The Effect of Gut Hormones on Metabolism and Energy Homeostasis

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Abstract

The increasing prevalence of obesity, and its associated morbidity and mortality, together with limited treatment options, underscores an urgent need for investigation into effective therapeutic interventions. Gut hormones have been identified as integral factors in the regulation of appetite. This thesis examines two signalling systems involved in the gastrointestinal regulation of energy homeostasis: Peptide YY (PYY) and Proglucagon-derived peptides.

PYY is a gut hormone released from the L cells of the intestine after the ingestion of food and elicits its effects via the Y2 receptor. The predominant circulating form of PYY, PYY3-36, has been shown to acutely reduce food intake when administered peripherally at physiological concentrations in both lean and obese rodents and humans. However, several groups have shown that continuous administration of PYY3-36 via osmotic mini-pumps results in a transient reduction in food intake. It has been suggested that an apparent desensitisation to the anorectic effects of PYY3-36 may be due to the physiological defence of body weight and counter regulatory mechanisms; however tolerance due to receptor downregulation may also occur. In this thesis, I aimed to elucidate if the transient anorectic effect of PYY3-36 is a result of direct tolerance to the peptide itself or if this is indirect, due to the homeostatic defence of body weight. I have shown that the anorectic effect of PYY3-36 is attenuated following prior exposure to a low dose, suggesting tolerance at the receptor level. In addition, animals which were food restricted preceding an infusion of PYY3-36 remained sensitive to the anorectic effects, suggesting that body weight change alone cannot result in the hyperphagic response. However, no change in Y2 mRNA receptor expression following PYY3-36 infusion was detected.

The proglucagon peptide family includes the hormones Glucagon like peptide-1 (GLP-1) and Glucagon (GCG). GLP-1 is release from the L-cells of the intestine in response to food intake and has known actions as a satiety factor and incretin hormone. GCG is released under fasting conditions and in response to adrenergic stimulation to stimulate gluconeogenesis and glycogenolysis, as well as to increase energy expenditure. Recent evidence suggests that simultaneous co-agonism of the GLP-1 and GCG receptors may be beneficial to the treatment of obesity and diabetes. In this thesis, I explore the development of a GLP-1R and GCGR co-agonist which reduces food intake and increases energy expenditure in rodents. Furthermore, to investigate the mechanism of GCG agonism on energy expenditure, I evaluate the effect of the specific dual agonist, GX6, on metabolic gene expression in brown adipose tissue and the liver.

Overall, this thesis evaluates the potential roles of PYY, GLP-1 and GCG receptor agonism as novel therapies for obesity.
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Declaration of Contributors

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

Chapter 2:

All animal studies and RIAs were done in collaboration with Dr. Victoria Salem. Help was provided by Joyceline Cuenco-Shillito, Dr. James Minnion and Dr. Jordan Baxter.

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Table of Contents

Abstract ................................................................................................................................. 2

Acknowledgments ............................................................................................................... 3

Declaration of Contributors .............................................................................................. 4

Table of Contents ................................................................................................................ 5

List of Figures ...................................................................................................................... 9

List of Tables ...................................................................................................................... 12

Abbreviations Used in this Thesis .................................................................................... 13

Chapter 1: General Introduction ....................................................................................... 16
  1.1 Obesity ......................................................................................................................... 17
  1.2 Energy Homeostasis .................................................................................................... 18
  1.3 Energy expenditure ...................................................................................................... 19
    1.3.1 Brown Adipose Tissue and Adaptive Thermogenesis ............................................ 19
  1.4 Central Control of Food Intake .................................................................................... 20
    1.4.1 The Hypothalamus ............................................................................................... 20
      1.4.1.1 The Arcuate Nucleus (ARC) ............................................................................ 21
      1.4.1.2 The Paraventricular Nucleus (PVN) ............................................................... 23
      1.4.1.3 The Ventromedial Nucleus (VMN) ................................................................ 23
      1.4.1.4 The Dorsomedial Nucleus (DMN) ................................................................. 24
      1.4.1.5 The Lateral Hypothalamus (LH) ................................................................. 25
    1.4.2 The Brainstem ......................................................................................................... 25
  1.5 Peripheral factors in the regulation of Food Intake ....................................................... 26
    1.5.1 Adipostat Factors .................................................................................................. 27
      1.5.1.1 Insulin ............................................................................................................. 27
      1.5.1.2 Leptin ............................................................................................................. 28
    1.5.2 Peripheral Signals from the Gastrointestinal (GI) Tract ........................................... 29
      1.5.2.1 Ghrelin ......................................................................................................... 29
      1.5.2.2 Cholecystokinin ............................................................................................ 31
      1.5.2.3 Pancreatic Polypeptide (PP)-fold family ...................................................... 33
      1.5.2.3.1 Pancreatic Polypeptide ........................................................................... 33
      1.5.2.3.2 Peptide YY ............................................................................................... 35
      1.5.2.4 Proglucagon derived signals ......................................................................... 39
        1.5.2.4.1 Glucagon-like-peptide 1 ........................................................................ 41
      1.5.2.4.2 Glucagon .................................................................................................... 42
      1.5.2.4.3 Oxyntomodulin ....................................................................................... 45

  1.6 Overview of Thesis ...................................................................................................... 47
Chapter 2: Investigation into Continuous Administration of PYY3-36 on Food Intake and Body Weight

2.1 Introduction

2.1.1 PYY3-36 and the Y2 receptor (Y2R)

2.1.2 Therapeutic potential of PYY as an Anti-Obesity Agent

2.1.3 Development of Tolerance to Anorectic Agents

2.1.4 Tolerance to anorectic effect of PYY3-36

2.2 Hypothesis and Aims

2.3 Methods

2.3.1 Peptide Synthesis

2.3.2 Animal Husbandry and General Experimental Conditions

2.3.3 The Effect of PYY3-36 Administration on Acute Food Intake

2.3.4 Effect of Continuous Subcutaneous Infusion of PYY3-36

2.3.4.1 Investigation into the Anorectic Effect of Continuous Administration of PYY3-36 for 7 days

2.3.4.2 Investigation into the Effect of 3 Day Continuous Low Dose Pre-treatment followed by 3 day High Dose Treatment of PYY3-36

2.3.4.3 Investigation into the Effect of Food Restriction on the Anorectic Effect of PYY3-36

2.3.5 Quantification of hypothalamic Y2 receptor using qPCR

2.3.5.1 Messenger RNA extraction

2.3.5.2 RNA Quantification and Assessment of Quality

2.3.5.3 Reverse Transcription

2.3.5.4 Quantitative Polymerase Chain Reaction (qPCR)

2.3.6 Radioimmunoassay

2.3.7 Statistical Analysis

2.4 Results

2.4.1 The Effect of PYY3-36 Administration on Acute Food Intake in Mice

2.4.2 Effect of 7 day Continuous Subcutaneous Infusion of PYY3-36 via Alzet osmotic minipump on Food Intake and Body Weight

2.4.2.1 Effect on Cumulative Food Intake

2.4.2.2 Effect on Daily Food Intake

2.4.2.3 Effect on Cumulative Body Weight Change

2.4.3 The Effect of 3 Day Pair Feeding to PYY3-36 on the Subsequent Ad Libitum Food Intake in C57BL/6 Mice

2.4.3.1 Effect on Cumulative Food Intake

2.4.3.2 Effect on Daily Food Intake

2.4.3.3 Effect on body weight

2.4.4 Plasma PYY3-36 levels following 7 day continuous infusion

2.4.4 Effect of 3 day low dose PYY pre-treatment on the effects of subsequent high dose treatment

2.4.4.1 Effect on cumulative food intake
Chapter 3: Investigation of Dual GLP-1/GCG Receptor Agonists on Energy Homeostasis

3.1 Introduction ........................................................................................................... 96
   3.1.1 Glucagon and Energy Homeostasis ............................................................... 96
   3.1.2 Exendin 4......................................................................................................... 98
   3.1.3 GLP-1 and Glucagon receptor co-agonism .................................................. 99
   3.1.4 Design of GLP-1 and Glucagon receptor co-agonists .................................. 100

3.2 Hypothesis and Aims ............................................................................................. 103

3.3 Methods ................................................................................................................... 104
   3.3.1 Peptide Synthesis ........................................................................................... 104
   3.3.2 Receptor Binding Assays (RBAs) ..................................................................... 104
      3.3.2.1 Cell and Tissue Membrane Preparation ................................................ 104
      3.3.2.2 Receptor Binding Assays ........................................................................ 105
   3.3.3 cAMP Accumulation Assay ........................................................................... 106
   3.3.4 In vivo studies .................................................................................................. 107
      3.3.4.1 Animals .................................................................................................... 107
   3.3.4.2 Acute Feeding Studies in Mice ................................................................... 108
   3.3.4.3 Acute Feeding Studies in Rats ................................................................... 108
   3.3.4.4 Pair Feeding ............................................................................................... 108
   3.3.4.5 Pharmacokinetic analysis ......................................................................... 109
   3.3.5 Radioimmunoassay ......................................................................................... 109
   3.3.6 Metabolic Gene Expression Analysis .............................................................. 110
      3.3.6.1 Animals and Tissues ................................................................................ 110
      3.3.6.2 mRNA extraction .................................................................................... 110
      3.3.6.3 RNA Quantification and Assessment of Quality ...................................... 112
      3.3.6.3.1 Spectrophotometry ............................................................................. 112
      3.3.6.3.2 Electrophoresis ................................................................................... 112
      3.3.6.4 Reverse Transcription ............................................................................ 113
      3.3.6.5 Quantitative Polymerase Chain Reaction (qPCR) .................................. 113
   3.3.7 Statistics ......................................................................................................... 114

3.4 RESULTS ................................................................................................................. 116
   3.4.1 Affinity of Glucagon-Ex4 Chimera analogues to the rGLP-1R, hGLP-1R, rGCGR and hGCGR ................................................................. 116
List of Figures

Figure 1.1: Schematic diagram in coronal section showing the neuropeptides expressed in arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN) and the lateral hypothalamus (LH). ............24

Figure 1.2. Peripheral signals regulating appetite. .................................................................32

Figure 1.3. The human amino acid sequences of the members of the human PP-fold family of peptides: NPY, PP and PYY. Numbers at top of diagram denote amino acid position, from position 1 at the N terminal to position 36 at the C-terminal........34

Figure 1.4. PYY concentrations along the human GI tract..........................................................36

Figure 1.5. A diagrammatic representation of the tissue-specific processing of preproglucagon in the pancreas and the CNS/intestine..............................................................40

Figure 1.6. Amino acid sequences of Proglucagon-derived peptides.................................43

Figure 2.1. The proposed mechanism of action of the anorectic effect of PYY3-36.......50

Figure 2.2. A schematic representation of an Alzet osmotic minipump .........................60

Figure 2.3. Timeline of the Investigation into the Anorectic Effect of Continuous administration of PYY 3-36 for 7 days. ..............................................................62

Figure 2.4. Timeline of the Investigation into the Effect of 3 Day Continuous Low Dose Pre-treatment followed by 3 day High Dose Treatment of PYY3-36............................63

Figure 2.5. Timeline of the investigation into the effect of food restriction (FR) on the subsequent anorectic effect of PYY3-36..............................................................64

Figure 2.6. Effect of vehicle control or PYY3-36 10, 100 or 1000 nmol/kg on 24 hour food intake in overnight fasted mice........................................................................71

Figure 2.7. Effect of 7 day continuous infusion of PYY3-36 on cumulative food intake in mice..................................................................................................................73

Figure 2.8. Effect of 7 day continuous infusion of PYY3-36 (375 nmol/kg/day) on daily interval food intake expressed as percent of vehicle controls in mice.................75

Figure 2.9. Effect of 7 day continuous infusion of PYY3-36 (375 nmol/kg/day) on body weight in mice...............................................................................................................76

Figure 2.10. The Effect of 3 Day Pair Feeding to PYY3-36 on the Subsequent Ad Libitum Food Intake in C57BL/6 Mice..............................................................78

Figure 2.11. Effects of pair-feeding a PYY-naive group to the food intake of a group of mice treated with continuous subcutaneous delivery of 375 nmol/kg PYY3-36 followed by ad-libitum feeding....79
Figure 2.12. Cumulative body weight change during pair feeding/food restriction on days 1-3 followed by ad libitum feeding on days 4-7. .......................................................... 80

Figure 2.13. Plasma levels of PYY3-36 immunoreactivity following 7 days of continuous subcutaneous infusion of PYY3-36 (375 nmol/kg/day) or vehicle via Alzet osmotic minipumps .......................................................... 81

Figure 2.14. Effect of continuous infusion of vehicle or low dose (50 and 200 nmol/kg/day) PYY3-36 from day 0-3 and vehicle or high dose (1500 nmol/kg) PYY3-36 from day 3-6 on cumulative food intake .......................................................... 83

Figure 2.15. Effect of continuous infusion of vehicle or low dose (50 and 200 nmol/kg/day) PYY3-36 from day 0-3 and vehicle or high dose (1500 nmol/kg) PYY3-36 from day 3-6 on daily interval food intake represented as daily food intake as percentage of vehicle ......................................................................................... 85

Figure 2.16. Cumulative food intake over 10 day period following PYY3-36 (250 nmol/kg/day) or vehicle infusion via Alzet osmotic minipump in mice ........................................................................ 87

Figure 2.17. Daily interval food intake over 10 day period following PYY3-36 (250 nmol/kg/day) expressed as % of vehicle controls (n=9). ................................................................. 88

Figure 2.18. Cumulative body weight change over 10 day period following PYY3-36 (250 nmol/kg/day) expressed as % of vehicle controls (n=9) ......................................................... 89

Figure 2.19. Quantification of hypothalamic Y2 receptor expression in mice treated with a continuous infusion of saline or 250 nmol/kg/day PYY3-36 after 28 days .......... 90

Figure 3.1. Schematic of proposed mechanism of action of dual agonist ...................... 100

Figure 3.3. Representative binding affinity curves of chimera peptides GX1-GX6 on the rat A) GLP-1R and B) GCGR. ........................................................................................................... 119

Figure 3.4. Representative binding affinity curves of chimera peptides GX1-GX6 on the human A) GLP-1R and B) GCGR. ........................................................................................................... 120

Figure 3.5. Interval food intake (expressed as percentage of saline controls on each study day) following subcutaneous administration of saline, Exendin 4 (50 nmol/kg), GLP-1 (500 nmol/kg), GCG (500 nmol/kg) in mice .................................................................. 122

Figure 3.6. cAMP accumulation in A) human GLP-1R and B) human GCGR expressing HEK 293 cells in response to incubation with GCG, Ex4, GLP-1 and analogues GX1-6. ........................................................................................................... 124

Figure 3.7 Interval food intake (expressed as percentage of saline controls on each study day) following subcutaneous administration of saline and GX1-6 (50 nmol/kg) in C57BL/6 mice .................................................................................................................................................. 126

Figure 3.8. Interval food intake (expressed as percentage of saline controls on each study day) following subcutaneous administration of saline and GX1-6 (50 nmol/kg) in Wistar rats .................................................................................................................................................. 128
Figure 3.9. Plasma concentration-time curve following subcutaneous administration of GX6 at 500 nmol/kg.

Figure 3.10. The effect of daily administration of saline, GX6 (30 nmol/kg) and pair-feeding controls (GX6-PF) on A) cumulative food intake (g) over 10 days in male Wistar rats and B) Final food intake (g) on day 10.

Figure 3.11. The effect of daily administration of GX6 (30 nmol/kg) and pair-feeding controls (GX6-PF) body weight change over 10 days in male Wistar rats A) Cumulative body weight change (g) from initial (g) over 10 days and B) Final body weight change (g) on day 10.

Figure 3.12. The effect of daily administration of GX6 (30 nmol/kg) and Exendin-4 (30 nmol/kg) and pair-feeding (GX6-PF and Ex-4-PF) in male Wistar rats on A) cumulative food intake (g) over 10 days and B) Final food intake (g) on day 10.

Figure 3.13. The effect of daily administration of GX6 (30 nmol/kg) and Exendin 4 (30 nmol/kg) and pair-fed controls (GX6-PF and Ex4-PF) body weight change over 10 days in male Wistar rats.

Figure 3.14. Relative expression levels for Pgc1α, Ucp1, Pparγ compared to saline normalised to internal controls β-actin and β2-myoglobulin.

Figure 3.15. Relative expression levels of liver G6Pase, Pck1a, Pparα, FGF21, PGC1α compared to saline normalised to internal controls β-actin and β2-myoglobulin.

Figure 3.16 Schematic of glucagon and GLP-1 receptor agonism ratios of compounds.

Figure 5.1. Electropherogram and gel-like images generated during the analysis of total RNA extracted from BAT and liver from Wistar rats.

Figure 6.1. Analogues sequences of GX1-GX6 Glucagon 1-16, GLP-1/Exendin 17-30.
List of Tables

**Table 2.1.** Investigation into the Anorectic Effect of Continuous administration of PYY3-36 for 7 days........................................................................................................................................... 61

**Table 2.2.** Investigation into the Effect of 3 Day Continuous Low Dose Pre-treatment followed by 3 day High Dose Treatment of PYY3-36........................................................................................................ 63

**Table 2.3.** Investigation into the Effect of food restriction (FR) on the subsequent anorectic effect of PYY3-36........................................................................................................................................ 64

**Table 3.1.** Summary of binding affinities of Glucagon-Exendin4 chimeras with substitutions at positions 17, 20, 24 and 27 to the rat and human GLP-1R and GCGR. ........................................................................................................................................ 118

**Table 3.2.** Summary of EC50 values of Glucagon-Exendin4 chimeras with substitutions at positions 17, 20, 24 and 27 at the rat and human GLP-1R and GCGR.................. 123

**Table 5.1.** Table outlining the general structure of an RIA ................................................. 155
Abbreviations Used in this Thesis

AcN  Acetonitrile
ACTH  Adreno-corticotrophic hormone
AgRP  Agouti related protein
AP  Area postrema
ARC  Arcuate nucleus
BAT  Brown adipose tissue
BBB  Blood brain barrier
BDNF  Brain derived neurotrophic factor
BMI  Body mass index
BSA  Bovine serum albumin
cAMP  Cyclic adenosine monophosphate
CART  Cocaine and amphetamine-regulated transcript
CCK  Cholecystokinin
CNS  Central nervous system
CRH  Corticotrophin-releasing hormone
CSF  Cerebrospinal fluid
CTA  Conditioned taste aversion
DMN  Dorsomedial hypothalamus
DPP-IV  Dipeptidyl peptidase-IV
EDTA  Ethylene diamine tetra-acetic acid
Ex-4  Exendin-4
Ex (9-39)  Exendin (9-39)
FGF21  Fibroblast growth factor 21
G6Pase  Glucose-6-phosphatase
GCG  Glucagon
GCGR  Glucagon receptor
GI  Gastrointestinal
GLP-1  Glucagon-like peptide-1
GLP-1R  Glucagon-like peptide-1 receptor
GPCR  G-protein coupled receptor
GTT  Glucose tolerance test
GX  Glucagon-Exendin-4 chimera
HEK  Human embryonic kidney
HPA  Hypothalmo-pituitary-adrenal
HPLC  High performance liquid chromatography
HPT  Hypothalmo-pituitary-thyroid
ICV  Intracerebroventricular
i.p.  Intraperitoneal
i.p.GTT  Intraperitoneal glucose tolerance test
i.v.  Intravenous
IR  Immunoreactivity
KO  Knock-out
LepR  Leptin receptor
LH  Lateral hypothalamus
MCH  Melanin-concentrating hormone
MCR  Melanocortin receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>Median eminence</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>OXM</td>
<td>Oxyntomodulin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pck1a</td>
<td>Phosphoenolpyruvate carboxykinase 1a</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>Peroxisome proliferating associated receptor-gamma coactivator 1 alpha</td>
</tr>
<tr>
<td>POA</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopio-melanocortin</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>Pparγ</td>
<td>Peroxisome proliferating associated receptor-gamma</td>
</tr>
<tr>
<td>Pparα</td>
<td>Peroxisome proliferating associated receptor-alpha</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RQ</td>
<td>Respiratory Quotient</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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Chapter 1:
General Introduction
1.1 Obesity

Obesity is a pandemic in the modern developed world presenting a substantial economic and social burden. Worldwide, there are 300 million obese humans, and projections suggest that more than half of the world’s population will be overweight or obese by 2015 (World Health Organisation, 2006). Being either overweight, which is defined as body mass index (BMI) of 25.0-29.9 kg/m$^2$, or obese, defined as BMI of 30 kg/m$^2$ or greater, substantially increases the risk of debilitating conditions. These include type 2 diabetes, hypertension, stroke, cardiovascular disease, respiratory problems, gallbladder disease, osteoarthritis and sleep apnoea, as well as certain cancers ((Kopelman, 2000; Calle et al., 1999).

Ultimately, obesity results from an imbalance of energy homeostasis, in which energy intake exceeds energy output. Energy intake is driven by appetite and the energy density of the diet while energy expenditure is dependent on resting metabolic rate and physical activity. While the aetiology of obesity is multi-factorial, it is ultimately a result of an interaction of environmental factors and genes which optimise energy conservation that were favoured for millions of years of relative famine (Ravussin and Bogardus, 2000; Weinsier et al., 1998). The disparity in the conditions under which these genetic systems evolved and the current environment of highly palatable and readily available food and the low requirement for physical activity promotes obesity. It is thought that the obesity-promoting ‘Western’ diet, which has resulted in widespread availability of calorie-dense, palatable food, is fuelling the obesity pandemic (Kopelman, 2000).

