regard to experimental design, image acquisition and image analysis. My research and that of others have established key concepts, and it is expected that the coming decade should see consolidation of these findings. We are fortunate that fMRI studies have allowed scientists to investigate for the first time in humans, the intricacies of appetitive processing. As this technology continues to evolve, it will remain an important tool for investigating basic physiology as well as evaluating new therapies.

APPENDIX 1

Principles of Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is a technique used to measure the concentration of an unknown quantity of peptide sample (in this case, the total PYY, PYY₃₋₃₆ or total GLP-1) in plasma. Unlabelled antigen on the peptide in the plasma sample and radiolabelled (using I-125) antigen compete for an antibody binding site. The specificity of the antibody is dependent upon its ability to recognise structural features of the antigen on the peptide. Antibodies for total PYY and total GLP-1 in my assays are produced by immunising rabbits with PYY and GLP-1 respectively. The antibody for PYY₃₋₃₆ used in my assay is produced commercially by immunising Guinea Pigs with PYY₃₋₃₆.

In the RIA, both the radiolabelled peptide and antibody are at specific, constant concentrations, with the concentration of antibody being limiting. The known amount of radiolabelled antigen will compete with unlabelled antigen (in the plasma sample of peptide) for antibody binding, with the amount of radiolabelled antigen bound to the antibody being inversely proportional to the amount of unlabelled antigen in the plasma peptide sample being assayed. A 'standard curve' is also set up, adding known concentrations of pure antigen to fixed amounts of antibody and radiolabelled antigen.

Tubes containing unknown concentrations of unlabelled sample antigen, radiolabelled antigen and antibody are incubated for 72 hours at 4°C, following which separation of the unbound radiolabelled antigen from the radiolabelled antigen-antibody complex is

performed. For GLP-1, separation by adsorption with charcoal is used (free radiolabelled antigen is bound by the charcoal pellet following centrifugation, with the supernatant then separated from the pellet using a Pasteur pipette). For PYY, separation of the free radiolabelled antigen from the radiolabelled antigen-antibody complex using a primary antibody / secondary antibody complex is used (free radiolabelled antigen is contained in the supernatant following centrifugation, which can then be separated from the radiolabelled antigen-antibody complex pellet using a Pasteur pipette). In my assay for total PYY, the secondary antibody was raised in sheep against the primary antibodies derived from rabbits. In the commercial assay that I used to measure PYY₃₋₃₆, the secondary antibody was raised in goats against the primary antibodies derived from Guinea Pigs.

Following separation of the free radiolabelled antigen from the antibody-bound radiolabelled antigen, radioactivity in both fractions is counted using a gamma counter. Using the standard curve tubes, the percentage binding of radiolabelled antigen to the antibody is plotted for different concentrations of pure 'standard' antigen. From this, the concentration of unlabelled antigen (and hence peptide) in the unknown plasma samples can be interpolated.

Principles of Enzyme- Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) as used in my work is based on the capture of active GLP-1 (GLP-1₇₋₃₆ amide and GLP-1₇₋₃₇) in the plasma sample by a monoclonal antibody, already immobilized in the wells of a microwell plate. The antibody binds specifically to the N-terminal region of active GLP-1 molecule. Plasma containing the unknown concentrations of active GLP-1 are added to the microwell plate containing the antibody and incubated for 24 hours at 4°C. Following washing to remove unbound materials, an anti GLP-1-alkaline phosphatase detection conjugate is added, which binds to the immobilized active GLP-1 that

is itself bound to the antibody. Following a further washing step to remove unbound conjugate, quantification of bound detection conjugate (and hence active GLP-1 that is bound to the antibody) is possible by adding MUP (methyl umbelliferyl phosphate), which in the presence of alkaline phosphatase, forms the fluorescent product umbelliferone. The amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the unknown plasma sample. Thus, using a fluorescence plate reader, the concentration of active GLP-1 in the plasma sample can be derived. As with the RIA technique, interpolation from a reference standard curve (generated in the same assay using reference standards of known concentrations of active GLP-1) is required in order to derive the concentration of active GLP-1 in each unknown plasma sample.