Lifestyle changes to reduce energy intake and increase energy output can promote weight loss, but there is resistance to broad adoption by many populations and promotion of such lifestyle changes has had little impact on the rising levels of obesity (Rennie and Jebb, 2005). There is thus an urgent requirement for the scientific community to elucidate the physiology of appetite and energy homeostasis, in order to facilitate the development of anti-obesity therapies. Such therapies would ideally be able to restore appetite control and reduce body weight through the use of specific endogenous satiety signals, rather than by acting on the ubiquitous, non-specific neurotransmitter
systems that many previous anti-obesity drugs acted upon (Padwal and Majumdar, 2007a). In recent years a number of scientific advances in our understanding of the regulation of appetite and energy expenditure have been made (Padwal et al., 2004). Identifying physiological drug targets and conducting physiological and pharmacological investigations into these systems will aid the design and development of effective therapeutic interventions for obesity.

1.2 Energy Homeostasis

Despite the increased incidence of obesity in the population, within the individual, energy balance is tightly regulated. For most people, the amount and composition of food eaten varies considerably from meal to meal and from day to day, but over time energy intake is matched to energy expenditure and body weight is tightly conserved (Schwartz et al., 2000). This involves a complex regulatory system comprising afferent and efferent signals. Afferent signals include peripheral signals of energy storage which mainly originate from adipose tissue and signals of satiety which primarily arise from the gut. The efferent signals regulating food intake include hypothalamic neural circuits which influence higher brain centres downstream.

Energy may be expended by physical activity or by thermogenesis, the production of heat. Thermogenesis can be further classified into obligatory thermogenesis, which consists of the basal heat produced by cellular and organ functions required for survival (Argyropoulos and Harper, 2002b) and adaptive thermogenesis, which is the additional production of heat in response to temperature or diet (Silva, 2001b; Lowell and Spiegelman, 2000a). The hypothalamic-pituitary-thyroid (HPT) axis and the sympathetic nervous system (SNS) are both efferent signals in the regulation of energy expenditure.

Thus, energy homeostasis is regulated by a combination of factors controlling both food intake and energy expenditure.
1.3 Energy expenditure

Energy homeostasis is a balance between energy intake and energy expenditure. Energy may be expended by physical activity or by the production of heat (thermogenesis). Thermogenesis can be further classified into obligatory thermogenesis, which consists of the basal heat produced by cellular and organ functions required for survival (Argyropoulos and Harper, 2002a), and adaptive thermogenesis, which is the additional production of heat in response to temperature or diet (Silva, 2001a; Lowell and Spiegelman, 2000b). The hypothalamic-pituitary-thyroid (HPT) axis and the sympathetic nervous system (SNS) are both involved in the regulation of energy expenditure.

1.3.1 Brown Adipose Tissue and Adaptive Thermogenesis

Brown adipose tissue (BAT) is a major site for adaptive thermogenesis. BAT consists of brown adipocytes which contain triglyceride droplets and many large mitochondria packed with cristae that contain uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 2004; Sell et al., 2004).

BAT depots are found in the interscapular, subscapular, axillary, intercostals, perirenal and periaortic regions in small mammals, and human and other large mammal newborns (Sell et al., 2004). Recent evidence suggests that BAT also plays a role in energy homeostasis in human adults (Cypess et al., 2009; Elabd et al., 2009; Lee et al., 2011). In response to cold exposure or the ingestion of high energy diets, thermogenesis is stimulated in BAT tissue (Rothwell and Stock, 1983). BAT thermogenesis is mediated by the SNS and by thyroid hormone stimulation. The SNS abundantly innervates BAT (Nnodim and Lever, 1988) and thyroid hormone receptors are highly expressed in this tissue (Bianco and Silva, 1987). Noradrenaline (NA) is released from SNS nerve endings and binds to α- and β-adrenergic receptors found on brown adipocytes, activating UCP1 gene expression to stimulate thermogenesis (Cannon and Nedergaard, 2004; Silva and Rabelo, 1997). NA binding to the α-adrenergic receptors also promotes the production of the thyroid hormone T₃ (Silva and Larsen,
1983) T₃ also has a stimulatory effect on UCP1 gene expression and acts synergistically with NA to increase thermogenesis in BAT (Silva and Rabelo, 1997).

The UCP1 proteins are located in the inner membrane of the mitochondria and play a central role in the thermogenesis (Nicholls and Locke, 1984). During oxidative-phosphorylation, the oxidation of fuel molecules such as glucose or free fatty acids generates the electron donors, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). The movement of electrons down the respiratory chain is associated with the pumping of protons from the mitochondrial matrix into the intermembrane space. This results in the formation of a proton gradient across the membrane, which in most tissues is used to drive the conversion of ADP to ATP, via ATP synthase. In brown adipocytes, however, UCP1 allows the leakage of protons back into the mitochondrial matrix, thereby uncoupling oxidative phosphorylation from ATP synthesis and causing the dissipation of energy as heat (Locke et al., 1982).

1.4 Central Control of Food Intake

The major areas of the central nervous system (CNS) regulating energy homeostasis are the hypothalamus and brainstem. These respond to humoral and neuronal signals arising from the periphery and inputs from the higher areas of the brain to provide co-ordinated regulation of food intake and energy expenditure.

1.4.1 The Hypothalamus

The hypothalamus is a brain area which occupies the ventral half of the diencephalon on both sides of the third ventricle and lies immediately above the pituitary gland. It is involved in the regulation of multiple homeostatic systems, including reproduction, thirst and temperature control, and is essential in the regulation of food intake and energy expenditure. The hypothalamus has extensive connections
with the major ascending and descending fibre systems which allow it to sense and influence activity in all major parts of the brain and spinal cord (Berthoud, 2002b). The hypothalamus is comprised of a number of discrete nuclei which contain distinct neuronal populations (Figure 1.1).

The hypothalamus was first implicated in the regulation of food intake and body weight in 1940 by experiments performed by Hetherington and Ranson. They introduced bilateral lesions in regions of the rat hypothalamus including the dorsomedial, ventromedial and arcuate nuclei. These rats developed obesity, doubling in body weight compared to control rats (Hetherington and Ranson, 1940; Hetherington and Ranson, 1940). Anand and Brobeck subsequently demonstrated that lesions in the rat lateral hypothalamic area led to decreased food intake and body weight and eventually to death by starvation (Anand and Brobeck, 1951; Elmquist et al., 1999b; Elmquist et al., 1998b). Thus, the hypothalamic ‘dual centre’ model was proposed, in which the ventromedial nucleus served as a ‘satiety centre’ and the lateral hypothalamic area served as the ‘feeding centre’.

However, over the subsequent years, several groups using refined experimental methodologies have shown that the hypothalamic control of appetite is far more complex than the ‘dual centre’ model. Energy homeostasis is controlled by several nuclei of the hypothalamus and brainstem. Signalling via specific neuropeptides allows communication between these regions to produce a coordinated regulation of energy homeostasis. Specific nuclei receive and integrate information about the body’s energy state and adiposity via blood-borne peripheral signals in order to maintain the regulation of energy intake and expenditure.

1.4.1.1 The Arcuate Nucleus (ARC)

The ARC is an arc-shaped, elongated collection of neuronal cell bodies, situated at the base of the hypothalamus on either side of the third cerebral ventricle. The ARC plays an integrative role in the regulation of energy balance. Both anorectic neuropeptides (which inhibit food intake) and orexigenic
neuropeptides (which stimulate food intake) are highly expressed in the ARC, as are receptors for many hormones and neuropeptides involved in the regulation of energy homeostasis.

Two neuronal populations in the ARC are of particular importance in energy homeostasis. The first consists of neurons containing the orexigenic peptides neuropeptide Y (NPY) and agouti related protein (AgRP) (Hahn et al., 1998) and the other population consists of neurons containing the anorectic peptides alpha-melanocyte stimulating hormone (α-MSH, derived from the cleavage of pro-opiomelanocortin (POMC)) and cocaine- and amphetamine- regulated transcript (CART) (Elias et al., 1998b; Jacobowitz and O'Donohue, 1978; Koylu et al., 1997). ARC neurons project to other hypothalamic nuclei that control energy homeostasis, including the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN) and lateral hypothalamic area (LHA) (Elias et al., 1998a; Elmquist et al., 1998a; Fekete et al., 2000; Cowley et al., 1999; Kalra et al., 1999a).

The brain and spinal cord are isolated by the blood-brain barrier (BBB), an interface situated between the blood stream and the CNS extracellular fluid that acts to limit and restrict the access of drugs, peptide hormones, antibodies, metabolites and exogenous drugs or toxins into the brain (Pardridge, 2001; Lee et al., 2001; Smith, 2000; Schinkel et al., 1994; Mann et al., 2003). There are, however, specific sites, called circumventricular organs, at which the BBB is modified to allow the access of signalling molecules with the bloodstream. One such circumventricular organ is the median eminence (ME), where the hypothalamus secretes pituitary releasing hormones into the hypophysial portal circulation. The ARC is in direct contact with the ME also allowing it to sense and integrate peripheral signals to regulate energy homeostasis (Duvernoy and Risold, 2007).
1.4.1.2 The Paraventricular Nucleus (PVN)

The PVN is richly supplied with neuronal projections from several CNS regions including the ARC, LHA and brainstem (Sawchenko and Swanson, 1983). Microinjection into the PVN of almost all known orexigenic peptides including NPY (Lambert et al., 1995; Stanley and Leibowitz, 1985), ghrelin (Lawrence et al., 2002a) and orexin-A (Edwards et al., 1999; Shirasaka et al., 2001) stimulate feeding, while microinjection of the anorectic signals leptin (Elmquist et al., 1997a), glucagon-like peptide-1 (GLP-1) (McMahon and Wellman, 1998) and cholecystokinin (CCK) (Hamamura et al., 1991) inhibit feeding. The PVN is also involved in the regulation of the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-adrenal (HPA) axes (Perello et al., 2010).

1.4.1.3 The Ventromedial Nucleus (VMN)

The VMN is thought to act generally as a satiety centre as bilateral VMN lesions result in hyperphagia and obesity (Brobeck, 1946). The VMN receives afferent neuronal projections from other hypothalamic nuclei involved in appetite regulation, including the ARC, LHA, DMN and PVN (Ter Horst and Luiten, 1987a) as well as from the nucleus of tractus solitarius (NTS) of the brainstem (Berthoud, 2002a). It also has efferent neuronal projections to the LHA and DMN (Ter Horst and Luiten, 1987b). The growth factor brain-derived neurotrophic factor (BDNF) is highly expressed in the VMN and believed to signal downstream of the melanocortin system to reduce food intake (Xu et al., 2003a). Food deprivation in rodents significantly decreases BDNF mRNA expression in the VMN while intracerebroventricular (ICV) administration of a melanocortin 4 receptor (MC4R) agonist significantly increases BDNF expression (Xu et al., 2003b). Mice heterozygous for the BDNF gene (Kernie et al., 2000) or with BDNF expression eliminated from the brain (Rios et al., 2001) are hyperphagic and develop obesity. Evidence also suggests that lower circulating levels of BDNF in humans results in childhood-onset obesity (Han et al., 2008).
Figure 1.1: Schematic diagram in coronal section showing the neuropeptides expressed in arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN) and the lateral hypothalamus (LH). The ARC is ideally positioned over the median eminence (ME) which allows the detection and integration of peripheral signals. Neuronal connections are present from the ARC to all the other hypothalamic nuclei (i.e. VMN, DMN, PVN and LHA) and within these nuclei to co-ordinate and control energy homeostasis. BBB: blood-brain barrier; αMSH: alpha-melanocyte stimulating hormone; CART: cocaine- and amphetamine-regulated transcript; AgRP: agouti-related peptide; NPY: neuropeptide Y; BDNF: brain-derived neurotrophic factor; NTS: nucleus tractus solitarius; AP: area postrema. Figure adapted from (Simpson et al., 2009).

1.4.1.4 The Dorsomedial Nucleus (DMN)

Destruction of the DMN results in hyperphagia and obesity, although, to a lesser extent than that seen following lesioning of the VMN (Bernardis and Bellinger, 1998). The DMN contains high levels of NPY immunoreactivity (Chronwall et al., 1985) and intra-nuclear injection of NPY and other factors including the orexigenic peptides galanin (Kyrkouli et al., 1990) and γ-aminobutyric acid (GABA) (Kelly et al., 1979) into the DMN increases food intake. Chronic food restriction in rats results in an increase in NPY gene expression in the DMN (Bi et al., 2003). The DMN interacts with other
hypothalamic nuclei, receiving projections from the VMN and LHA and projecting to the PVN (Elmquist, 1998; Kalra et al., 1999b).

1.4.1.5 The Lateral Hypothalamus (LH)

Introduction of discrete lesions in the LHA suppresses food intake and results in a dramatic loss of body weight in rodents (Leibowitz et al., 1981; Aravich and Sclafani, 1983). Conversely, electrical stimulation of the LHA increases food intake in rats (Mogenson and Wu, 1982). The LHA contains neurons expressing the orexigenic peptides melanin-concentrating hormone (MCH) (Bittencourt et al., 1992) and orexin A and B (Sakurai et al., 1998). Furthermore, the large number of glucose-sensing neurons in the LHA detect low plasma glucose levels to elicit a strong hyperphagic response (Bernardis and Bellinger, 1996). The LHA is therefore believed to increase food intake when plasma glucose levels are low.

1.4.2 The Brainstem

The brainstem is the stem-like part of the brain connected to the spinal cord. The brainstem integrates signals from the periphery and interacts with other CNS regions to regulate energy homeostasis. The area postrema (AP) is a circumventricular organ that lies within the brainstem in close anatomical proximity to the nucleus tractus solitarius (NTS) (Brightman and Broadwell, 1976). The relatively permeable nature of the BBB at the AP allows the brainstem to detect and respond to peripheral circulating signals (Sawchenko, 1983). The brainstem then conveys these signals to other regions of the CNS to elicit appropriate responses. Extensive reciprocal connections exist between the hypothalamus and the brainstem, particularly the NTS (Ter Horst et al., 1989; van der Kooy et al., 1984; Ricardo and Koh, 1978). The brainstem is involved in mediating the effects of the gut hormones ghrelin and GLP-1 on feeding (Williams et al., 2003a; Grill et al., 2004; Faulconbridge et
The NTS expresses NPY and high levels of the NPY receptors Y1R, Y2R and Y5R (Glass et al., 2002; Dumont et al., 1998). NPY release from the NTS is dependent on nutritional status (Yoshihara et al., 1996) suggesting a physiological role for brainstem NPY in energy homeostasis. NPY-expressing neurons also project from the NTS to the hypothalamic PVN (Sawchenko et al., 1985). POMC-expressing neurons and the MC4R are also found in the NTS (Mountjoy et al., 1994; Bronstein et al., 1992; Fodor et al., 1996; Kawai et al., 1984) POMC expression in the NTS is stimulated by feeding (Fan et al., 2004), again suggesting a physiological role in energy homeostasis.

The brainstem also receives information about the mechanical or chemical stimulation of the GI tract via afferent fibres of the abdominal vagus nerve (Kalia and Sullivan, 1982; Schwartz, 2000b). Gastric loads stimulate the vagal fibres (Schwartz et al., 1991; Mathis et al., 1998) and are believed to cause suppression of feeding independent of the content of the food ingested (Phillips and Powley, 1996). The vagus nerve is also important in gut hormone signalling. Receptors for many appetite regulating gastrointestinal (GI) hormones are expressed on the vagus nerve (Moran and Kinzig, 2004b; Date et al., 2002b) and vagotomy abolishes or attenuates the actions of the anorectic signals cholecystokinin (CCK) (Moran and Kinzig, 2004a), peptide YY (PYY) (Koda et al., 2005c; Abbott et al., 2005c), GLP-1 (Abbott et al., 2005b) and pancreatic polypeptide (PP) (Asakawa et al., 2003c) on feeding. The brainstem thus integrates peripheral hormonal and vagal signals and relays these messages to the hypothalamus.

1.5 Peripheral factors in the regulation of Food Intake

Energy homeostasis is regulated by the interaction of peripheral signals with hypothalamic and brainstem feeding circuits. These peripheral signals originate from adipose tissue, the pancreas and GI tract, and provide information on the body’s nutritional status to regulate energy balance (Fig. 1.2). All of the known peripheral signals have anorectic effects with the exception of the gastric hormone
ghrelin. Some peripheral signals, including leptin and insulin, also relay information to the brain regarding long-term energy stores.

1.5.1 Adipostat Factors

1.5.1.1 Insulin

In addition to its primary role in regulating glucose homeostasis, insulin is also a signal of body adiposity (Schwartz et al., 1992a). Insulin is secreted rapidly after a meal by the β cells of pancreatic islets of Langerhans (Ashcroft, et al, 1989). Circulating levels are proportional to body fat content. (Polonsky et al. 1988). This is because the levels of insulin secreted are dependent on peripheral insulin sensitivity which is related to both total body fat and to fat distribution, with visceral fat being a key determinant (Porte, Jr. et al., 2002).

Insulin crosses the BBB from the circulation via a receptor-mediated transport mechanism (Baura et al., 1993). ICV administration of insulin reduces food intake and body weight in rodents and primates (McGowan et al., 1990; Woods et al., 1979). The insulin receptor is expressed in several nuclei involved in energy homeostasis, including the ARC, where it is expressed on POMC neurons (Benoit et al., 2002a), the DMN and PVN (Corp et al., 1986; Marks et al., 1990). ICV administration of insulin increases hypothalamic POMC mRNA expression and prevents the fasting-induced increase in NPY mRNA expression in the ARC in food deprived rats (Air et al., 2002; Schwartz et al., 1992b). The anorectic effects of ICV insulin are blocked by a melanocortin antagonist (Benoit et al., 2002b). Furthermore, POMC expression is markedly reduced in insulin-deficient diabetic rats (Havel et al., 2000), while NPY expression is increased and returns to near control levels following insulin replacement (Sipols et al., 1995; White et al., 1990; Williams et al., 1989). The effects of insulin, therefore, appear to be brought about via the modulation of both the NPY and melanocortin systems. Studies of peripheral insulin administration are complicated by resultant hypoglycaemia, which in
itself stimulates food intake. However, hyperinsulinaemic, euglycaemic clamp studies have demonstrated an anorectic effect of circulating insulin in both rodents and baboons (Woods et al., 1984; Nicolaidis and Rowland, 1976).

### 1.5.1.2 Leptin

Leptin is a hormone released from adipocytes in white adipose tissue and which acts as a signal of long-term energy stores (Kolaczynski et al., 1996a). Leptin circulates at levels proportional to body adiposity (Considine et al., 1996b), as obese rodents and humans exhibit high levels of circulating plasma leptin (Maffei et al., 1995b). Plasma leptin levels also reflect short term feeding status; leptin levels fall dramatically with fasting and increase within hours after a meal in rodents (Harris et al., 1996; Maffei et al., 1995a; Ahima et al., 2000), and after several days of overeating in humans (Kolaczynski et al., 1996b). The decrease in leptin levels following a fast occurs much faster than would be expected from a decrease in body weight. It has therefore been suggested that the transient decrease in leptin levels following a fast may act as a signal to stabilise body weight, by promoting feeding and reducing energy expenditure, before energy stores become substantially depleted (Havel, 2001).

Leptin signals via the long form of the leptin receptor, Ob-Rb, which is expressed widely within the hypothalamus but is found particularly in the ARC, VMH, DMH, LHA and medial preoptic area (MPOA) (Fei et al., 1997; Elmquist et al., 1999a) Ob-Rb mRNA is also expressed in appetite-modulating pathways of the brainstem (Mercer et al., 1998). Peripheral leptin administration alters neuronal activity in these hypothalamic and brainstem regions (Elmquist et al., 1997b); (Hosoi et al., 2002). In the ARC, Ob-Rb is expressed on NPY (Baskin et al., 1999), AgRP (Wilson et al., 1999), POMC (Cheung et al., 1997) and CART (Kristensen et al., 1998) expressing neurons. Circulating leptin crosses the BBB by a saturable transport system and reduces the activity of several orexigenic peptides, including AgRP and NPY, while increasing the release of the anorectic peptides α-MSH, CART and CRH (Schwartz, 2000a; Morton et al., 2006; Cowley et al., 2001). Microinjection of leptin
into the ARC, VMH and LHA decreases food intake in rats, with the intra-ARC injection of leptin having the most profound effect (Satoh et al., 1997b; Satoh et al., 1997a). The ob/ob mouse, which lacks functional leptin, and the db/db mouse model, which lacks functional leptin receptors, are hyperinsulinaemic, hyperphagic and very obese (Zhang et al., 1994; Tartaglia et al., 1995; Coleman, 1973; Coleman and Hummel, 1969). Peripheral or ICV administration of leptin to ob/ob mice reduces food intake and body weight, and increases energy expenditure (Pellemounter et al., 1995; Campfield et al., 1995; Halaas et al., 1995).

Although a small subset of obese human subjects have a relative leptin deficiency, the majority of obese animals and humans have a proportionally high circulating leptin (Considine et al., 1996a; Maffei et al., 1995c). This suggests resistance to the actions of leptin. Indeed, recombinant leptin administered subcutaneously to obese human subjects has only a modest effect on bodyweight (Fogteloo et al., 2003; Heymsfield et al., 1999). Leptin resistance appears to be the result of a combination of factors, including reduced transport across the blood-brain barrier (Schwartz, 2000c; Van et al., 1996; Banks et al., 1999) and reduced leptin signalling in leptin-sensitive hypothalamic neurons (Sahu et al., 2002; El-Haschimi et al., 2000). Although leptin deficiency has profound effects on body weight, the effect of high leptin levels seen in obesity and the therapeutic administration of leptin are much less potent at restoring body weight. It has thus been proposed that the role of leptin may be to signal starvation rather than excess food intake.

### 1.5.2 Peripheral Signals from the Gastrointestinal (GI) Tract

#### 1.5.2.1 Ghrelin

Ghrelin is a potent orexigenic peptide hormone secreted primarily by the stomach, but also produced at low levels by the duodenum, ileum, caecum and colon (Date et al., 2000; Sakata et al., 2002). Circulating ghrelin exists in two major forms: bioactive n-octanoyl modified ghrelin and the non-modified form, des-n-octanoyl ghrelin (Hosoda et al., 2000). The octanoylated form of ghrelin binds
and activates the growth hormone secretagogue receptor (GHS-R) and triggers the release of growth hormone (GH). Ghrelin also stimulates food intake via GHS-R (Kojima et al., 1999).

Central and peripheral administration of ghrelin increases food intake and body weight gain in rodents (Wren et al., 2001b; Tschop et al., 2000) and central administration of anti-ghrelin antibodies attenuates re-feeding in food deprived animals (Nakazato et al., 2001a). Ghrelin infusion in healthy human volunteers increases in food intake as well as hunger scores (Wren et al., 2001a). Diurnal variations in ghrelin levels are seen in humans and rodents. Basal levels are highest during the morning and lowest at night in man (Cummings et al., 2001b) and peak at the end of the light and dark periods in rodents, coinciding with the periods of highest food intake in rodents (Murakami et al., 2002). Ghrelin levels increase on fasting and fall on re-feeding, and sharp peaks are observed before a meal, suggesting a role for ghrelin in meal initiation (Cummings et al., 2001a). One mechanism by which ghrelin stimulates food intake is via the modulation of energy homeostasis pathways in the hypothalamus. In rats in which the ARC has been ablated, peripheral administration of ghrelin does not increase food intake (Tamura et al., 2002b). Peripheral administration of ghrelin activates NPY neurons of the ARC (Tamura et al., 2002a) and NPY/AgRP double KO mice do not respond to ghrelin administration (Chen et al., 2004). Evidence also suggests that the brainstem is involved in the actions of ghrelin. Central administration of ghrelin activates neurons in the NTS and AP (Lawrence et al., 2002b; Nakazato et al., 2001b). The GHS-R is expressed on the vagus nerve and vagotomy abolishes the orexigenic effects of ghrelin in rats (Date et al., 2002a; Williams et al., 2003b) and in humans (Le Roux et al., 2005).

Circulating ghrelin levels may also be reflective of long-term energy stores, showing an inverse correlation with adiposity in humans (Otto et al., 2001; Cummings et al., 2002). Peripheral administration of ghrelin in rats has been shown to promote adipogenesis (Thompson et al., 2004; Tsubone et al., 2005) and inhibit lipid oxidation (Theander-Carrillo et al., 2006). Endogenous ghrelin is therefore thought to act as a peripheral orexigenic and adiposity signal.
1.5.2.2 *Cholecystokinin*

Cholecystokinin (CCK) was the first gut hormone shown to inhibit food intake following exogenous peripheral administration in rodents (Gibbs et al., 1973) and has since been shown to inhibit food intake in man (Kissileff et al., 1981). CCK is a peptide hormone widely distributed in the GI tract (Larsson and Rehfeld, 1978), but primarily released from the mucosal endocrine I-cells of the upper small intestine (Polak et al., 1975). After a meal, CCK is released locally and into the circulation in which levels remain elevated for up to five hours (Liddle et al., 1985).

The long term effects of CCK on food intake and body weight are unclear. In animals, chronic pre-prandial administration of CCK does reduce food intake, but results in a compensatory increase in meal frequency, which prevents any resultant effect on body weight (West et al., 1984; West et al., 1987). Continuous infusion of CCK becomes ineffective after the first 24 hours (Crawley and Beinfeld, 1983). Rats which lack CCK\(\alpha\) receptors are hyperphagic and obese (Moran et al., 1998; Schwartz et al., 1999) and chronic administration of CCK antibodies or CCK\(\alpha\) antagonists results in weight gain in rodent models, although without a significant increase in food intake (McLaughlin et al., 1985; Meereis-Schwanke et al., 1998). However, CCK\(\alpha\) receptor knock-out mice are not obese (Lo et al., 2010). The long-term effects of CCK on body weight may partially result from an interaction with signals of adiposity such as leptin, which enhance the satiating effect of CCK (Matson et al., 2000). The evidence for a role of CCK in long-term body-weight regulation, and hence as a potential therapy for obesity, remains elusive.
Figure 1.2. Peripheral signals regulating appetite. Appetite is controlled by peripheral signals from adipose tissue, pancreas and the gastrointestinal tract. Peripheral signals from the gut include peptide tyrosine-tyrosine (PYY), oxyntomodulin (OXM), ghrelin, pancreatic polypeptide (PP), glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK). These gut-derived peptide and adiposity signals influence central circuits in the hypothalamus and brainstem in order to produce a negative or positive energy balance. (Adapted from Wynne, et al, 2005)
1.5.2.3 *Pancreatic Polypeptide (PP)-fold family*

The PP-fold family consists of neuropeptide Y (NPY), and the gut hormones pancreatic polypeptide (PP) and peptide tyrosine-tyrosine (PYY) (See Figure 1.3 for sequences). Each of these peptides is thought to have a distinct role in appetite regulation. The common structure shared by the PP hormones is a characteristic U-shaped fold in their tertiary structure, which is necessary for receptor binding (Glover et al., 1983). Peptides of the PP-fold family mediate their effects via the Y-receptors, a superfamily of G-protein-coupled receptors which include Y1R, Y2R, Y4R, Y5R and Y6R.

1.5.2.3.1 Pancreatic Polypeptide

Pancreatic polypeptide (PP) is produced primarily in the PP cells in the islets of Langerhans of the pancreas, but also in the colon and rectum (Larsson et al., 1975). It is released following a meal in proportion to the calorie intake with levels remaining elevated for up to 6 hours post-prandially (Adrian et al., 1976) and has potent anorectic effects.

Peripheral administration of PP acutely decreases food intake and increases energy expenditure in rodents (Asakawa et al., 2003b). Repeated administration of PP over six days reduces body weight gain and ameliorates insulin resistance in *ob/ob* mice (Asakawa et al., 2003a; Malaisse-Lagae et al., 1977). PP over-expressing mice have a reduced daily food intake and exhibit a lean phenotype (Ueno et al., 1999). PP reduces food intake and hunger scores over a 24 hour period following i.v. infusion in healthy human volunteers (Jesudason et al., 2007; Batterham et al., 2003e). However, the effect of PP on food intake in obese subjects has yet to be investigated.

PP binds to all the PP-fold receptors but exhibits the highest affinity for the Y4R (Larhammar, 1996). The Y4R has been shown to be expressed in the ARC, PVN, DMN and VMN of the hypothalamus and in the AP of the brainstem (Parker and Herzog, 1999b). The anorectic effects of peripherally administered PP are abolished in Y4R KO mice (Batterham et al., 2009), suggesting that the Y4R is mediating the effects of PP on energy homeostasis.
**Figure 1.3.** The human amino acid sequences of the members of the human PP-fold family of peptides: NPY, PP and PYY. Numbers at top of diagram denote amino acid position, from position 1 at the N terminal to position 36 at the C-terminal.
1.5.2.3.2 Peptide YY

Peptide YY (PYY) is a 46 amino acid peptide, named for the presence of a tyrosine residue at each terminal. PYY was first isolated from the porcine intestine in 1980 (Tatemoto and Mutt, 1980). PYY is a 36 amino acid peptide hormone postprandially secreted from L cells of the gastrointestinal tract in proportion to food intake. As well as acting as a potent peripheral satiety signal, PYY has direct effects on the digestive system. Endogenous PYY exists in two major forms: full length PYY1-36 and the truncated form PYY3-36. PYY3-36 is believed to be the major circulating form (Grandt et al., 1994; Batterham et al., 2006b) and is formed by the removal of the N-terminal tyrosine-proline dipeptide by the ubiquitously expressed enzyme dipeptidyl peptidase IV (DPP-IV) (Mentlein et al., 1993). While full length PYY1-36 has a high affinity to the Y1R, Y2R and Y5R, PYY3-36 shows a relatively selective affinity to the Y2R (Michel et al., 1998).

PYY is located primarily within the enteroendocrine cells of the pancreas and GI tract (Ekblad and Sundler, 2002; Rindi et al., 2004). A gradual increase in PYY levels is observed distally along the gut with lowest levels found proximally in the stomach and the highest concentrations found in the colonic mucosa and rectum (Figure 1.4) (Adrian et al., 1982; Ferri et al., 1988b). PYY is also found in the CNS, where immunoreactivity has been detected in the medulla oblongata, nucleus reticularis, dorsal medulla, NTS, hypothalamus, pons, medulla and spinal cord, and in the peripheral nervous system (PNS), where it is expressed in enteric neurons (Ekblad and Sundler, 2002; Pieribone et al., 1992).
Figure 1.4. PYY concentrations along the human GI tract. PYY concentrations (pmol/g of tissue) obtained from (Adrian et al., 1982; Ferri et al., 1988a)

Circulating levels of PYY increase after the ingestion of food, peaking 1-2 hours after a meal, and remaining at a plateau level for around 6 hours (Adrian et al., 1985b). The enteroendocrine L-cells are thus thought to sense the presence of glucose, lipids, short-chain fatty acids, amino acids, gastric acid and bile acids into the small bowel and colon and to release PYY in response (Onaga et al., 2002; Ballantyne, 2006). However, fat and protein seem particularly powerful stimuli. The amount of PYY secreted by L-cells has been shown to rise in proportion to an increased ratio of fat to carbohydrate and protein in a meal (Batterham et al., 2006c). Unlike other gut hormones, PYY is not released in response to gastric distension (Pedersen-Bjergaard et al., 1996).

Evidence suggests that the majority of circulating PYY is released from the distal small intestine and/or large intestine. Ileocoloectomy has been found to abolish the release of PYY in response to the administration of a fatty acid into the duodenum, and basal PYY levels are low in patients who have undergone colonic resection and ileostomy (Oh et al., 2005). However, since a meal given
intruduodenally increases circulating PYY before nutrients are able to reach the L-cells of the distal gastrointestinal tract, a neuro-hormonal response has been proposed to underlie its immediate release. The vagus nerve complex may mediate this proposed neural regulation, while regulatory gut peptides such as vasoactive intestinal peptide (VIP), CCK, gastrin and GLP-1 have been suggested as humoral mediators of release (Greeley, Jr. et al., 1989; Ballantyne, 2006; Naslund et al., 1999; McGowan and Bloom, 2004). A considerable number of gastrointestinal diseases associated with appetite loss are characterized by elevated PYY levels, which may indicate a protective role for this peptide in reducing food intake during diseased states (Liu et al., 1996).

PYY may be involved in the pathogenesis of obesity. One study has reported that obese patients exhibit significantly lower levels of PYY compared to people with a normal BMI, though this has not been confirmed by other studies (Batterham et al., 2003d). Another group found that PYY plasma levels were similar between normal-weight and obese patients using the same assay, but that obese patients did show blunted postprandial PYY3-36 plasma levels (Stock et al., 2005). Interestingly postprandial PYY levels are elevated in obese patients that have undergone Roux-en-Y gastric bypass surgery (Le Roux et al., 2006). It has been proposed that abnormal delivery of undigested fats to the small bowel, common in Roux-en-Y and gastrointestinal disease, may be responsible for this increase in PYY release. Such as response may result in heightened anorectic signalling during diseased states when this response may be advantageous (Korner et al., 2005; Vincent and Le Roux, 2008).

Since the 1980s, endogenous PYY has been known to play a fundamental role in gastric motility and secretion (Pappas et al., 1985a). PYY is a mediator of the ‘ileal brake’, the slowing of gastric emptying in response to nutrients in the distal small intestine (Spiller et al., 1988). PYY also inhibits glucose-induced insulin secretion, and gastric and pancreatic secretion, reduces gallbladder emptying, enhances vasoconstriction in the GI tract and pancreas, and augments salt and water absorption in the colon (Adrian et al., 1985; Bottcher et al., 1989). At supra-physiological doses, PYY3-36 was recently found to inhibit the diarrhoea induced by prostaglandin-E2 through the reduction of intestinal fluid secretion and slowing of colonic transit (Moriya et al., 2010).
PYY3-36 has been shown to acutely reduce food intake when administered peripherally at physiological concentrations in both normal and obese rodents (Chelikani et al., 2006; Batterham et al., 2002; Chelikani et al., 2005). Initially, other laboratories were unable to reproduce these results (Tschop et al., 2004). Subsequently, the effects of PYY3-36 on food intake have been shown to be susceptible to stress and careful acclimatization of animals to the injection and handling procedure is necessary before feeding studies are performed (Halatchev et al., 2004; Chaudhri et al., 2008; Abbott et al., 2006). Chronic peripheral administration of PYY3-36 has been shown to reduce food intake and body weight gain or adiposity in normal-weight and obese rodents (Chelikani et al., 2007; Batterham et al., 2002; Abbott et al., 2005). IV administration of PYY3-36 significantly reduced 24h caloric intake in normal-weight and obese humans (Batterham et al., 2003c). Supraphysiological doses of PYY3-36 produce conditioned taste aversion in rats and nausea in humans (Chelikani et al., 2006; Le Roux et al., 2008). PYY null mice exhibit hyperphagia and increased adiposity, which is reversed by treatment with PYY3-36 (Batterham et al., 2002). However, PYY3-36 has not produced a chronic anorectic effect in all studies using different administration and dosing regimens. Intravenous infusion has proven the most reproducible route for PYY3-36 administration to effectively reduce food intake (Chelikani et al., 2006; Scott et al., 2005).

The administration of PYy and PYY3-36 into CNS regions which include the third, fourth and lateral ventricles and the PVN, causes an orexigenic effect (Marsh et al., 1999; Hagan, 2002). This orexigenic effect is believed to occur via the Y1R and Y5R as Y1R, and Y5R KO mice show attenuated hyperphagia to ICV administration of PYY3-36 (Kanatani et al., 2000). Interestingly, when administered directly into the ARC PYY3-36 causes an anorectic effect in rats (Batterham et al., 2002g). The differences in feeding effects could be explained by the differences in doses administered in each of the studies. It is possible that only very high doses of hypothalamically administered PYY3-36 are capable of activating the Y1R and Y5R and inducing an orexigenic response. The striking contrast between central and peripheral administration highlights the complex and sensitive nature of the PP-fold family multi-receptor/multi-ligand system.
In humans, circulating PYY levels are dependent on nutritional status, suggesting a role in satiety signalling. PYY plasma levels are low in the fasted state and increase rapidly after food intake. Within 15 minutes after a meal, plasma levels begin to rise, peaking 1-2 hours later and remaining elevated for up to 6 hours (Adrian et al., 1985a). Despite original controversy over the effectiveness of reducing food intake in rodents (Tschop et al., 2004), peripheral administration of PYY3-36 has been shown to decrease food intake and body weight gain in rats and mice (Halatchev et al., 2004a; Chelikani et al., 2006; Pittner et al., 2004a; Batterham et al., 2002f; Sileno et al., 2006; Koegler et al., 2005). In addition, PYY KO mice are hyperphagic and obese, a phenotype which is attenuated by a single daily i.p. injection of PYY3-36, strongly suggesting a physiological role for PYY3-36 in the control of food intake (Batterham et al., 2006a). PYY1-36 has also been shown to decrease food intake following i.v. infusion into rodents but with a lower potency than PYY3-36 (Chelikani et al., 2004a). It is possible that the anorectic effects of the infused PYY1-36 may be a result of the in vivo conversion to PYY3-36 by DPP-IV. This is supported by a study in which the anorectic effects of PYY1-36 were abolished in DPP-IV deficient rats (Unniappan et al., 2006b).

PYY3-36 may also elicit at least part of its anorectic effects by peripheral action on the GI tract. PYY has paracrine effects within the GI tract to delay gastric emptying and gut transit time (Savage et al., 1987). PYY also inhibits secretions from the stomach and pancreas (Hosotani et al., 1989; Symersky et al., 2005) and increases fluid and electrolyte absorption in the jejunum and ileum (Bilchik et al., 1993). Although most of the studies investigating GI tract function were carried out using full length PYY, Chelikani et al. have shown that PYY3-36 is more potent than PYY1-36 at delaying gastric emptying in rats (Chelikani et al., 2004b).

1.5.2.4 Proglucagon derived signals

The structure of the mammalian proglucagon gene was discovered in 1983 and the post-translational processing of the hormone established three years later (Bell et al., 1983; Mojsov et al., 1986). The proglucagon gene product undergoes tissue specific post-translational processing in the intestine,
pancreas and CNS (see figure 1.5) (Drucker, 2005). In pancreatic islet α-cells, proglucagon is cleaved by prohormone convertase 2 to produce the 29 amino acid hormone glucagon and a larger unprocessed polypeptide called the “major proglucagon fragment” (Patzelt and Schiltz, 1984). In the enteroendocrine L cells of the small intestine and in the brain, proglucagon is cleaved by prohormone convertase 1 to generate glicentin (enteroglucagon), oxyntomodulin and glucagon-like-peptide-1 and 2 (GLP-1 and GLP-2).

![Diagram of proglucagon gene and processing](image)

**Figure 1.5.** A diagrammatic representation of the tissue-specific processing of preproglucagon in the pancreas and the CNS/intestine. GLP-1 (glucagon-like peptide 1); GLP-2 (glucagon-like peptide 2); GRPP (glicentin-related pancreatic polypeptide); SP-1 (spacer peptide-1).
1.5.2.4.1 Glucagon-like-peptide 1

Glucagon-like-peptide 1 (GLP-1) is a 30 amino acid that undergoes further post-translational modification to produce the truncated bioactive peptides GLP-1\textsubscript{7-37} and GLP-1\textsubscript{7-36} amide (Herrmann et al., 1995; Orskov et al., 1993a) (See Figure 1.6 for sequences). GLP-1 is a potent incretin, physiologically augmenting postprandial glucose-dependent insulin release and inhibiting the secretion of glucagon. GLP-1 also modulates digestion by delaying gastric emptying (Holst et al., 1987; Naslund et al., 1999b; Kreymann et al., 1987).

GLP-1 is also involved in the regulation of energy homeostasis. Central or peripheral administration of GLP-1 acutely inhibits food intake in rodents (Turton et al., 1996a), and repeated daily administration reduces body weight in rats (Meeran et al., 1999). Peripheral administration also inhibits food intake in humans (Verdich et al., 2001d). The anorectic effect of GLP-1 is likely to be mediated via hypothalamic and brainstem centres, as well as via delayed gastric emptying (Shughrue et al., 1996b). Abbott, et al, showed that these anorectic effects were abolished following vagotomy or transection of the brainstem-hypothalamic pathway in rodents (2005).

GLP-1 signals via the GLP-1 receptor (GLP-1R), which is widely expressed peripherally (Brubaker and Drucker, 2002) and within the CNS, this receptor is localised to the ARC and PVN of the hypothalamus, and to the brainstem (Wei and Mojsov, 1995; Shughrue et al., 1996a). Inhibiting the actions of endogenous GLP-1, by the central administration of the GLP-1 receptor antagonist, Exendin (9-39) (Ex9-39), increases food intake (Turton et al., 1996b). GLP-1R expression in the hypothalamus and brainstem changes with nutritional status, with expression levels lower in the fed than the fasted state (Zhou et al., 2003). Intracerebroventricular (ICV) administration of GLP-1 increased oxygen consumption and core temperature in anaesthetised rats suggesting it has a stimulatory effect on energy expenditure (Osaka et al., 2005). However, this effect has not been reported following peripheral administration in rodents or in humans.
Endogenous levels of GLP-1 are inversely correlated with body mass in humans (Naslund et al., 1999a; Verdich et al., 2001b). There is evidence suggesting that GLP-1 secretion is reduced in obese subjects and that weight loss normalises these levels (Nauck et al., 1993; Ranganath et al., 1996). Reduced secretion of GLP-1 may therefore contribute to the pathogenesis of obesity, and replacement may restore satiety and aid weight control. In lean and obese human volunteers, i.v. infusion of GLP-1 dose-dependently decreases food intake (Verdich et al., 2001a). However, the anorectic effects of GLP-1 are small when it is infused at doses that mimic physiological post-prandial circulating levels (Flint et al., 2001; Verdich et al., 2001c). Obese subjects, s.c. administered GLP-1, reduced their calorie intake by 15% and lost approximately 0.5 kg in body weight over a 5 day study period (Naslund and Hellstrom, 1998). Although GLP-1 may be a useful treatment for type 2 diabetes, Edwards and colleagues reported that it caused hypoglycaemia in non-diabetic subjects (1998). Therefore, it may be limited as a potential therapy for non-diabetic obese individuals. There have also been reports of nausea and vomiting at high doses, potentially limiting the maximum tolerated dose in humans. Additionally, GLP-1 has a very short half life of one to two minutes in humans, due to the rapid breakdown by the enzymes DPP-IV and neutral endopeptidase (NEP) (Orskov et al., 1993b; Deacon et al., 1995).