APPENDIX 2

Results of mouse toxicology study

PYY₃₋₃₆ and GLP-1₇₋₃₆ amide, each at a dose greater than 10 times the maximum intended for humans (in pmol/kg body weight), were administered by intra-peritoneal injection to 20 mice, and saline was administered to a control group of 10 mice. The mice were coded as detailed below. Half the animals in each group were sacrificed by CO₂ asphyxiation after 48 hours, and the other half after 14 days. The heart, lungs, kidneys, liver, spleen, pancreas, brain and gut were dissected, fixed and sent for examination by an independent histopathologist. Histology results are detailed below.

CODE:

1 - 5 = saline injection, sacrificed at 48 hours

6 - 10 = saline injection, sacrificed at 14 days

11 - 15 = PYY₃₋₃₆ injection, sacrificed at 48 hours

16 - 20 = PYY₃₋₃₆ injection, sacrificed at 14 days

21 - 25 = GLP-1₇₋₃₆ amide injection, sacrificed at 48 hours

26 - 30 = GLP-1₇₋₃₆ amide injection, sacrificed at 14 days

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

11. 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 Brunner's glands, normal pancreas with normal islets, normal colon.
12. Normal lungs, normal thymus gland, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's glands, normal pancreas with normal islets, normal colon.
13. 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 Brunner's 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 Brunner's 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 Brunner's glands, normal pancreas with normal islets, normal colon. The brain may have slight asymmetric 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 Brunner's glands, normal pancreas with normal islets, normal colon.
18. Normal lungs, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's 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 Brunner's 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 Brunner's glands, normal pancreas with normal islets, normal colon.
21. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's glands, normal pancreas with normal islets, normal colon.
22. Normal lungs, normal thymus gland, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's glands, normal pancreas with normal islets, normal colon.
23. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's 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 Brunner's glands, normal pancreas with normal islets, normal colon.
25. Normal lungs, normal bronchi, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's glands, normal pancreas with normal islets, normal colon.
26. 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 Brunner's glands, normal pancreas with normal islets, normal colon.

27. 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 Brunner's glands, normal pancreas with normal islets, normal colon.
28. Normal lungs, normal trachea, normal oesophagus, normal heart, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's 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 Brunner's glands, normal pancreas with normal islets, normal colon. is possibly subtle dilatation of the lateral ventricles although the third ventricle appears relatively normal.
29. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's glands, normal pancreas with normal islets, normal colon. There is possibly subtle dilatation of the lateral ventricles although the third ventricle appears relatively normal.
30. Normal lungs, normal trachea, normal oesophagus, normal heart, normal brain, normal spleen, normal liver, normal kidney, normal stomach, normal duodenum with Brunner's glands, normal pancreas with normal islets, normal colon.

APPENDIX 3

Publications arising from thesis

The gut hormones PYY 3-36 and GLP-1 7-36 amide reduce food intake and modulate brain activity in appetite centers in humans. **De Silva A**, Salem V, Long CJ, Makwana A, Newbould RD, Rabiner EA, Ghatei MA, Bloom SR, Matthews PM, Beaver JD, Dhillon WS. *Cell Metab.* 2011 Nov 2;14(5):700-6.

The use of functional MRI to study appetite control in the CNS. **De Silva A**, Salem V, Matthews PM, Dhillon WS. *Experimental Diabetes Research* 2012 (2012:764017.Epub 2012 May 8).

Gut Hormones and Appetite Control: A Focus on PYY and GLP-1 as Therapeutic Targets in Obesity. **De Silva A** & Bloom SR. *Gut and Liver* 2012 Jan; 6(01): 10-20.

Imaging the neuroendocrinology of appetite. Salem V, **De Silva A**, Matthews PM, Dhillon WS. *Adipocyte* 2012 Jan; 1(1): 68-72.

Translational studies on PYY as a novel target in obesity. Zac-Varghese S, **De Silva A**, Bloom SR. *Curr Opin Pharmacol.* 2011 Dec;11(6):582-5.

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