1.5.2.4.2 Glucagon

Glucagon (GCG) is a 29 amino acid peptide released from the α-cells of pancreatic islets. The best characterised function of glucagon is the regulation of hepatic glucose production. Glucagon opposes the actions of insulin at the hepatocytes, promoting de novo glucose synthesis, called gluconeogenesis (Freychet et al., 1988; Beuers and Jungermann, 1990). The catabolic action of glucagon to promote the breakdown of glycogen to glucose (glycogenolysis) functionally opposes the anabolic action of insulin to promote glucose storage as glycogen, enabling glucagon and insulin to tightly regulate blood glucose levels (Beale et al., 1984). Since its discovery in the early 1920s by Kimball and Murlin...
**Figure 1.6. Amino acid sequences of Proglucagon-derived peptides.** Boxed regions denote areas of structure homology.
(1923), glucagon’s physiological role in experimental studies has been defined in the context of its inimical actions to insulin in the regulation of blood glucose homeostasis. Even today, its primary therapeutic use is to rescue insulin-induced hypoglycaemia in type-1 diabetes (Freychet et al., 1988).

Glucagon binds to the glucagon receptor (GCGR), a GPCR linked to the activation of cyclic AMP (cAMP) (Jelinek et al., 1993). The GCGR shares 42% homology with the GLP-1R (Runge et al., 2007). Glucagon binding sites have been identified in the liver, kidney, intestinal smooth muscle, skeletal muscle, brain, adipose tissue and pancreatic α-cells (Moens et al., 1996). Circulating levels of glucagon are low in the post-prandial state and increase during fasting (Ramnanan et al., 2011; Geary and Smith, 1982). Glucagon receptors are also found on the β cells of the pancreas where they are linked to the post prandial increase in insulin release. This is thought to allow the newly available circulating glucose to be taken up by insulin dependent tissues such as the liver and muscle. High circulating levels of glucagon alongside high circulating insulin levels (insulin resistance) has been reported to play a major role in the development of type 2 diabetes mellitus and the metabolic syndrome (Spellman, 2007a).

The role of glucagon is of interest to those studying the aetiology of type 2 diabetes. (Spellman, 2007b). One of the hallmarks of the metabolic dysfunction which occurs in type-2 diabetes is a failure of post-prandial glucagon suppression and elevated circulating glucagon levels, in addition to the well recognised problems with insulin deficiency/insulin resistance (Unger and Orci, 1975). As a result, glucagon antagonists have been utilised to attenuate glucagon-stimulated hepatic glucose production in the short term. In fact, Exenatide (exendin-4) and Pramlintide (an analogue of amylin, co-secreted with insulin) exert their anti-diabetic actions in part due to the inhibition of glucagon secretion (Schmitz et al., 2004).

However, emerging evidence suggests additional functions for this well-known hormone. In addition to being highly expressed in the liver, the GCGR is expressed in the kidney, heart, adipose tissue,
skeletal muscle, adrenals and hypothalamus (Ritter et al., 1986; Hoosein and Gurd, 1984; Watanabe et al., 1998). The expression of GCGR in these extrahepatic tissues suggests a broader physiological role beyond glucose homeostasis.

Glucagon has been shown to be involved in the regulation of food intake and energy expenditure. Studies from the 1950s indicated that exogenous glucagon administration diminishes feelings of hunger in humans and acutely decreases food intake in humans and rats (STUNKARD et al., 1955; SCHULMAN et al., 1957; PENICK and HINKLE, Jr., 1961). Pharmacological infusion of glucagon has also been shown to increase oxygen consumption in rats and in humans (DAVIDSON et al., 1957b; SALTER et al., 1957). In vitro and in vivo studies have confirmed that a physiological dose of glucagon increases energy expenditure by stimulating BAT activated thermogenesis via the sympathetic nervous system (Billington et al., 1991; Edwards and Howland, 1986; Billington et al., 1991). Nevertheless, the molecular mechanisms by which glucagon exert its affects on food intake, body weight and energy expenditure remain poorly understood.

1.5.2.4.3 Oxyntomodulin

Oxyntomodulin (OXM) is another peptide product of proglucagon cleavage (Fig. 1.4). Following a meal, OXM is secreted from intestinal L-cells into the bloodstream in proportion to the calories ingested (Ghatei et al., 1983; Le et al., 1992). Central and peripheral administration of OXM significantly reduces food intake in rodents, and chronic administration decreases in body weight gain and adiposity (Dakin et al., 2004; Dakin et al., 2002b; Dakin et al., 2001). I.v. infusion of OXM in healthy humans decreases food intake and hunger scores (Cohen et al., 2003), and in a four week study in obese individuals, s.c. administration of OXM prior to each meal resulted in a substantial reduction in body weight (Wynne et al., 2005b). Evidence also suggests that OXM increases in energy expenditure. Chronic administration in rodents resulted in greater weight loss than predicted by reduced food intake alone (Dakin et al., 2002a). As well as reducing energy intake, peripherally
administered OXM increased in activity-related and overall energy expenditure while reducing energy intake in over-weight human volunteers (Wynne et al., 2006). The anorectic effects OXM are thought to be mediated by the activation of the GLP-1R as the effect of OXM on food intake is abolished in GLP-1R KO mice and the hypothalamic administration of the GLP-1 antagonist, Ex(9-39) blocks the anorectic actions of both GLP-1 and OXM (Baggio et al., 2004a).
1.6 Overview of Thesis

This thesis evaluates the potential roles of PYY, GLP-1 and GCG receptor agonism as novel therapies for obesity. Chapter 2 investigates the homeostatic maintenance of body weight and, in particular, the effect that a continuous subcutaneous infusion of PYY3-36 administration has on food intake. Chapter 3 examines the design and development of synthetic GLP-1R and GCGR dual agonists and their effect on energy intake and energy expenditure.
Chapter 2: 
Investigation into 
Continuous Administration 
of PYY3-36 on Food Intake 
and Body Weight
2.1 Introduction

2.1.1 PYY3-36 and the Y2 receptor (Y2R)

PYY3-36 has been shown to acutely reduce food intake when administered peripherally at physiological concentrations in both normal and obese rodents (Chelikani et al., 2006; Batterham et al., 2002; Chelikani et al., 2005). These affects are thought to be mediated via the Y2R, as PYY3-36 does not reduce food intake in Y2R knockout mice. The Y2R is a 381 amino acid protein found predominantly in the brain. Antagonist and knockout studies have implicated Y2R in functions and responses including delayed gastric emptying, bone formation, angiogenesis, vasoconstriction, memory enhancement, circadian rhythms, analgesia, anorexia and anxiety (Berglund et al., 2003; Parker and Balasubramaniam, 2008; Lindner et al., 2008). *In situ* hybridization in the rat brain demonstrated Y2R mRNA expression in the hippocampus, hypothalamus, amygdala and brainstem. In the periphery, Y2R is found in sympathetic and parasympathetic neurons of the peripheral nervous system (Gustafson et al., 1997b).

The Y2R is thought to be a presynaptic autoinhibitory receptor and is expressed on most NPY-positive neurons in the hypothalamus (Broberger et al., 1997a). In the hypothalamus, the Y2R is found in the ARC, preoptic nucleus (PON) and dorsomedial nucleus (DMN). More than 80% of arcuate NPY neurons co-express the Y2R (Broberger et al., 1997; Wolak et al., 2003; Gustafson et al., 1997). The Y2R is confined to the medial portion of the ARC, which contains the NPY neurons (Parker and Herzog, 1999a). In the human brain, the Y2R is expressed predominately over other Y receptor subtypes, and is additionally found in the dentate gyrus and cerebral cortex (Caberlotto et al., 1998). Brainstem regions that express the Y2R include the nucleus of the solitary tract (NTS) and the lateral reticular nucleus; areas which relay information between the hypothalamus and spinal cord. Furthermore, the Y2R is the only Y receptor expressed in the lateral septum, an area involved in the integration of efferent and afferent neuronal signalling within the limbic system, which may be an important downstream mediator of the homeostatic regulation of appetite (Cota et al., 2006).
Peripheral administration of PYY3-36 does not inhibit food intake in the Y2R knock out mouse (Batterham et al., 2002h). Hypothalamic administration of a Y2R agonist decreases food intake in rodents (Leibowitz and Alexander, 1991) and Y2R KO mice have increased food intake, adiposity and body weight (Naveilhan et al., 1999). Administration of PYY3-36 into the ARC potently and dose-dependently reduces food intake during the dark phase and during re-feeding of fasted rats. Furthermore, administration of a specific Y2R antagonist into the ARC prevents the effects of exogenous PYY3-36 on food intake in rats and increases food intake in satiated rats, implying that endogenous PYY3-36 regulates food intake (Batterham et al., 2002; Abbott et al., 2005).

**Figure 2.1 The proposed mechanism of action of the anorectic effect of PYY3-36.** Adapted from Wynne and Bloom, 2006.

Initial investigations into the pathways involved in the anorectic effect of PYY3-36 suggested that PYY3-36 inhibits NPY expression and dis-inhibits aMSH expression. PYY3-36 was reported to
decrease NPY release and increase αMSH release from hypothalamic explants (Batterham et al., 2002e). More recent studies, however, have shown that PYY3-36 reduced food intake when peripherally administered to POMC (Challis et al., 2004) or MC4R (Halatchev et al., 2004b) KO mice. The anorectic effects of PYY3-36 may thus be primarily mediated via the inhibition of NPY expression as the Y2R, rather than via indirect effects on melanocortin signalling (Broberger et al., 1997b; Chen et al., 1997; King et al., 2000).

Whether the effect of PYY3-36 on Y2R-expressing neurons in the ARC is direct or indirect is unknown. The ARC is relatively permeable, and PYY3-36 can be transported across the BBB via a non-saturable mechanism (Peruzzo et al., 2000; Nonaka et al., 2003). There is also evidence to suggest that peripheral PYY3-36 may transmit satiety signals via the vagus-brainstem neural pathway. The Y2R is expressed in the NTS of the brainstem (Gustafson et al., 1997a) and the nodose ganglion of the vagus nerve (Koda et al., 2005b) and vagotomy or the transectioning of the brainstem-hypothalamic neuronal pathways abolishes the anorectic effects of PYY3-36 in rats (Koda et al., 2005a; Abbott et al., 2005a). In humans, infusion of PYY3-36 to mimic postprandial PYY3-36 concentrations activates neuronal areas in the CNS including the brainstem, mid-brain areas and higher cortical regions involved in reward processing (Batterham et al., 2007). This further supports the notion that the brainstem is involved in the PYY3-36 induced effects on food intake, but also suggests PYY3-36 can influence food intake via modulation of hedonic brain circuits.

The NTS and AP are directly innervated by vagal sensory neurons from the gut, which express the Y2R, and are stimulated by PYY3-36 (Koda et al., 2005). Meal related satiety signals may therefore be transmitted via the vagal neurons to increase activity within neuron populations of the NTS and AP, which project to the hypothalamus. Since the Y2R is expressed in the NTS and AP, PYY3-36 may also directly activate these neuronal populations. The ARC is linked to the NTS via descending pathways, and the NTS is linked to the ARC via an ascending noradrenergic pathway, through which, for example, the orexigenic gastric hormone ghrelin has been suggested to exert its effects (Blevins et al., 2008; Date et al., 2006). However, it is unclear whether neuronal activation in the ARC precedes or follows the activation of the NTS.
2.1.2 Therapeutic potential of PYY as an Anti-Obesity Agent

Batterham et al. first reported that the i.v. infusion of PYY3-36 at a dose that mimicked physiological levels in non-obese human volunteers resulted in a 36% reduction in acute calorie intake at a meal presented 2 hours post-infusion when compared to saline controls, and a 33% reduction in calorie intake over the 24 hour post-infusion period (Batterham et al., 2002d). Caloric intake following i.v. PYY3-36 infusion was also similarly decreased in obese human volunteers (Batterham et al., 2003b). Degen and colleagues investigated the effect of graded doses of PYY3-36 on appetite and calorie intake when infused in healthy non-obese volunteer subjects. In this study, the lowest dose was shown to raise circulating PYY to levels similar to those observed following a high caloric meal, whereas the higher doses produced supra-physiological circulating levels and resulted in adverse effects, including nausea and abdominal discomfort (Degen et al., 2005c).

Le Roux et al. investigated the effects of i.v. infusion of pharmacological doses of PYY3-36 in healthy volunteers. A short lived feeling of nausea (≤ 30 min) was experienced by 5 of the 6 study participants following the infusion. In addition, no further reduction in food intake over those reported in previous studies was observed (Le Roux et al., 2008a). Sloth and colleagues investigated the effect of i.v. infusion as well as s.c. administration of PYY3-36 on appetite and food intake (Sloth et al., 2007h; Sloth et al., 2007a). In agreement with the other PYY3-36 human infusion studies, a low dose of 0.2 pmol/kg/min had no effect on food intake, did not cause changes in appetite ratings and generally had no adverse effects on lean and obese volunteer subjects. On the other hand, 0.8 pmol/kg/min PYY3-36 significantly reduced food intake but also caused adverse effects including nausea, abdominal discomfort and hot flushes in 5 of the 9 subjects taking part in the study (Sloth et al., 2007g). Graded doses of PYY3-36 (25 - 100 pmol/kg lean body mass) s.c. injected in obese male volunteers over 5 days caused a significant decrease in satiety and hunger scores associated with either no or mild adverse effect, but no significant decrease in food intake. It was speculated in this study that the lack of an anorectic effect and the minimal aversive effects may have been due to the development of tolerance as graded doses of PYY3-36 were given daily over 5 days (Sloth et al., 2007b). However, it is also possible that the doses used in this study were not high enough to elicit an
anorectic effect as peak plasma PYY levels following s.c administration were shown to be lower than those following i.v. infusion of an anorectic dose of PYY3-36 (0.8 pmol/kg/min) (Sloth et al., 2007f; Sloth et al., 2007c).

Merck Research Laboratories has investigated the effect of repeated intranasal administration of PYY3-36 at doses of 200 and 600 µg for 12 consecutive weeks on food intake of obese subjects. PYY3-36 was administered using a nasal spray 20 minutes before breakfast, lunch and dinner. A decrease of about 2.11 kg in body weight was achieved with the 200 µg dose over the study period. However, 29.5% of patients experienced at least one episode of nausea, and 9.1% experienced at least one episode of vomiting. In the 600 µg group, almost 80% of patients experienced nausea and about 50% experienced at least one episode of vomiting. More than 70% of patients in the 600 µg dose group discontinued the study before completion due to the side effects experienced. The small number of patients left in this group prevented the accurate measurement of the effect of 600 µg of intranasally administered PYY3-36 on body weight in the obese subjects (Gantz et al., 2007b).

Although further research is required to better characterise the effect of long-term administration of PYY3-36 on body weight in humans, it is clear that PYY3-36 has a narrow therapeutic window. Only higher doses that have aversive effects cause robust reductions in food intake. In addition, PYY3-36 appears to be rapidly cleared from the circulation. PYY3-36 infused in humans to mimic post-prandial concentrations levels were reported to have declined to baseline levels within 30 minutes after infusion (Batterham et al., 2002c). Following s.c. administration of PYY3-36 (25 - 100 pmol/kg lean body mass) in human subjects, PYY plasma levels peaked 30-45 minutes later and by 240 minutes dropped down to near control levels (Sloth et al., 2007d). The metabolic half-life of PYY1-36 is about 11 minutes in dogs (Pappas et al., 1985b) and about 8 minutes in the rat (Lluis et al., 1989) and peak plasma levels after i.p. administration were observed as early as 15 minutes after injection in rats (Batterham et al., 2002b).
2.1.3 Development of Tolerance to Anorectic Agents

Many appetite suppressing agents appear to lose their appetite-suppressing efficacy when taken for extended periods of time. With the recent advances in the knowledge of the neurophysiological organization of appetite control circuits in the CNS (Schwartz, 2000c; Berthoud, 2002b), this knowledge is rarely applied to the investigation of the mechanisms underlying appetite regulation (Grudell and Camilleri, 2007). It is unclear why anorectic agents appear to lose efficacy with repeated administration, however Fenstrom and Choi described three potential mechanisms underlying this tolerance: 1) the homeostatic/physiological defence of body weight set point, 2) motivation and reward mechanism involved in food intake, and 3) a behavioural adaptation to the drug (Fernstrom and Choi, 2008).

The most widely accepted hypothesis is the homeostatic view. It has been suggested that the true action of an anorectic agent is to alter the brain in a manner that resets the body weight set point to a lower value (STUNKARD, 1982; Levitsky et al., 1981). Most of the evidence supporting this hypothesis is derived from rodent studies given the drug fenfluramine (FEN), sibutramine and rimonabant (Choi et al., 2002; Pi-Sunyer et al., 2006; Levin and Dunn-Meynell, 2000). Consistently, the administration of appetite suppressants chronically to rodents causes an immediate suppression of food intake, which typically lasts only during the first 3–6 days of treatment, and is followed by a rebound to pre-treatment levels, despite continued drug treatment (Fernstrom and Choi, 2008). In addition, rats also exhibit an initial rapid reduction in body weight that reaches a plateau, which corresponds in time roughly with the recovery of food intake. However, there is little and inconsistent data available regarding the development of tolerance to gut hormones, such as GLP-1 and PYY.
2.1.4 Tolerance to anorectic effect of PYY3-36

It is important in the development of obesity drugs to determine the effect of chronic/continuous administration and whether this can produce a sustained reduction in daily food intake and ultimate decrease body weight in experimental animals.

Several groups have shown that continuous administration of PYY3-36 via osmotic minipumps results in a transient reduction in food intake (Chelikani et al., 2007; Chelikani et al., 2005b; Unniappan and Kieffer, 2008b; Unniappan et al., 2006a; Reidelberger et al., 2008a; Reidelberger et al., 2011). This apparent desensitisation to the anorectic effects of PYY3-36 may limit its therapeutic potential as an anti-obesity drug and requires investigation. One explanation was that an acute decrease in circulating leptin levels following the initial change in body weight after PYY3-36 administration is a potential mechanism behind the apparent tachyphylaxis. Unniappan and colleagues confirmed that there was an acute decrease in leptin following administration of PYY3-36 and a co-infusion of leptin and PYY3-36 indeed extended the anorectic effect of PYY by one day (Unniappan and Kieffer, 2008c). However, this alone is unlikely to be the mechanism behind the apparent tachyphylaxis.

Another mechanism underlying the transient anorectic effect is receptor downregulation. At present, this has not been investigated in vivo. Extensive research has been done into the activity and internalisation The Y1, Y2, Y4 and Y5 receptors, and has demonstrated that all of these receptors are susceptible to internalisation in vitro following prolonged agonist stimulation (Parker et al., 2007a). However, the Y2 receptor is internalised at a slower rate than the other receptors of the Y family (Parker et al., 2001a). The Y2 receptor has been shown to exhibit slow cycling and low levels of endocytosis, as well as attachment of ligands to the internalisation complex (Parker et al., 2001b; Gicquiaux et al., 2002). While it is presumed that PYY3-36 exhibits its anorectic effects primarily via the Y2 receptor, actions on the other Y receptors cannot be ruled out. It is currently unknown whether internalisation or desensitisation of the Y2 receptor is responsible for the apparent diminished efficacy of exogenous PYY3-36 on food intake and body weight following chronic administration.
2.2 Hypothesis and Aims

Hypothesis:

Resistance to the anorectic effect of PYY3-36 that occurs during continuous infusion is the result of the downregulation of Y2R expression and is independent from the homeostatic stimulation of appetite following weight loss.

Aims:

- To distinguish between the anorectic effect of acute and chronic PYY3-36 administration
- To investigate whether exposure to low dose PYY3-36 decreases sensitivity to the anorectic effect of a high dose PYY3-36 infusion
- To investigate whether food restriction and the resulting decrease in body weight results in a decreased sensitivity to the anorectic effect of PYY3-36
- To quantify expression of hypothalamic Y2R following continuous PYY3-36 infusion
2.3 Methods

2.3.1 Peptide Synthesis

PYY3-36 used in the following studies was synthesized by and purchased from Bachem, Ltd. PYY3-36 had a purity > 95%.

The peptide used in the studies described in this chapter is based on the human sequence, and all sequence information refers to the human form, unless otherwise stated. In humans the predominant circulating active form of PYY is the truncated form PYY3-36, and it is this form which was used in all experiments unless otherwise stated.

PYY3-36 was synthesized using an automated fluorenylmethoxycarbonyl solid phase peptide synthesis methodology with each amino acid added sequentially from the C to the N terminus.

Bachem, Ltd. purified peptides by reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. The purified product was subsequently analysed by reverse phase HPLC and by matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS).

2.3.2 Animal Husbandry and General Experimental Conditions

Male C57BL/6 mice (Charles River Ltd., Margate, Kent, UK) were obtained at 4 weeks of age. All were group housed (5 mice per cage) upon arrival and allowed to acclimatise to laboratory conditions for a minimum of 7 days. Mice were handled daily for three days prior to their first study in order to acclimatise the animals to the handling procedure. Mice were given two sham injections 2-4 days before the start of the study to acclimatise them to the injection procedure and to minimise any potential non-specific effects due to stress. Mice were maintained under controlled temperature (21-23°C) with, unless otherwise stated, ad libitum access to standard chow (RM1 diet, SDS Ltd, Witham,
Essex, UK) and to tap water. The light cycle for all animals was ‘dawn’ at 07:00, lights fully on at 07:30, ‘dusk’ at 19:00 with lights fully off at 19:30. The light cycle is particularly relevant to feeding studies as rodents are nocturnal feeders and eat only a small proportion of their daily caloric intake during the light phase, demonstrating a dramatic increase in food intake after the onset of darkness (Lu et al., 2002b).

For acute studies, lean C57BL/6 mice, weighing between 20-25 g were singly housed and maintained on a standard chow diet (RM1 diet, SDS Ltd, Witham, Essex, UK). Mice were all purchased from the same supplier and housed under the same conditions, and the same equipment was used as described above for all studies. All mice feeding studies were completed under conditions where external disturbances were minimised.

For chronic studies (longer than 7 days), the diet induced obesity (DIO) mouse model was used. This is a commonly used model of the metabolic changes associated with obesity, as it develops resistance to both insulin and leptin (Moraes et al, 2003). Mice were given *ad libitum* access to tap water and a high fat containing diet (60 % energy from fat, 20% from carbohydrate, 20% from protein, D12492, Research Diets, New Brunswick, NJ, USA) for 10 weeks prior to the start of the study and remained on this diet throughout the study. Seven days prior to the start of the experiment, mice were placed into individual cages.

All animal procedures performed were approved under the British Home Office Animals (Scientific Procedures) Act 1986 (Project license number 70/6402).
2.3.3 The Effect of PYY3-36 Administration on Acute Food Intake

To evaluate the effect of a single injection of PYY3-36 on acute food intake, a dose response was performed. PYY3-36 was dissolved in sterile saline (0.9%). The acute feeding study was carried out during the early light phase (0800-1000h), in mice which had been fasted from 1600h the preceding day. Mice were fasted before the study as the agents tested were expected to have anorectic effects, and the higher food intake of mice following a fast is more likely to successfully demonstrate such effects. Subcutaneous injections were administered to mice via a 0.5 ml insulin syringe with a 29-gauge needle (Bd Micro-Fine U-100 Insulin Syringe 0.5ml, VWR International, UK). Mice were randomised into groups (n=8) of approximately equal mean body weight to receive vehicle, 10, 100 or 1000 nmol/kg of PYY3-36.

Immediately following injection, mice were returned to their home cage with a pre-measured amount of food. Food was reweighed at 1, 2, 4, 8 and 24 hour intervals post-injection: food was removed from the hopper, a visual inspection of the cage was made to check for any food spillage/hoarding and food was weighed using balances accurate to 0.01 g. Mouse body weight was measured 24 hour post-injection.

2.3.4 Effect of Continuous Subcutaneous Infusion of PYY3-36

Alzet® Osmotic Minipumps (Durect Corporation, CA, USA) are an established method of chronic delivery and have previously been used for administration of appetite-regulating peptides, including PYY3-36 (Reidelberger et al., 2008c; Unniappan and Kieffer, 2008d). These implantable pumps provide the advantage of continuous administration without the need for connectors or frequent handling. Prior to implantation, the peptide was injected into a flexible central reservoir within the pump. This reservoir is separated by an impermeable membrane from an outer chamber with high salt concentration. Once implanted into the animal, fluid enters via a semi-permeable cellulose outer membrane due to the high osmotic pressure of the salt. This mechanically compresses the central
reservoir and results in delivery of the treatment agent at a fixed rate (dependent on outer membrane permeability) via a stainless steel flow moderator (see figure 2.2).

Different pumps were utilised in the experiments for different periods. All pumps had a length of 150 mm, circumference of 0.6 mm and weight of 0.4 g. The models used in the following experiments were 3 day, model 1003D (release rate of 1 µl/hour); 7 day, model 1007D (release rate of 0.5 µl/hour); and 28 day, model 1004 (release rate of 0.11 µl/hour).

Figure 2.2. A schematic representation of an Alzet osmotic minipump utilised in the experiments in this chapter. Image from Alzet.com.
On the morning of surgery, pumps were loaded with either PYY3-36 reconstituted in sterile water (5%), 0.1M acetic acid (18%) and 0.9% sterile saline (77%), or vehicle control. Pumps were implanted subcutaneously below the scapulae in mice anesthetised with 4% inhaled isoflurane (Abbott Laboratories, Ltd) and the incision was closed with a single uninterrupted cruciate suture. Post-operative antibiotic Baytril (enrofloxacin 5mg/kg intraperitonealy (Bayer Healthcare)) and analgesic Rimadyl (carprofen 4mg/kg subcutaneously (Pfizer Animal Health)) was administered. Delivery of pump content was confirmed by extracting and measuring the residual volume of the reservoir at the end of the study and comparing it to the expected volume which was calculated.

### 2.3.4.1 Investigation into the Anorectic Effect of Continuous Administration of PYY3-36 for 7 days

To investigate the effect of PYY3-36 delivered as a chronic subcutaneous infusion over a seven day period, lean C57BL/6 mice, *ad libitum* fed, were randomised into groups of equivalent mean body weight (n=9) to receive pumps delivering vehicle or PYY3-36 at 375 nmol/kg. A group implanted with a vehicle pump was pair-fed to the mean of the PYY3-36 food intake for days 0-3 and was *ad libitum* fed for days 4-7, as described in table 2.1

<table>
<thead>
<tr>
<th>Group</th>
<th>Pump contents</th>
<th>Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Ad libitum</td>
</tr>
<tr>
<td>PYY375</td>
<td>PYY3-36 375 nmol/kg</td>
<td>Ad libitum</td>
</tr>
<tr>
<td>PYY-PF</td>
<td>Vehicle</td>
<td>Pair Fed to PYY375 on days 0-3</td>
</tr>
</tbody>
</table>

**Table 2.1. Investigation into the Anorectic Effect of Continuous administration of PYY3-36 for 7 days.**

Alzet osmotic minipumps were implanted on day 0 (Pump 1) and on day 3 (Pump 2) to deliver either vehicle or PYY3-36

Model 1007D pumps were used in this study. These have a reservoir volume of 90 μL and set flow rate of 0.5 μl/hour, and can thus last for up to 180 hours. Pumps were inserted during early light phase (0900h) of day 0 (Fig. 2.3). Food intake and body weight was then measured daily at the start
of early light phase. At the start of day 7, animals were killed by CO₂ asphyxiation. Blood was taken via cardiac puncture and collected into heparin coated tubes. Samples were centrifuged (Sigma Laboratory centrifuges 3 K18, Rotor No. 19777-H) and the plasma separated and stored at -20°C for later analysis using radioimmunoassay.

![Timeline of the Investigation into the Anorectic Effect of Continuous administration of PYY 3-36 for 7 days](image)

**Figure 2.3.** Timeline of the Investigation into the Anorectic Effect of Continuous administration of PYY 3-36 for 7 days. Alzet osmotic minipumps were implanted on day 0 delivering either vehicle or PYY3-36 at 375 nmol/kg.

2.3.4.2 **Investigation into the Effect of 3 Day Continuous Low Dose Pretreatment followed by 3 day High Dose Treatment of PYY3-36**

To investigate whether prior exposure to lower doses of PYY3-36 attenuated the anorectic response to higher doses, mice underwent two pump insertion procedures. The first pump, inserted on day 0, was a 1003D model which delivered either vehicle, a subanorectic low dose of PYY3-36 (50 nmol/kg) or an anorectic low dose of PYY3-36 (200 nmol/kg) on days 0-3 of the experiment. In the late light phase (1700h) of day 3, mice underwent a second procedure in which a second 1003D model pump was inserted infusing either vehicle or high dose PYY3-36. Mice were randomised into groups of equivalent mean body weight (n=9) as described in table 2.2.
<table>
<thead>
<tr>
<th>Group</th>
<th>Pump 1 contents</th>
<th>Pump 2 contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Vehicle/1500</td>
<td>Vehicle</td>
<td>PYY3-36 1500 nmol/kg</td>
</tr>
<tr>
<td>PYY50/PYY1500</td>
<td>PYY3-36 50 nmol/kg</td>
<td>PYY3-36 1500 nmol/kg</td>
</tr>
<tr>
<td>PYY200/PYY1500</td>
<td>PYY3-36 200 nmol/kg</td>
<td>PYY3-36 1500 nmol/kg</td>
</tr>
</tbody>
</table>

Table 2.2. Investigation into the Effect of 3 Day Continuous Low Dose Pre-treatment followed by 3 day High Dose Treatment of PYY3-36. Alzet osmotic minipumps were implanted on day 0 (Pump 1) and on day 3 (Pump 2) to deliver either vehicle or PYY3-36.

Pumps were inserted during early light phase of day 0 and late light phase of day 3 (Figure 2.4). Food intake and body weight was then measured daily at the start of early light phase. At the start of day 7, animals were killed by CO2 asphyxiation. Blood was taken via cardiac puncture and collected into tubes coated with heparin. Samples were centrifuged and the plasma separated and stored at 20°C for later measurements using radioimmunoassay.

![Timeline of the Investigation into the Effect of 3 Day Continuous Low Dose Pre-treatment followed by 3 day High Dose Treatment of PYY3-36. Alzet osmotic minipumps were implanted on day 0 (Pump 1) and on day 3 (Pump 2) to deliver either vehicle or PYY3-36.](image)

2.4.4.3 Investigation into the Effect of Food Restricction on the Anorectic Effect of PYY3-36

To evaluate the effect of a period of food restriction has on the sensitivity to PYY3-36, DIO mice weighing 33-43 grams, maintained on a high fat diet, underwent two minipump implantation...
procedures. On day 0, mice were implanted pump model number 1007D, which infused either vehicle or PYY3-36 at 250 nmol/kg (n=6). One group, receiving vehicle, was food restricted to the median food intake of the PYY3-36 group on days 0-6 (FR) (n=10). On day 6, all mice underwent a second implantation procedure. Vehicle controls and PYY3-36 groups were received vehicle pumps (model 1003D), while the FR group was split into equal groups (n=5) to receive infusions of either vehicle or PYY3-36 at 250 nmol/kg and had access to ad libitum food from this point forward (see table 2.3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Pump 1 contents</th>
<th>Pump 2 contents</th>
<th>Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Ad libitum</td>
</tr>
<tr>
<td>PYY250</td>
<td>PYY3-36 250 nmol/kg</td>
<td>Vehicle</td>
<td>Ad libitum</td>
</tr>
<tr>
<td>FR/Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Pair Fed to PYY250 on days 0-3</td>
</tr>
<tr>
<td>FR/PYY250</td>
<td>Vehicle</td>
<td>PYY3-36 250 nmol/kg</td>
<td>Pair Fed to PYY250 on days 0-3</td>
</tr>
</tbody>
</table>

Table 2.3. Investigation into the Effect of food restriction (FR) on the subsequent anorectic effect of PYY3-36. Alzet osmotic minipumps were implanted on day 0 (Pump 1) and on day 6 (Pump 2) to deliver either vehicle or PYY3-36.

![Timeline of the investigation into the effect of food restriction (FR) on the subsequent anorectic effect of PYY3-36](image)

Figure 2.5. Timeline of the investigation into the effect of food restriction (FR) on the subsequent anorectic effect of PYY3-36. Alzet osmotic minipumps were implanted on day 0 (Pump 1) and on day 6 (Pump 2) to deliver either vehicle or PYY3-36.

Food intake and body weight was measured daily at the start of early light phase. Blood was taken via cardiac puncture and collected into heparin coated tubes. Samples were centrifuged (Sigma Laboratory centrifuges 3 K18, Rotor No. 19777-H) and the plasma separated and stored at -20°C for later
analysis using radioimmunoassay. Hypothalami were isolated and snap frozen in liquid nitrogen and stored at -80°C until RNA extraction.

2.3.5 Quantification of hypothalamic Y2 receptor using qPCR

Quantitative PCR (qPCR) was used to verify the expression of the Y2R in dissected hypothalami from mice infused with PYY3-36 or vehicle for 28 days.

2.3.5.1 Messenger RNA extraction

Total RNA was extracted from the hypothalamus using the RNEasy Mini Kit column (Qiagen, UK). Approximately 30 - 40 mg of tissue was added to a 2 mL Eppendorf containing 600 µL ‘RLT’ lysis buffer with 12 µL 2 M dithiothreitol (DTT) and a steel cone bead (7 mm diameter, Retch, Qiagen, UK). Samples were homogenised by high speed shaking using a Qiagen Tissuelyser II (Retch, Qiagen, UK) for 2 x 2 min at 20 Hz. Samples were centrifuged at max speed (>8000g, 13000 rpm, Biofuge Pico centrifuge, Heraeus, UK) for 3 minutes and supernatant (approximately 700 µL) was removed and placed into a fresh 1.5 mL eppendorf. One volume of 70% ethanol was added to the lysate and mixed by pipetting. Then, 700 µL of the sample, including any precipitates, was added to an RNeasy Mini spin column placed in a 2 mL collection tube. The column was centrifuged at maximum speed for 15s and the flow through was discarded. This was repeated with the remainder of the sample. Then, 350 µL of wash buffer ‘RW1’ was added to the RNeasy spin column and centrifuged for 15s at maximum speed. The flow through was discarded.

On column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). To prepare the DNase I stock solution, 500 µL RNase-free water was injected into the DNase I vial using an RNase-free needle and syringe. The vial was mixed gently by inverting, and aliquotted for long term storage at -20°C. Then, 70 µL of Buffer RDD was added to 10 µL of DNase I stock and mixed gently
by inverting and briefly centrifuging. The DNase I incubation mix (80 μL) was added directly onto the RNeasy column membrane and left at room temperature (20-30°C) for 15 minutes.

350 μL Buffer RW1 was added to the spin column, centrifuged for 15s at maximum speed and flow through was discarded. Then, 500 μL of Buffer RPE was added to the spin column, centrifuged at maximum speed for 15 s, and flow through discarded. Again, 500 μL of Buffer RPE was added once again and centrifuged at maximum speed for 2 minutes. The RNeasy spin column was then transferred to a new 2 mL collection tube and centrifuged at full speed for 2 minutes to dry. Finally, to elute the RNA, the spin column was placed into a new 1.5 mL eppendorf and 30 μL of THE RNA storage solution (1 mM sodium citrate, pH 6.4; Ambion) was added directly to the membrane and centrifuged for 1 minute. To increase the yield of RNA, this elution step was repeated using the same collection tube resulting in a total volume of 60 μL. Each RNA sample was vortexed and divided into a 50 μL aliquot, for long term storage at -80°C, and a 10 μL aliquot for integrity analysis.

### 2.3.5.2 RNA Quantification and Assessment of Quality

#### 2.3.5.2.1 Spectrophotometry

RNA concentration and purity was determined using a Nanodrop 3300 spectrophotometer (Thermo Fischer Scientific Inc.). The 260/280 nm and 260/230 nm absorption ratios are measures of relative purity of a nucleic acid sample and assess the presence of protein or other contaminants. RNA samples are regarded “pure” when these ratios are ~2.0 and in the following experiments, only samples with ratios of >1.8 were bioanalysed.

#### 2.3.5.2.2 Electrophoresis

Due to the omnipresence of ribonucleases (RNases) and the instability of RNA, it is important to quantify and check the integrity of the RNA prior to qPCR. The Agilent 2100 Bioanalyzer (Agilent
Biotechnologies Inc.), was used to generate an RNA Integrity Number (RIN), quantification estimate, ribosomal ratios of the total RNA sample and an entire electrophoretic trace of the sample.

To avoid decomposition of the RNA samples, the bioanalyser electrodes were first decontaminated for 1 minute using 350 µL of RNaseZap reagent (Ambion, Inc). To prepare the gel-dye mixture, 1 µL of dye was added to 65 µL of filtered gel, vortexed for 10 seconds and centrifuged at 13000xg for 10 minutes. Then, 9 µL of the gel-dye mixture was carefully added to the designated wells of a RNA Nano 6000 chip and primed for 30 seconds using a chip Priming Station. Each remaining well then received 5 µL of the loading RNA Nano marker. RNA samples to be analysed were diluted using THE to a concentration of 5-500 ng/µL and 2 µL of each sample, including an RNA ladder, were then denatured for 2 minutes at 70°C. Next, 1 µL of the denatured RNA ladded and of each sample was loaded into the wells of the chip and vortexed for 60s at 2400 rpm. The chip was then placed into the bioanalyser so that the electrodes fit into each well, and was analysed using the Agilent 2100 Expert Software.

**2.3.5.3 Reverse Transcription**

RNA samples were reverse transcribed into cDNA using the Superscript VILO cDNA synthesis kit (Invitrogen, UK). Firstly, each RNA sample was diluted with THE to a final concentration of 200 ng/µL. Each sample was reverse transcribed in triplicate in a 96 well plate, with each well containing:

- 2 µL 5x VILO Reaction Mix
- 1 µL 10x SuperScript Enzyme Mix
- 3 µL RNA sample (600 ng)
- 4 µL RNase free water

A foil seal was placed on the plate and samples were placed on a shaker for 3 minutes before centrifuging for 15 seconds. The plate was placed in a thermal cycler (to incubate at 25°C for 10 minutes, 42°C for 120 minutes and 85°C for 5 minutes, before being stored at -20°C.
2.3.5.4 Quantitative Polymerase Chain Reaction (qPCR)

The relative expression of metabolic genes of interest was quantified using TaqMan® Gene Expression Assays (Applied Biosystems, Life Technologies, UK). An inventoried primer containing a predesigned, preformulated primer and probe set targeting the mouse Y2 receptor was used (Mm01218209_m1).

TaqMan® Gene Expression Assay allows accurate quantification of gene expression by incorporating a reporter-tagged probe, which is complementary to part of the targeted cDNA sequence, into the amplification process. During PCR, the probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. The DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye to allow fluorescence of the reporter.

The following constituents were mixed in each well for each qPCR reaction: 1µl of cDNA template (600ng), 10µl master mix, 1µl of specific Gene Expression Assay, and GDW to give a 20µl reaction volume. Each reaction was mixed before the plate was sealed. Each reaction was assayed in triplicate.

The sealed plate was loaded onto a thermal cycler (Applied Biosystems 7900HT Real-Time PCR System), on the ΔΔCt programme, under the following conditions: 15 seconds incubation at 95°C followed by 1 minute incubation at 60°C, repeated for 40 cycles. The amplification data for each sample was corrected against its respective endogenous control.

2.3.6 Radioimmunoassay

PYY-like immunoreactivity (PYY-IR) was measured with a specific and sensitive radioimmunoassay, as described in Appendix D. The assay measured both the major functional fragment (PYY3-36) and
the full-length hormone (PYY1-36). The antiserum (Y21) was produced in rabbits against purified porcine PYY coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:50,000. This antibody does not cross-react with PP, NPY, or other known gastrointestinal hormones. The $^{125}\text{I}$-PYY was prepared by the direct iodination method and purified by reverse phase high-pressure liquid chromatography. The specific activity of the $^{125}\text{I}$-PYY label was 54 Bq/fmol. The assay was performed in a total volume of 0.7 ml of 0.06 M phosphate buffer, pH 7.4, containing 0.3% bovine serum albumin. The assay was incubated for 3 days at 4°C before separation of the free and antibody-bound label by goat anti-rabbit antibody.

2.3.7 Statistical Analysis

All data are expressed as the mean value +/- SEM.

Interval food intake was compared by one-way ANOVA followed by post-hoc Dunnett correction. This is a method often used to study longitudinal data which consists of repeated measures of an individual or group of individuals taken over time. These repeated measures from any one individual or group are correlated with each other and are therefore no longer independent.

Generalized Estimation Equations (GEE) estimate the correlation between a single individual or group’s response and provide a estimate of each effect's variance. Chronic and continuous data were analysed using a GEE, and, if significantly different, further analysed by a non-parametric two-sample Wilcoxon rank-sum (Mann-Whitney U) test comparing the control group to the treatment group. Prism (Version 5, GraphPad Software Inc. San Diego, USA) was used for ANOVA statistical analysis and Stata Intercooled, version 9 (Statacorp Ltd, College Station, Texas) for GEE and Wilcoxon rank-sum analysis. For qPCR, a t-test followed by a Wilcoxon matched pairs test was used. For all cases, values of p<0.05 were considered statistically significant.
2.4 Results

2.4.1 The Effect of PYY3-36 Administration on Acute Food Intake in Mice

Subcutaneous administration of PYY3-36 at 10, 100 and 1000 nmol/kg significantly reduced food intake compared to vehicle controls (see Figure 2.6). Food intake in the first hour was significantly inhibited at all doses of PYY3-36 [food intake (g): 0.93 +/- 0.08 (vehicle); 0.71 +/- 0.14 (10 nmol/kg (p<0.05 vs. vehicle)); 0.52 +/- 0.08 (100 nmol/kg (p<0.001 vs. vehicle)); 0.54 +/- 0.07 (1000 nmol/kg (p<0.001 vs. vehicle))]. In the 1-2 hour interval, food intake was significantly inhibited by the 100 and 1000 nmol/kg doses [food intake (g): 0.36 +/- 0.06 (vehicle); 0.1g +/- 0.03 (100 nmol/kg (p<0.01 vs. vehicle)); 0.11g +/- 0.02 (1000 nmol/kg (p<0.001 vs vehicle))]. In the 2-4 hour interval after administration, only the highest dose caused a significant reduction in food intake [food intake (g): 0.44 +/- 0.07 (vehicle); 0.07 +/-0.03 (1000 nmol/kg (p<0.001 vs. vehicle))]. There was no significant effect of any doses of PYY3-36 on food intake after 4 hours at any time point investigated.
Figure 2.6. Effect of vehicle control or PYY3-36 10, 100 or 1000 nmol/kg on 24 hour food intake in overnight fasted mice. Data shown as mean +/- SEM food intake in C57BL/6 mice (n=8-10) for 0-1, 1-2, 2-4, 4-8 and 8-24 hour intervals after single subcutaneous injection of vehicle or PYY3-36 at 10, 100 or 1000 nmol/kg. Statistics: One way ANOVA with Dunnett’s post-hoc test *=p<0.05, **=p<0.01, ***=p<0.001 vs. vehicle.
2.4.2 Effect of 7 day Continuous Subcutaneous Infusion of PYY3-36 via Alzet osmotic minipump on Food Intake and Body Weight

2.4.2.1 Effect on Cumulative Food Intake

*Ad libitum* fed male C57BL/6 mice underwent surgery on day 0 and an Alzet osmotic minipump was implanted subcutaneously. Minipumps delivered a continuous infusions of either PYY3-36 (375 nmol/kg/day) or vehicle for 7 days. At the study end, mice that received vehicle had consumed 29.7 +/- 1.20 g (n=8) and those that received PYY3-36) had consumed 27.9 +/- 1.13 g (n=8) of chow (Figure 2.7). GEE analysis followed by Mann-Whitney U test showed that the group that received PYY3-36 had consumed significantly less than vehicle controls, from day 1 until day 6 (p<0.01, n=9).
Figure 2.7. Effect of 7 day continuous infusion of PYY3-36 on cumulative food intake in mice compared to vehicle controls. Data shown as mean +/- SEM food intake in C57/BL6 mice (n=8). Statistics: GEE with Mann Whitney U post hoc test : * = p<0.05, ** = p<0.001 vs. vehicle.
2.4.2.2 Effect on Daily Food Intake

Figure 2.8 represents mean daily food intake of animals receiving PYY3-36 (375 nmol/kg/day) as a percentage of vehicle controls. A transient reduction in food intake was observed for the 3 days after pump implantation. PYY significantly reduced food intake on days 1 and 2 compared to vehicle controls. On Day 1, the PYY treated group consumed significantly less food than the vehicle group [food intake (g) Day 1: 4.45 +/- 0.13 (vehicle); 1.30 +/- 0.21 (PYY (p<0.001 vs. vehicle))]. On days 4 and 5, PYY significantly increased food intake compared to vehicle controls [food intake (g) Day 4: 4.73 +/- 0.12 (vehicle); 5.35 +/- 0.21 (PYY (p<0.001 vs. vehicle)); Day 5: 4.35 +/- 0.15 (vehicle); 5.33 +/- 0.26 (PYY (p< 0.05 vs. vehicle))]. There was no significant difference between the mean food intake of the PYY treated group and that of the vehicle group on days 3, 6 or 7.
Figure 2.8. Effect of 7 day continuous infusion of PYY3-36 (375 nmol/kg/day) on daily interval food intake expressed as percent of vehicle controls in mice. Data shown as mean +/- SEM food intake in C57/BL6 mice (n=8). Statistics: One way ANOVA with Dunnet’s post hoc test: *p<0.05, **p<0.01, ***p<0.001 vs. vehicle.
2.4.2.3 Effect on Cumulative Body Weight Change

A transient significant reduction in body weight was observed in mice receiving PYY3-36 (375 nmol/kg/day) on days 1-4 (figure 2.9). There was no significant difference in body weight between the vehicle treated and PYY3-36 treated groups on days 5, 6 or 7. Continuous subcutaneous infusion of PYY3-36 over a 7 day period did not result in a significant difference in body weight compared to vehicle controls (vehicle: 23.33 +/- 0.34; PYY3-36: 23.44 +/- 0.58 g).

Figure 2.9. Effect of 7 day continuous infusion of PYY3-36 (375 nmol/kg/day) on body weight in mice compared to vehicle controls. Statistics: GEE followed by Mann Whitney U post-hoc test. *** p<0.001, * p<0.05 PYY vs Saline.
2.4.3 The Effect of 3 Day Pair Feeding to PYY3-36 on the Subsequent Ad Libitum Food Intake in C57BL/6 Mice

2.4.3.1 Effect on Cumulative Food Intake

To investigate the effects of the initial 3-day anorectic phase on the subsequent orexigenic phase seen in the PYY3-36 treated mice, we utilised the pair-feeding model of food restriction.

On days 1-6, there was a significant reduction in the cumulative food intake in the group receiving PYY3-36 infusions compared to vehicle controls. The PYY PF group was given a vehicle infusion and received a quantity of food for each 24 hour period equivalent to the mean food intake of the group which received an infusion of PYY3-36 from the previous 24 hour period. Unsurprisingly, there was therefore a significant difference on days 1-4 between the vehicle infused group and the PYY PF group (p<0.001). On day 3, the vehicle treated group consumed a mean of 12.25 +/- 0.18 g, the PYY3-36 group consumed 7.69 +/- 0.21 and the PYY PF group consumed 8.01 +/- 0.03 g (Figure 2.10).

After day 3, the food restriction of the PYY PF group was removed and the animals returned to ad libitum feeding. There was an increase in food intake from day 4 onward in the PYY PF group and food intake returned to that of control levels. On days 5-7, the cumulative food intake in the PYY PF group was significantly greater than that of the PYY3-36 group (p<0.01). At the study termination, at day 7, the vehicle treated group had consumed a mean of 29.78 +/- 0.32 g, the PYY3-36 group consumed 27.91 +/- 0.44 and the PYY PF group consumed 30.51 +/- 0.13 g.
Figure 2.10. The Effect of 3 Day Pair Feeding to PYY3-36 on the Subsequent Ad Libitum Food Intake in C57BL/6 Mice. Cumulative food intake over a 7 day period of mice treated with Alzet osmotic minipump infusions of vehicle (black solid), PYY3-36 (375 nmol/kg/day) (blue solid) or vehicle and PYY PF (pair fed to PYY group for days 1-3, ad libitum fed for days 4-7 (light blue dotted). Statistics: GEE followed by Mann Whitney U test.* p < 0.05 PYY375 vs vehicle; *** p < 0.001 PYY375 vs vehicle; ††† p <0.001 Pair-fed vs vehicle; $$ p<0.01 PYY375 vs PYY PF. Dotted line at t=3 days represents initiation of ad libitum feeding phase of the PYY PF group.
2.4.3.2 Effect on Daily Food Intake

Figure 2.11 represents the daily food intake of the PYY3-36 and PYY PF groups expressed as percentage of vehicle control and demonstrates the significant decrease in daily food intake in the PYY3-36 and PYY PF groups compared to vehicle controls on days 1-3 (p<0.001), which is followed by a significant increase in food intake on day 4 and 5 compared to vehicle controls (p<0.001). On day 4, the PYY PF group consumed significantly more than the PYY3-36 group (p<0.001) before returning to a similar level on days 5-7.

Figure 2.11. Effects of pair-feeding a PYY-naive group to the food intake of a group of mice treated with continuous subcutaneous delivery of 375 nmol/kg PYY3-36 followed by ad-libitum feeding. Dotted line at t=3 days represents initiation of ad libitum feeding phase of the PYY PF group. Statistics: One way ANOVA with Dunnet’s post hoc test: ** p <0.01 PYY375 vs vehicle; *** p < 0.001 PYY375 vs vehicle; ††p <0.01 Pair-fed vs vehicle; ††† p <0.001 Pair-fed vs vehicle; $$$ p<0.001 PYY375 vs PYY PF.
2.4.3.3  *Effect on body weight*

There was a significant reduction in body weight in the PYY3-36 treated group on days 1 to 4 compared to vehicle controls (Figure 2.12). There was no significant difference in body weight between the vehicle treated and PYY3-36 treated groups on days 5, 6 or 7. Continuous subcutaneous infusion of PYY3-36 over a 7 day period did not result in a significant difference in body weight compared to vehicle controls (vehicle: 23.33 +/- 0.34; PYY3-36: 23.44 +/- 0.58 g). Pair fed mice exhibited a significant decrease in body weight on days 1 to 3 and returned to control levels during the *ad libitum* feeding phase.

![Graph showing body weight changes](image.png)

*Figure 2.12. Cumulative body weight change during pair feeding/food restriction on days 1-3 followed by *ad libitum* feeding on days 4-7. Statistics: GEE followed by Mann Whitney U test: * p<0.05 PYY3-36 vs vehicle controls; *** p <0.001 PYY375 vs vehicle controls, ††† p < 0.001 3-day Pair Fed group vs. vehicle controls. Dotted line at t=3 days represents initiation of *ad libitum* feeding phase of the PYY PF group.*
2.4.4 Plasma PYY3-36 levels following 7 day continuous infusion

RIA analysis of plasma PYY3-36 levels at the end of the 7 day infusion indicate a concentration of 10579 +/- 1145 pmol/L (n=9) immunoreactive PYY3-36 in the treatment group and 231 +/- 54 pmol/L (n=9) in the vehicle controls (p<0.001 PYY-IR vs. Vehicle) (Figure 2.13).

Figure 2.13. Plasma levels of PYY3-36 immunoreactivity following 7 days of continuous subcutaneous infusion of PYY3-36 (375 nmol/kg/day) or vehicle via Alzet osmotic minipumps as determined by RIA analysis (n=9). Statistics: One way ANOVA with Dunnett’s post-hoc analysis; ***p<0.001 PYY3-36 vs Vehicle.
2.4.4 Effect of 3 day low dose PYY pre-treatment on the effects of subsequent high dose treatment

2.4.4.1 Effect on cumulative food intake

To investigate if mice receiving continuous subcutaneous infusions of PYY3-36 exhibit a decreased sensitivity to the peptide, mice were given a low dose of PYY3-36 at either 50 or 200 nmol/kg/day or vehicle for days 0-3 and second infusion pump administering a high dose of PYY3-36 (1500 nmol/kg/day) on days 3-7 (Figure 2.14). Prior to second pump insertion, 3-day vehicle and 3 day 50 nmol/kg/day groups exhibited no significant difference in food intake from vehicle control groups. Prior to second pump insertion, the 3-day PYY3-36 200 nmol/kg/day consumed significantly less than vehicle controls (Day 3, vehicle: 13.11 +/- 1.81 g; Day 3, 200 nmol/kg/day: 10.65 +/- 1.04 g (p<0.001 (n=9)).

Following implantation of a second pump containing 1500 nmol/kg/day PYY3-36 on day 3, all treatment groups exhibited a significant reduction in food intake compared to vehicle controls, as determined using GEE analysis and Mann Whitney U post test. (Day 4, vehicle: 17.96 +/- 2.12 g; Day 4, vehicle + 1500 nmol/kg/day: 13.60 +/- 1.08 g (p<0.001 (n=9)); Day 4, 50 + 1500 nmol/kg/day: 14.45 +/- 1.19 g (p<0.001 (n=9)); Day 4, 200 + 1500 nmol/kg/day: 13.02 +/- 0.99 g (p<0.001 (n=9)).

Day 5, vehicle: 22.66 +/- 1.98 g; Day 5, vehicle + 1500 nmol/kg/day: 14.83 +/- 2.04 g (p<0.001 (n=9)); Day 5, 50 + 1500 nmol/kg/day: 15.93 +/- 1.11 g (p<0.001 (n=9)); Day 5, 200 + 1500 nmol/kg/day: 15.95 +/- 1.93 g (p<0.001 (n=9)). Day 6, vehicle: 27.19 +/- 1.72 g; Day 6, vehicle + 1500 nmol/kg/day: 16.42 +/- 1.27 g (p<0.001 (n=9)); Day 6, 50 + 1500 nmol/kg/day: 19.21 +/- 2.79 g (p<0.001 (n=9)); Day 6, 200 + 1500 nmol/kg/day: 19.20 +/- 2.96 g (p<0.001 (n=9)).]
Figure 2.14. Effect of continuous infusion of vehicle or low dose (50 and 200 nmol/kg/day) PYY3-36 from day 0-3 and vehicle or high dose (1500 nmol/kg) PYY3-36 from day 3-6 on cumulative food intake in lean C57BL/6 mice. Dotted line represents time of 2nd pump (1500 nmol/kg/day) implantation. *** p<0.001 PYY200 3d+ PYY1500 vs. Vehicle; $$$ p<0.001 PYY50 3d+ PYY1500 vs. Vehicle; ††† p<0.001 Vehicle 3d+ PYY1500 vs. Vehicle.
2.4.4.2 Effect on Daily Food Intake

Figure 2.15 represents the daily food intake following 3 day infusions of vehicle or PYY3-36 at 200 nmol/kg or 50 nmol/kg followed by a second 3 day infusion of vehicle or PYY3-36 at 1500 nmol/kg expressed as percent of vehicle control. This view highlights that there is a significant decrease in daily food intake in the PYY200 3d group compared to vehicle controls on days 1-3 (p<0.01). PYY3-36 at 1500 nmol/kg infusion significantly reduced food intake in the rats previously given 200 nmol/kg and 50 nmol/kg on days day 4 and 5 (p<0.01) compared to vehicle controls. PYY3-36 at 1500 nmol/kg significantly reduced food intake in rats which had previously received a vehicle infusion (Vehicle 3d + PYY1500) on days 4, 5 and 6 (p<0.001) compared to vehicle controls and on day 6 compared to rats previously given PYY3-36 at 200 nmol/kg.
Figure 2.15. Effect of continuous infusion of vehicle or low dose (50 and 200 nmol/kg/day) PYY3-36 from day 0-3 and vehicle or high dose (1500 nmol/kg) PYY3-36 from day 3-6 on daily interval food intake represented as daily food intake as percentage of vehicle. Statistics: One Way ANOVA with Dunnett’s post-hoc analysis: **p<0.01 PYY 200 3d vs Vehicle; †† PYY 50 3d vs vehicle; $$$ p<0.001 vehicle 3d+PYY1500 vs vehicle; & PYY 200 3d vs Vehicle 3d. Dotted line represents time of 2nd pump implantation.
2.4.5 The Effect of Food Restriction on the Subsequent Anorectic Effect of PYY3-36 Infusion

2.4.5.1 Effect on Cumulative Food Intake

To investigate if a period of controlled food restriction attenuates the anorectic effect of a subsequent infusion of PYY3-36, mice were introduced to reduced food for a period analogous to the initial anorectic phase of anorexia seen during continuous PYY3-36 administration (Figure 2.16). Mice which received a PYY3-36 at 250 nmol/kg/day via osmotic minipump infusion exhibited a significant decrease in food intake for the first 5 days of treatment compared to vehicle controls [food intake (g): vehicle: 11.34 +/- 0.58; PYY3-36: 7.59 +/- 0.62 (n=9) (p<0.05)]. Overall PYY3-36 (250 nmol/kg/day) infusion significantly decrease food intake compared to vehicle controls over the 10 day study period [food intake (g): vehicle: 27.73 +/- 0.81; PYY3-36: 23.06 +/- 0.77 (n=9) (p<0.05)].

A third, group (n=16) was food restricted by pair feeding to the median of the PYY3-36 (250 nmol/kg/day) group on days 1-5. On day 6, a 2nd minipump delivering either PYY3-36 or Vehicle was implanted into the food restricted mice and were fed ad libitum. The group which received PYY3-36 following food restriction exhibited a significant decrease in food intake compared to the group which received a pump infusing vehicle on days 7-10 [food intake (g): FR/Vehicle: 19.58 +/- 0.09; FR/PYY3-36: 14.58 +/- 0.15 (n=9) (p<0.01)].
Figure 2.16. Cumulative food intake over 10 day period following PYY3-36 (250 nmol/kg/day) or vehicle infusion via Alzet osmotic minipump in mice. A third group (FR; n=16) was pair fed to the median food intake of PYY3-36 group for days 0-6 and then received either PYY3-36 (FR/PYY3-36; n=8) at 250 nmol/kg/day or vehicle (FR/Vehicle; n=8) infusion on day 6 (denoted by arrow). Data expressed as mean +/- SEM. Statistics: GEE with Mann Whitney U test. * p<0.05 PYY3-36 vs Vehicle controls; †† p<0.01 FR/PYY3-36 vs FR/Vehicle.
2.4.5.2 Effect on Daily Food Intake

Figure 2.17 illustrates the daily changes of food intake during the experimental period. PYY3-36 infusion resulted in decreased food intake on days 1-5 and food intake returned to similar levels as vehicle controls. From days 7-10, mice which were food restricted on days 1-5 and received a subsequent PYY3-36 infusion exhibited a significant reduction in food intake compared to vehicle controls. Mice which received a vehicle infusion and *ad libitum* feeding following food restriction exhibited a pronounced increase in food intake compared to both vehicle controls and the FR/PYY3-36 group.

![Graph showing daily food intake](image)

**Figure 2.17.** Daily interval food intake over 10 day period following PYY3-36 (250 nmol/kg/day) expressed as % of vehicle controls (n=9). A third group (FR; n=16) was pair fed to the median food intake of PYY3-36 group for days 0-6 and then received either PYY3-36 (FR/PYY3-36; n=8) at 250 nmol/kg/day or vehicle (FR/Vehicle; n=8) infusion on day 6 (denoted by arrow). Data expressed as mean +/- SEM. Statistics: One Way ANOVA with Tukey post-hoc. *p<0.05, **p<0.01, ***p<0.001 PYY3-36 vs Vehicle; ††† p<0.001 PF/Saline vs Vehicle.
2.4.5.3 Effect on Body Weight

Both PYY3-36 infusion and food restriction resulted in a significant decrease in body weight in mice compared to vehicle controls until day 6. From day 6 onward, there was no significant change in body weight in the PYY3-36 treated group and the FR/Vehicle group. However, the mice which received a PYY3-36 infusion following food restriction (FR/PYY3-36 group) exhibited a significant decrease in body weight compared to both vehicle controls and the FR/Vehicle group (Fig. 2.18).

Figure 2.18. Cumulative body weight change over 10 day period following PYY3-36 (250 nmol/kg/day) expressed as % of vehicle controls (n=9). A third group (FR; n=16) was pair fed to the median food intake of PYY3-36 group for days 0-6 and was then divided to received either PYY3-36 (FR/PYY3-36; n=8) at 250 nmol/kg/day or vehicle (FR/Vehicle; n=8) infusion from day 6 (denoted by arrow). Data expressed as mean +/- SEM. Statistics: GEE with Mann Whitney U test.
2.4.6 Effect of 28 day PYY3-36 infusion on hypothalamic mY2 receptor expression

There was no change in the levels of hypothalamic mY2 receptor mRNA following 28 days of continuous PYY3-36 infusion via osmotic minipump compared to vehicle controls (Fig. 2.19).

Figure 2.19. Quantification of hypothalamic Y2 receptor expression in mice treated with a continuous infusion of saline or 250 nmol/kg/day PYY3-36 after 28 days. Expression levels normalised to mouse actin internal control of each sample.
2.5 Discussion

The experiments described in this chapter aimed to elucidate the mechanism underlying the apparent transient anorectic effect seen with continuous PYY3-36 administration. I hypothesised that the development of resistance to the anorectic effect of PYY3-36 observed during continuous infusion is independent from the stimulation of appetite resulting from weight loss, i.e. that the loss of sensitivity to PYY3-36 results from a desensitization to PYY itself and not from the homeostatic defence of body weight. I aimed to confirm this hypothesis by conducting a series of experiments in which PYY3-36 was infused via Alzet osmotic minipump under various conditions.

Firstly, I first verified the potent anorectic effect of a single, subcutaneous injection of three doses of PYY3-36 over a 24 hour period. This was performed to establish that administration PYY3-36 is capable of decreasing food intake in an acute setting. This effect is in line with published data and with previous experiments conducted in our lab (Abbott et al., 2005d).

Mice receiving a 7 day continuous infusion of PYY3-36 via osmotic minipump exhibited a biphasic anorectic profile. As expected, there was a significant reduction of food intake during the first 3 days of the infusion, followed by an orexigenic “overswing” on day 4 and 5. This characteristic transient anorectic effect has been described by several groups (Unniappan and Kieffer, 2008a; Reidelberger et al., 2008b; Chelikani et al., 2005a; Pittner et al., 2004b). However the mechanism underlying this attenuation of anorectic effect has not previously been investigated. This biphasic profile of food intake can be described as consisting of an “anorectic phase”, during the first 3 days following administration, followed by an orexigenic “rebound phase”.

To distinguish the degree of rebound increase in food intake, a pair feeding protocol was utilised. Pair feeding is an effective method of distinguishing if the effects of PYY3-36 are due to direct pharmacological actions or simply secondary effects related to anorexia. In this experimental design, one control group receiving a vehicle infusion minipump received a single portion of food each day equal to the average amount consumed by the PYY3-36 treated animals.
The pair fed group was given the mean food intake of the PYY3-36 group during the anorectic phase and was given ad libitum access to food from day 4 onward. Interestingly, this group exhibited a significantly greater degree of hyperphagia on day 4 compared to that receiving PYY3-36. Both the PYY3-36 treated and the pair fed/ad libitum groups lost a similar degree of body weight during the anorectic phase of the experiment (days 0-3). However, the subsequent overswing which resulted on day 4 was greater in the group not receiving PYY3-36. This suggests that PYY3-36 is continuing to exert a relative anorectic effect which may be masked by the animals’ compensatory drive to conserve body weight.

While pair feeding methodology is widely used in metabolic research, it is not without confounding factors. One group found that the pair feeding procedure alone affected the diurnal pattern of food intake in rats in that the food restricted animals consume their daily allotment within the first few hours and remain food deprived during the remainder of the 24 hour period (Russell et al., 2008). This feeding results in an intermittent feeding and fasting schedule which may have secondary physiological effects, such as changes to glucose homeostasis and lipid mobilisation from tissues.

To investigate if continuous exposure to PYY3-36 results in desensitisation, a dual pump procedure was employed. Mice were implanted first with pumps delivering a subanorectic low dose (50 nmol/kg/day), and anorectic low dose (200 nmol/kg/day). At the end of the anorectic phase on day 3, these groups were implanted with pumps delivering a high dose (1500 nmol/kg/day). There was a dose dependent reduction in the anorectic effect of high dose PYY3-36 dependent on the previous dose infused. Hence, mice which received 200 nmol/kg/day of PYY were less sensitive to 1500 nmol/kg/day than those which received 50 nmol/kg/day. High dose PYY3-36 had the greatest anorectic effect in mice which had received a 3 day vehicle infusion alone. This suggests that continuous 3 day stimulation of the Y2 receptor may result in some form of desensitisation, such as receptor downregulation, that is exhibited in a dose dependent loss of effect. However, the small but significant reduction of body weight may be contributing to the decreased effect of high dose PYY3-36 diminishing further weight loss, particularly in the group which was infused with 200 nmol/kg/day. While there was no change in hypothalamic Y2R mRNA levels following 28 days of continuous
PYY3-36 infusion, desensitization does not necessarily require a change in expression levels and it is possible that there were changes in Y2R expression in the brainstem and the periphery.

To further investigate if the decrease in anorectic effect is due to PYY3-36 exposure or to body weight loss alone, in experiment 4, mice were divided into 2 groups which were given either a PYY3-36 infusion or were food restricted by being pair fed to the median food intake of the PYY3-36 group for 6 days, during the anorectic phase. Due to the longer duration of this experiment, DIO mice were utilised instead of lean mice in order to obtain a sufficient degree of weight loss during the anorectic phase. At the end of the anorectic phase, the food restricted group was divided into subgroups which were implanted with either a 2nd vehicle pump or a PYY3-36 infusing pump and were allowed ad libitum access to food. Unsurprisingly, the group allowed to feed ad libitum exhibited a pronounced overswing hyperphagia on days 7 and 8. Surprisingly, PYY3-36 exerted a similarly potent anorectic effect in the mice which were previously food restricted to that observed in the PYY3-36 controls. It therefore appears that mice can retain sensitivity to the anorectic effects of PYY3-36 despite having lost approximately 16% of their starting body weight within a short period of time. Based on this experiment, one can infer that the resistance to the anorectic effect of continuous infusion of PYY3-36 is not simply due to the homeostatic defence of body weight, but due to a direct desensitisation to the effects of PYY3-36. However, it is important to note that while there is a transient anorectic effect of PYY3-36, it is lacking in the orexigenic overswing seen in mice which are food restricted and then allowed to eat freely. It is clear that continuous PYY3-36 administration does not result in complete desensitisation, preserving sufficient bioactivity to prevent the compensatory hyperphagia that would otherwise follow a period of decreased food intake.

Based on the results described in this chapter, dosing strategy is critical to maintaining sensitivity to PYY3-36. I hypothesise that during continuous infusion, the Y2 receptor is overstimulated and becomes at least partially desensitized. However it may be possible to overcome the homeostatic defence of body weight by giving frequent, but intermittent administration of PYY3-36, thus maintaining a pulsatile dosing profile in the blood. This would mimic the physiological release patterns of endogenous PYY3-36 and may therefore be a more effective pharmacotherapy for obesity.
Central homeostatic control mechanisms maintain body weight (or fat mass or energy content) at a given level, opposing change, and restoring it following any significant deviation. Hence, if body weight drops, the brain senses the change and acts to oppose it by, for example, by slowing metabolic rate or stimulating hunger and food seeking behaviours rate, to restore body weight to the set point value (Keesey and Powley, 1986; Levin and Keesey, 1998). In the experiments described in this chapter, I have shown that, while there is a homeostatic drive to restore body weight and food intake to baseline levels, the pharmacological administration of the anorectic peptide hormone PYY3-36 can override this physiological control mechanism. This apparent transient anorectic effect observed with continuous PYY administration has not previously been investigated by directly comparing the rebounding hyperphagia immediately following an anorectic period. This direct comparison has determined that PYY3-36 is continuing to exert an appetite suppressing action to some degree, preventing the profound orexigenic overswing usually observed following prolonged food restriction. Thus, PYY3-36 and the Y2 receptor system may be a feasible pharmacotherapeutic target for the treatment of obesity.
Chapter 3:
Investigation of Dual GLP-1/GCG Receptor Agonists on Energy Homeostasis
3.1 Introduction

3.1.1 Glucagon and Energy Homeostasis

Emerging evidence suggests novel roles for glucagon in addition to its highly researched effects on glucose homeostasis. Glucagon is specifically expressed in the alpha cells of the pancreas and once secreted travels via the hepatic-portal circulation to target tissues, most notably the liver where its action via a specific extracellular receptor leads to increased glucose production (Ritter et al., 1986). The expression of the GCGR in extrahepatic tissues, including brown and white adipose tissue, skeletal muscle, adrenals and the hypothalamus, suggests a broader physiological role beyond glucose homeostasis.

Recent studies have reported that glucagon influences energy management. However, the molecular mechanisms by which glucagon exerts its reported affects on food intake, body weight, energy expenditure, and thermogenesis remain poorly understood. It has been proposed that glucagon increases energy expenditure via brown adipose tissue (BAT) mediated thermogenesis. Cold exposure increases circulating plasma glucagon levels in rats, which may implicate a role for glucagon in non-shivering thermogenesis (Edwards and Howland, 1986). *In vitro* administration of high concentrations of glucagon directly to brown adipose tissue was associated with a rise in oxygen consumption and heat production (Joel, 1966; Kuroshima and Yahata, 1979). Administration of glucagon in rats resulted in an increase in whole body oxygen consumption and core body temperature as well as an increase in BAT size and temperature (Doi and Kuroshima, 1982; Billington et al., 1991). This putative role of BAT in mediating the effects of glucagon on energy expenditure is of particular interest as recent evidence has emerged of the importance of BAT to energy metabolism in humans (Cypess et al., 2009). Pharmacological infusions of glucagon into rats and humans increases oxygen consumption and resting energy expenditure (DAVIDSON et al., 1957a; Nair, 1987). Interestingly, hyperinsulinemia was found to attenuate the thermogenic effect of glucagon (Calles-Escandon, 1994; Markovic et al., 1995).
However, the physiological relevance of glucagon stimulated BAT thermogenesis is unclear as the doses used to elicit these effects are pharmacological. Only a single study has reported that infusion of GCG at physiological levels for 5 days in rats stimulated GDP binding in the mitochondria of brown fat, an indirect measure of UCP1 activity (Billington et al., 1991). Additionally, another study failed to demonstrate an increase in BAT temperature following GCG administration at physiological levels (Dicker et al., 1998). Therefore, it has been postulated that multiple systems may be involved in mediating the effects of glucagon on energy expenditure and thermogenesis, such skeletal muscle and WAT.

As mentioned, the mechanism by which GCG increases energy expenditure remains unclear. GCG may stimulate BAT mediated thermogenesis by activating UCP1 or by activating the transcriptional regulators: peroxisome proliferator-activated receptor gamma (PPARγ) or PPARγ coactivator-1α (PGC1α). PPARγ belongs to a family of transcriptional regulators which are involved in both white and brown adipocyte differentiation. PPARγ has also been reported to increase mitochondrial biogenesis in white adipocytes and treatment of obese mice with PPARγ agonists result in the formation of more insulin-sensitive adipocytes that may improve metabolic disease (Lindgren et al., 2004; Petrovic et al., 2010). PGC1α is abundantly expressed in BAT and is further increased during cold exposure and noradrenergic activation (Esterbauer et al., 1999). PGC1α appears to be required for the activation of brown fat thermogenesis, as UCP1 activation following β-adrenergic stimulation is abolished in PGC1α knockout mice (Uldry et al., 2006). PGC1α is also expressed in the liver, where it is an important regulator of gluconeogenesis during fasting. PGC1α has been shown to stimulate genes of fatty acid oxidation in hepatocytes, and induction is associated with an increase in fatty acid oxidation in adipocytes and in the liver (Puigserver and Spiegelman, 2003).

Glucagon may also play a role in the short term regulation of appetite. Early studies showed that hepatic-portal infusions of glucagon decreased meal size and food intake in rats (Martin and Novin, 1977). Similarly, i.v. infusion of glucagon resulted in a dose-dependent decrease in meal size in humans (SCHULMAN et al., 1957; PENICK and HINKLE, Jr., 1961). Further investigation into
glucagon-induced satiety showed that endogenous levels of glucagon increase during a meal and that intraperitoneal injections of anti-glucagon antibodies resulted in an increase in both meal size and meal duration in rats (Langhans et al., 1982). Given its effects on energy expenditure and food intake, glucagon agonism may be useful in the treatment of obesity and the metabolic syndrome.

### 3.1.2 Exendin 4

Exendin-4 (Ex-4) is a naturally occurring, DPP-IV resistant, 39 amino acid GLP-1 receptor agonist originally isolated from the venom of the *Heloderma suspectum* “Gila Monster” lizard (Eng et al., 1992). Ex-4 shares 53% sequence homology with mammalian GLP-1, and shares the glucoregulatory actions of GLP-1 which are mediated by the GLP-1R (Xu et al., 1999). These glucoregulatory actions of Ex-4 include the enhancement of glucose-dependent insulin secretion and the inhibition of inappropriately high glucagon secretion (Egan et al., 2002; Parkes et al., 2001; Young et al., 1999; Drucker and Nauck, 2006). In addition, Ex-4 potently inhibits food intake and gastric emptying (Szayna et al., 2000). There is also convincing evidence that exendin-4 promotes pancreatic β-cell proliferation and islet neogenesis *in vivo* and *in vitro* (Xu et al., 1999; Tourrel et al., 2001). As a result, Ex-4 has been extensively utilised for the treatment of type 2 diabetes (Schepp et al., 1994; Goke et al., 1993). Synthetic exendin-4 (Exenatide) treatment has been shown to significantly reduce HbA1c levels, a marker of long term glucose levels, in poorly controlled diabetics treated with metformin and sulphonylurea (Kendall et al., 2005). In addition, two daily subcutaneous injections of Exenatide prevented weight gain and in some cases caused modest weight loss over a 30 week treatment period in a phase III clinical trial (Blonde et al., 2006). However, similar to GLP-1, treatment with Ex-4 was associated with nausea, thereby potentially limiting the dose that can be administered.
3.1.3 GLP-1 and Glucagon receptor co-agonism

Two prominent studies published in 2009 reported that glucagon agonism may be beneficial to the pharmacological treatment of obesity and, potentially, diabetes when combined with simultaneous GLP-1 receptor agonism (Kendall et al., 2005; Day et al., 2009; Pocai et al., 2009). Both studies utilised synthetic GLP-1/GCG receptor dual agonists and found that co-agonism significantly decreased food intake, body weight and fat mass compared to a GLP-1R agonist monotherapy. Substantial decreases in blood glucose and insulin levels and improved glucose tolerance were also reported. Pocai and colleagues reported that a GLP-1R/GCGR dual agonist increased ketogenesis and decreased plasma lipids after 1 month of chronic treatment. Chronic treatment with this dual agonist also resulted in superior weight loss in DIO mice compared to a GLP-1R agonist alone. Instead of causing hyperglycemia as might be expected by GCGR agonism, glucose levels were normalised and glucose tolerance was comparable between both the dual agonist and GLP-1R agonist groups. Most notably, improvements in metabolic parameters such as plasma insulin, leptin, and adiponectin were more pronounced following chronic treatment with the dual agonist than with GLP-1R only agonist (Pocai et al., 2009). It is hypothesized that GLP-1R/GCGR dual agonists act directly on the CNS to reduce food intake, on the adipose tissue to stimulate lipolysis and on the liver to exert effects on gluconeogenesis and glycogenolysis (Figure 3.1).
Figure 3.1. Schematic of proposed mechanism of action of dual agonist. In addition to the known effects associated with GLP1R activation, hepatic GCGR activation increases liver glucose production and stimulates fatty acid oxidation. In WAT, pharmacological activation of GCGR and GLP1R may stimulate hydrolysis of triglycerides. In BAT, GCGR activation may enhance UCP1 mediated thermogenesis (Pocai et al., 2009).

3.1.4 Design of GLP-1 and Glucagon receptor co-agonists

GLP-1 is a short-acting signal and is therefore rapidly degraded by endopeptidases in the circulation (Drucker and Nauck, 2006). Degradation by the enzyme dipeptidyl peptidase IV (DPP-IV) is understood to be the main reason for the very short half-life of GLP-1. DPP-IV cleaves between positions 2 and 3 to render the molecule inactive (Deacon et al., 1998). DPP-IV is selective with regard to the residue present at position 2 of a peptide. Peptides like GLP-1 with an alanine residue at
this position are hydrolysed at a considerably faster rate than those, like glucagon, with a serine at this position (Bongers et al., 1992). DPP-IV cleaves GLP-1 at a rate many hundred times faster than it does exendin-4 (Hupe-Sodmann et al., 1995). The difference is likely to be due to in part to the nature and number of available cleavage sites and is partly responsible for the extended half-life of exendin-4. This could itself be at partially attributed to changes in the nature and number of available cleavage sites on the molecule (Hupe-Sodmann et al., 1995).

It is also possible that the increased helicity provided by an exendin-like central helix region may sterically hinder enzymatic interactions. Exendin-4 interacts with the GLP-1 receptor via its central helix and a 9 amino acid C-terminal extension that is not present in endogenous GLP-1. NMR analysis suggests that this tail region forms a compact folding unit called a ‘Trp-cage’ (Neidigh et al., 2001). This region may be exploited as a potential prosthetic extension to dual agonist analogues to increase their binding activity (Al-Sabah and Donnelly, 2003). Additionally, it has been shown that Exendin-4 contains considerably more α-helical regions than GLP-1 when studied in an aqueous solution (Wang et al., 2011; Neidigh et al., 2001), which may play a role in stability in vivo.

Chimeric peptides of GLP-1 and glucagon have been utilised to identify the regions of these peptides that confer specific selectivity for the two receptors. Selective recognition of the GCGR is conferred by the N-terminal of GCG and the GLP-1R by the C-terminal of GLP-1 (Runge et al., 2007). This was supported by the observation that a chimeric peptide consisting of the N-terminal part of the GCG molecule joined to the C-terminal of GLP-1 molecule is recognised with high affinity by both receptors (Runge et al., 2007; Hjorth et al., 1994). Furthermore, the high affinity GLP-1 receptor binding of the N-terminally truncated Exendin(9-39) peptide indicates that the N-terminal region is unnecessary for binding (Thorens et al., 1993; Goke et al., 1993). The toleration of these receptors to substitutions at the less vital terminal of their endogenous ligands conveniently allows the simultaneous replacement of the vulnerable DPP-IV cleavage sites in GLP-1 with a degradation resistant sequence. Such a molecule might therefore form a basis for the design of a long acting dual agonist.
In addition, it has been shown that systematic substitutions introduced in divergent residues of the molecules are well tolerated and may act to increase binding, activation and proteolytic resistance. For example, Gelvanov and colleagues reported that a substitution of a leucine (Leu) for the endogenous methionine residue at position 27 eliminates oxidative degradation and that the introduction of a lysine (Lys) at positions 20 or 24 increased potency at both receptors (2005). Hjorth and colleagues found that amino acid modifications in the C-terminal part of the glucagon molecule had a minimal effect on GCGR recognition but vastly improved affinity for the GLP-1R (1994). Additionally, the introduction of residues present in the glucagon sequence in a GLP-1 like molecule at positions 20 and 24 increased binding to the GCGR without compromising GLP-1R activity (Hjorth et al., 1994).

A combination of these different strategies could lead to the development of a long acting, enzyme resistant, dual agonist capable of inhibiting food intake, improving glucose tolerance and increasing energy expenditure in obese diabetic patients.
3.2 Hypothesis and Aims

Hypothesis:
Specific modification to the amino acid sequence of a GCG-Ex-4 (GX) chimera peptide may result in a dual agonist at the GCG and GLP-1 receptors which decreases food intake and increases energy expenditure in rodents.

Aims:
- To evaluate the GCG-Ex-4 chimeras relative binding affinity and activation at the GLP-1 and GCG receptors
- To evaluate the GCG-Ex-4 chimeras acute effects on food intake in mice and rats
- To assess the circulating half-life of a leading GCG-Ex-4 chimera
- To assess the effect of this leading GCG-Ex-4 chimera on energy expenditure in rats
3.3 Methods

3.3.1 Peptide Synthesis

GLP-1(7-37), Exendin-4, Oxyntomodulin (OXM) and Glucagon were purchased from Bachem Ltd. GX chimera analogues were synthesized by Advanced Biotechnology Services, Imperial College London (London, UK) and Biomol International LP (Exeter, UK). Peptides were synthesised and purified using the approach described below. Peptides were synthesized on resins derivatised with one of a number of cleavable linkers, using an Fmoc/t-butyl-based solid-phase synthesis strategy (Wellings and Atherton, 1997). Temporary Nα-amino group protection was afforded by the Fmoc-group, with t-butyl ethers being utilised for the protection of Tyr, Ser and Glu residues. His and Lys side chains were protected as their Nτ and Nε-tertbutyloxy-carbonyl (Boc) derivatives respectively, cysteine as its S-trityl derivative, and arginine guanidine moiety as its Pbf derivative. Where Nε-acylation of Lys was required, orthogonal protection was afforded by the incorporation of 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)3-methyl-butyl Lys (Lys(ivDde)), which was deprotected on solid phase using 2% hydrazine in DMF. Peptides were purified by reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. The purified product was subsequently analysed by reverse-phase HPLC and matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-ToF MS). For GX peptide sequences, see Figure 6.1 (page 191).

3.3.2 Receptor Binding Assays (RBAs)

3.3.2.1 Cell and Tissue Membrane Preparation

The binding affinity of a peptide is its ability to displace the radio-labelled endogenous ligand from a receptor. To measure the affinity of synthetic chimera peptides for the human GLP-1 and GCG receptors, cell membrane (200ng) from human embryonic kidney (HEK) 293 cells over-expressing the human GLP-1 receptor (hGLP-1R) and the human GCG receptor (hGCGR) were used for RBAs.
The GLP-1R and GCGR over-expressing cell membranes were kindly provided by Dr James Minnion, Section of Investigative Medicine, Imperial College London.

To isolate membranes expressing the rat GLP-1 and GCG receptors, lung and liver samples from rats were used for their high expression of GLP-1 and GCG receptors respectively. Tissues were removed from the animal immediately after death and snap frozen in liquid nitrogen before being stored at -80°C until used for membrane preparations. For the preparation of a membrane suspension from a tissue, the tissue was first homogenised in ice cold homogenisation buffer with sucrose (50mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.25M sucrose, with protease inhibitors using, the Ultra-Turrax motor-driven homogeniser. The homogenised tissue was then centrifuged at 1000g at 4°C for 20 minutes. The supernatant was kept and centrifuged again at 1000 g at 4°C for 1 hour. The supernatant was discarded and the pellet re-suspended in homogenisation buffer without sucrose before a further centrifugation at 1000 g at 4°C for 1 hour. The supernatant was discarded, with the pellet retained and re-suspended to a final protein concentration of 1-2mg/ml. Protein concentration was measured using the Bradford assay, and the membrane aliquotted and stored at -80°C.

### 3.3.2.2 Receptor Binding Assays

To measure the affinity to the GLP-1R, a range of concentrations of unlabeled peptide were made in assay buffer and added to siliconised polypropylene microtubes (Sigma-Aldrich, Dorset, UK). $^{125}$I-GLP-1 in assay buffer (50µl) (See Appendix B) at 1000 counts (Bq) per second was added to the tubes, followed by 10µl of the membrane suspension (at protein concentration 1-2µg/ml). The range of concentrations of peptide was also added to various tubes to form a binding curve. All concentrations were assayed in duplicate or triplicate. Tubes were each vortexed and incubated at room temperature for 90 minutes. Tubes were then centrifuged at 15700g (Sigma Laboratory centrifuges 3 K18, Rotor No. 19777-H) at 4°C for 3 minutes, the supernatant was removed and discarded, and another 500µl of assay buffer added to wash the pellet of unbound radio-ligand, before
being centrifuged as before. The supernatant was again discarded and the γ radiation in the pellets were counted for 240 seconds (Gamma counter, NE 1600, NE Technology Ltd, Reading, UK). The inhibition coefficients (IC$_{50}$) were then calculated and compared for each peptide tested. IC$_{50}$ values were calculated using the Prism 5.01 program (GraphPad Software Inc. San Diego, USA) using the following regression fit equation:

$$Y = Bottom + \frac{(Top - Bottom)}{(1+10^{(\frac{LogEC50}{X})})}$$

For measuring the affinity for the GCGR, the assay was completed as above with the exception of using specific GCGR RBA assay buffer (see Appendix B). The radiolabel used was $^{125}$I-Glucagon (specific activity 34 Bq/fmol).

### 3.3.3 cAMP Accumulation Assay

This assay measured cyclic adenosine monophosphate accumulation in cells in response to incubation with putative receptor agonists. HEK293 cells overexpressing the hGLP-1R or hGCGR were plated onto 24 well plates one day prior to assay at 50,000 cells/well in 500 uL of standard media (DMEM 6429, with L-glutamine, 4.5 g glucose, sodium pyruvate, Sigma-Aldrich, UK) supplemented with 1% fetal calf serum (FCS) and 5 mL PenStrep 20/20 (penicillin and streptomycin antibiotics). On the following day, the media was removed and replaced with FCS-free DMEM media and cells incubated for 1 hour. An appropriate range of concentrations of a peptide to be tested were made in serum free DMEM containing 1mM of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich, UK). The serum free media was removed from each well and replaced with incubation media containing the test peptide. The cells were incubated for a further 30 minutes. The media was removed and replaced with 110ul lysis buffer (0.1M HCl with 0.5% Triton-X) and incubated for 10 minutes. The lysis buffer now containing accumulated cAMP was then transferred into a microtube and centrifuged at 15800 g for 3 minutes. The supernatant was diluted at a ratio of 1:10 and assayed using a ‘Direct cyclic AMP Enzyme-linked Immunosorbant assay (ELISA) kit’
(ADI-900-066, EnzoLifeSciences, UK) as described in the assay manual. Briefly, 50 uL of Neutralising Reagent (provided) was added to each well except the total and blank wells. Provided standards (100 µl) and samples (100 µl) were added to appropriate wells before adding 50 µl of blue conjugate and 50 µl of the yellow antibody into each well. The plate was then sealed and left to incubate at room temperature for 2 hours on a plate shaker (~500 rpm). Contents were emptied from wells and washed three times with 400 µl of wash buffer. ‘Substrate solution’ (200 µl) was added to each well and left to incubate on bench top for 1 hour. After this incubation, 50 µl of ‘stop solution’ was added into each well, and optical density was read at 405 nm on a plate reader (Multiskan RC 381, Labsystems, MA, USA).

3.3.4 In vivo studies

3.3.4.1 Animals

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402). Adult male C57BL/6 mice (Harlan, UK) weighing 20-25g or adult male Wistar rats (Charles River, UK) weighing 300-400g were maintained in individual cages under controlled temperature (21-23°C) and lights (12:12 h light:dark cycle, lights on at 0700h). The light cycle is particularly relevant to feeding studies as rodents are nocturnal feeders and eat only a small proportion of their daily caloric intake during the light phase, demonstrating a dramatic increase in food intake after the onset of darkness (Lu et al., 2002a). Animals had ad libitum access to water and RM1 diet (Special Diet Services Ltd, Witham, Essex, UK). Animals were handled daily for three days prior to their first study in order to acclimatise the animals to the handling procedure. Animals were given two sham injections 2-4 days before the start of the study to acclimatise them to the injection procedure and to minimise any potential non-specific effects due to stress.
3.3.4.2 Acute Feeding Studies in Mice

The acute feeding studies in mice were carried out during the early light phase (0900h). Mice were fasted from the 1500h the previous day. Mice were randomised into groups of approximately equal average body weight and administered a SC injection of either saline or test peptide (50 nmol/kg). Peptides were dissolved in a maximum volume of 50µl saline. Body weight was recorded before injection. Mice were returned to their cages after injection with access to a pre-weighed amount of food. Food was then weighed at 1, 2, 4, 8 and 24h intervals post-injection and the body weight recorded at 24 h post-injection.

3.3.4.3 Acute Feeding Studies in Rats

The acute feeding studies in rats were carried out during the early light phase (0900h). Rats were fasted for 24 hours prior to injection. Rats were randomised into groups of approximately equal average body weight and administered a SC injection of either saline or test peptide (50 nmol/kg). Peptides were dissolved in a maximum volume of 50 µl saline. Body weight was recorded before injection. Rats were returned to their cages after injection with access to a pre-weighed amount of food. Food was then weighed at 1, 2, 4, 8 and 24 hour intervals post-injection and the body weight recorded at 24 hours post-injection.

3.3.4.4 Pair Feeding

Pair feeding is an experimental method in which food intake is limited in one group to the average level of another group. As a result, energy intake is controlled between groups. Any changes in body weight or other parameters observed between the groups can then be assumed to be due to effects independent of food intake elicited by the test substance itself. Male Wistar rats weighing an average of 530 g were randomised into groups of 8 to receive 50 µl daily subcutaneous injections of: Saline,
the dual agonist GX6 (30 nmol/kg) or Exendin-4 (30 nmol/kg). A further 2 groups (n=8) received saline injections and received a quantity of food equivalent to the mean amount consumed (to the nearest 0.1 g) rats receiving GX6 or Exendin-4 in the previous 24 hours. Injections were administered daily during the late light phase (1700-1800h) for 10 days, at which point food and body weights were recorded.

3.3.4.5 Pharmacokinetic analysis

A study was carried out to determine the plasma clearance of the chimera GX6 following subcutaneous administration. Male Wistar rats were randomised by body weight into a group of 5 and each administered 500 nmol/kg of GX6 at time 0. At 0 hours prior to injection and at 4, 24, 36 hours and 3 days following administration, blood was collected by superficial venesection of the lateral tail vein. Eppendorfs were flushed with 1:10 heparin:saline prior to sample collection to prevent clotting. No more than 300 uL of whole blood was collected into an eppendorf and placed on ice. Samples were centrifuged for at 4°C for 10 minutes at 15,000 x g (Sigma Laboratory centrifuges 3 K18, Rotor No. 19777-H) and the upper plasma layer was isolated and stored at -20°C until RIA analysis.

3.3.5 Radioimmunoassay

GX6-like immunoreactivity was measured with a specific and sensitive radioimmunoassay, as described in Appendix B. The antiserum (R2461) was produced in rabbits against purified GX6 coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:1500. This antibody cross reacts to the N-terminal sequence of GX6 and does not cross-react with other known gastrointestinal hormones. $^{125}$I-GCG was prepared by the direct iodination method and purified by reverse phase high-pressure liquid chromatography. The assay was performed in a total volume of 0.7 ml of 0.06 M phosphate buffer, pH 7.4, containing 0.3% bovine serum albumin. The assay was incubated for 3 days at 4°C before charcoal separation of the free and antibody-bound label.
3.3.6 Metabolic Gene Expression Analysis

3.3.6.1 Animals and Tissues

Male Wistar rats were subcutaneously injected with saline, GX6 (30 nmol/kg), Ex4 (30 nmol/kg), GCG (900 nmol/kg), CL316 (1mg/ml) (a β3-adrenergic agonist, Sigma-Aldrich, UK) (n=8) and returned to their cages. After 6 hours, rats were culled by CO₂ asphyxiation and approximately 100 mg of intrascapular BAT, inguinal white adipose tissue (WAT), liver and skeletal muscle (gastrocnemius) immediately excised, cut into pieces no larger than 5mm³ and placed in RNALater stabilisation reagent (Ambion, UK) and stored at -20°C:

3.3.6.2 mRNA extraction

Total RNA was extracted from liver and BAT samples using the RNEasy Mini Kit column (Qiagen, UK). Approximately 30 - 40 mg of tissue was added to a 2 mL Eppendorf containing 600 μL RLT lysis buffer with 12 μL 2 M dithiothreitol (DTT) and a steel cone bead (7 mm diameter, Retch, Qiagen, UK). Samples were homogenised by high speed shaking using a Qiagen Tissuelyser II (Retch, Qiagen, UK) for 2 x 2 min at 20 Hz. Samples were centrifuged at max speed (>8000g, 13000 rpm, Biofuge Pico centrifuge, Heraeus, UK) for 3 minutes and supernatant (approximately 700 μL) was removed and placed into a fresh 1.5 mL Eppendorf. One volume of 70% ethanol was added to the lysate and mixed by pipetting. Then, 700 μL of the sample, including any precipitates, was added to an RNeasy Mini spin column placed in a 2 mL collection tube. The column was centrifuged at maximum speed for 15s and the flow through was discarded. This was repeated with the remainder of the sample. Next, 350 μL of wash Buffer RW1 was added to the RNeasy spin column and centrifuged for 15s at maximum speed. The flow through was discarded.

On column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). To prepare the DNase I stock solution, 500 μL RNase-free water was injected into the DNase I vial using an RNase-free needle and syringe. The vial was mixed gently by inverting, and aliquoted for long term
storage at -20°C. Next, 70 µL of Buffer RDD was added to 10 µL of DNase I stock and mixed gently by inverting and briefly centrifuging. The DNase I incubation mix (80 µL) was added directly onto the RNeasy column membrane and left at room temperature (20-30°C) for 15 minutes.

Following this, 350 µL Buffer RW1 was added to the spin column, centrifuged for 15s at maximum speed and the flow through was discarded. Then, 500 µL of Buffer RPE was added to the spin column, centrifuged at maximum speed for 15 s, and flow through discarded. Again, 500 µL of Buffer RPE was added once again and centrifuged at maximum speed for 2 minutes. The RNeasy spin column was then transferred to a new 2 mL collection tube and centrifuged at full speed for 2 minutes to dry. Finally, to elute the RNA, the spin column was placed into a new 1.5 mL eppendorf and 30 µL of THE RNA storage solution (1 mM sodium citrate, pH 6.4; Ambion) was added directly to the membrane and centrifuged for 1 minute. To increase the yield of RNA, this elution step was repeated using the same collection tube resulting in a total volume of 60 µL. Each RNA sample was vortexed and divided into a 50 µL aliquot for long term storage at -80°C, and a 10 µL aliquot for integrity analysis.

Brown adipose tissue was extracted using the procedure described above, with several exceptions. Lysis and homogenisation was performed in 1 mL of the phenol/guanidine-based QIAzol lysis buffer (provided in the RNeasy Lipid Tissue Minikit, Qiagen, UK), followed by a 5 minute incubation at room temperature. Chloroform (200 µL) was then added followed and samples were vortexed for 15 seconds. Following this, all samples were centrifuged for 15 minutes at 4°C (Sigma Laboratory centrifuges 3 K18, Rotor No. 19777-H). The uppermost aqueous layer (approximately 600 µL) was removed and transferred to a new 1.5 mL tube and 1 volume of 70% ethanol was added. Extraction proceeded using the RNeasy spin column as described above.
3.3.6.3 RNA Quantification and Assessment of Quality

3.3.6.3.1 Spectrophotometry

RNA concentration and purity was determined using a Nanodrop 3300 spectrophotometer (Thermo Fischer Scientific Inc.). The absorption ratios, 260/280 nm and 260/230 nm, are measures of relative purity of a nucleic acid sample and assess the presence of protein or other contaminants. RNA samples are regarded “pure” when these ratios are ~2.0 and in the following experiments, only samples with ratios of >1.8 were bioanalysed.

3.3.6.3.2 Electrophoresis

Due to the omnipresence of ribonucleases (RNases) and the instability of RNA, it is important to quantify and check the integrity of the RNA prior to qPCR. The Agilent 2100 Bioanalyzer (Agilent Biotechnologies Inc.), was used to generate an RNA Integrity Number (RIN), quantification estimate, ribosomal ratios of the total RNA sample and an entire electrophoretic trace of the sample.

To avoid decomposition of the RNA samples, the bioanalyser electrodes were first decontaminated for 1 minute using 350 μL of RNaseZap reagent (Ambion, Inc). To prepare the gel-dye mixture, 1 μL of dye was added to 65 μL of filtered gel, vortexed for 10 seconds and centrifuged at 13000xg for 10 minutes. Next, 9 μL of the gel-dye mixture was carefully added to the designated wells of a RNA Nano 6000 chip and primed for 30 seconds using a chip Priming Station. Each remaining well then received 5 μL of the loading RNA Nano marker. RNA samples to be analysed were diluted using THE to a concentration of 5-500 ng/μL and 2 μL of each sample, including an RNA ladder, were then denatured for 2 minutes at 70°C. Next, 1 μL of the denatured RNA ladder and of each sample was loaded into the wells of the chip and vortexed for 60s at 2400 rpm. The chip was then placed into the bioanalyser so that the electrodes fit into each well, and was analysed using the Agilent 2100 Expert Software. See Appendix D for sample traces.
3.3.6.4 Reverse Transcription

RNA samples were reverse transcribed into cDNA using the Superscript VILO cDNA synthesis kit (invitrogen). Firstly, each RNA sample was diluted with THE to a final concentration of 200 ng/μL. Each sample was reverse transcribed in triplicate in a 96 well plate, with each well containing:

2 μL 5x VILO Reaction Mix
1 μL 10x SuperScript Enzyme Mix
3 μL RNA sample (600 ng)
4 μL RNase free water

A foil seal was placed on the plate and samples were placed on a shaker for 3 minutes before centrifuging for 15 seconds. The plate was placed in a thermal cycler (MJ Research, Programmable Thermal Cycler 200, GRI, Inc, UK) to incubate at 25°C for 10 minutes, 42°C for 120 minutes and 85°C for 5 minutes before being stored at -20°C.

3.3.6.5 Quantitative Polymerase Chain Reaction (qPCR)

The relative expression of metabolic genes of interest was quantified using TaqMan® Gene Expression Assays (Applied Biosystems, Life Technologies, UK). Inventoried primers containing a predesigned, preformulated primer and probe set targeting the following rat gene were purchased:

- PPARγ (Peroxisome proliferator activated receptor gamma), Rn00440945_m1
- PPARα (Peroxisome proliferator activated receptor alpha), Rn00566193_m1
- PGC1α (PPARγ coactivator 1-alpha), Rn00580241_m1
- UCP1 (Uncoupling protein 1), Rn00562126_m1
- G6P (Glucose-6-Phosphatase), Rn00689876_m1
- PCK1a (Phosphoenolpyruvate carboxykinase 1), Rn01529014_m1
- FGF21 (Fibroblast growth factor 21), Rn00590706_m1
- Endogenous controls:
  - ACTB (β-Actin), Rn00667869_m1
  - B2M (β2-microglobulin), Rn00560865_m1

TaqMan® Gene Expression Assay allows accurate quantification of gene expression by incorporating a reporter-tagged probe, which is complementary to part of the targeted cDNA sequence, into the amplification process. During PCR, the probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. The DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye to allow fluorescence of the reporter.

The following constituents were mixed per well of a 384-well plate for each qPCR reaction: 1µl of cDNA template (600ng/µl), 10µl of 2 x TaqMan® Gene Expression Master Mix, 1µl of specific 20 x TaqMan® Gene Expression Assay, and GDW to give a 20µl reaction volume. Each reaction was mixed before the plate was sealed. Each reaction was assayed in triplicate.

The sealed plate was loaded onto a thermal cycler (Applied Biosystems 7900HT Real-Time PCR System), on the ΔΔCt programme, under the following conditions: 15 seconds incubation at 95°C followed by 1 minute incubation at 60°C, repeated for 40 cycles. The amplification data for each sample was corrected against its respective endogenous control.

### 3.3.7 Statistics

All data are expressed as the mean value +/- SEM. Interval food intake was compared by one-way ANOVA followed by post-hoc Dunnett’s correction. This is a method used to study longitudinal data which consists of repeated measures of an individual or group of individuals taken over time. These repeated measures from any one individual or group are correlated with each other and are therefore
no longer independent. Generalized Estimation Equations (GEE) use the data to estimate the correlation between a single individual or group’s response and provide a correct estimate of each effect's variance. Chronic and continuous data were analysed using a GEE, and if significantly different, further analysed by a non-parametric two-sample Wilcoxon rank-sum (Mann-Whitney U) test comparing the control group to the treatment group. Prism (Version 5, GraphPad Software Inc. San Diego, USA) was used for ANOVA statistical analysis and Stata Intercooled, version 9 (Statacorp Ltd, College Station, Texas) for GEE and Wilcoxon rank-sum analysis. For all cases, values of p<0.05 were considered statistically significant. For qPCR analysis, a two-way ANOVA followed by Kruskal Wallis post-hoc was performed.
3.4 RESULTS

3.4.1 Affinity of Glucagon-Ex4 Chimera analogues to the rGLP-1R, hGLP-1R, rGCGR and hGCGR

The chimera GX1 bound to the rat and human GLP-1 receptors with comparable affinity as native GLP-1 (1.23 fold lower affinity for rGLP-1R and 0.93 fold affinity hGLP-1R compared to GLP-1). However, affinity for the GCGR in both species was decreased (43.3 fold lower for rGCGR and 83.2 fold lower for hGCGR compared to GCG).

The introduction of Leu at position 27 in GX2 slightly decreased affinity for the rGLP-1R and hGLP-1R, (8.08 fold lower for rGLP-1R and 2.11 fold lower for hGLP-1R compared to GLP-1). Affinity was also decreased for the GCGR (19.37 fold lower for rGCGR and 74.7 fold lower for hGCGR compared to GCG).

Introduction of Lys at position 17 in GX3 slightly decreased the affinity for the rGLP-1R compared to native GLP-1 but slightly enhanced the affinity to the hGLP-1 receptor (3.85 fold lower for rGLP-1R and 0.89 fold affinity for hGLP-1R compared to GLP-1). However, affinity for the GCG receptor was decreased (81.94 fold lower for rGCGR and 188.13 fold lower for hGCGR compared to GCG).

Presence of His at position 20 in GX4 slightly decreased the affinity for both species of GLP-1R (4.54 fold lower for rGLP-1R and 2.51 fold lower affinity for hGLP-1R compared to GLP-1), and decreased affinity for the GCGR (34.14 fold lower for rGCGR and 118.13 fold lower for hGCGR compared to GCG).

Introduction of Gln at position 24 in GX5 had little effect on binding affinity to the GLP-1R in both rat and human species (2.31 fold lower for rGLP-1R and 1.42 fold lower affinity for hGLP-1R)
compared to GLP-1) and resulted in decreased affinity for the GCGR (17.03 fold lower for rGCGR and 88.73 fold lower for hGCGR compared to GCG).

Finally, the introduction of Gln at position 17 in the chimera GX6 resulted in modestly decreased affinity for the GLP-1 receptors (4.54 fold lower for rGLP-1R and 1.64 fold lower affinity for hGLP-1R compared to GLP-1) and slightly lower affinity for the GCGR (4.67 fold lower for rGCGR and 8.38 fold lower for hGCGR compared to GCG). Binding affinities are summarised in Table 3.1 and binding curves are presented in Figures 3.3 and 3.4.
<table>
<thead>
<tr>
<th>peptide</th>
<th>rGLP-1R IC50 (nM)</th>
<th>fold binding x GLP 1</th>
<th>hGLP-1R IC50 (nM)</th>
<th>fold binding x GLP 1</th>
<th>rGCGR IC50 (nM)</th>
<th>fold binding x GCG</th>
<th>hGCGR IC50 (nM)</th>
<th>fold binding x GCG</th>
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<td>--</td>
<td>--</td>
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<tr>
<td>Exendin 4</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<td>1.00</td>
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<td>1.24</td>
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<td>5.09</td>
<td>4.67</td>
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Table 3.1: Summary of binding affinities of Glucagon-Exendin4 chimeras with substitutions at positions 17, 20, 24 and 27 to the rat and human GLP-1R and GCGR. A receptor binding assay measuring analogue binding at each receptor was used to determine the IC50 of each analogue vs. either GLP-1 or GCG. * denotes chimera of interest which maintains affinity to all receptors tested.
Figure 3.3. Representative binding affinity curves of chimera peptides GX1-GX6 on the rat A) GLP-1R and B) GCGR. The mean GLP1 and GCG binding assay (n=3) (black, on each axis) is shown as reference. Increasing concentrations of GX-chimeras were incubated with a fixed concentration of $^{125}$I GLP-1 and rat GLP-1R or $^{125}$I GCG and rat GCGR expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I GLP-1 or $^{125}$I GCG bound with and without unlabelled competing peptide. Dotted line represents 50% maximum binding from which IC50 value was extrapolated.
Figure 3.4. Representative binding affinity curves of chimera peptides GX1-GX6 on the human A) GLP-1R and B) GCGR. The mean GLP1 and GCG binding assay (n=3) (black, on each axis) is shown as reference. Increasing concentrations of GX-chimeras were incubated with a fixed concentration of $^{125}$I GLP-1 and human GLP-1R or $^{125}$I GCG and human GCGR expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I GLP-1 or $^{125}$I GCG bound with and without unlabelled competing peptide. Dotted line represents 50% maximum binding from which IC50 value was extrapolated.
3.4.2 Effect of GLP-1, GCG and Ex-4 on Acute Food Intake in C57BL/6 Mice

To confirm the acute anorectic effects of the proglucagon peptides, mice were randomised to receive a subcutaneous injection of saline, 500 nmol/kg of GCG, 500 nmol/kg of GLP-1 or 50 nmol/kg of Ex-4. Administration of all peptides significantly decreased food intake at 0-1 hours (GCG, Ex-4: p<0.001; GLP-1: p<0.01, vs. saline). In the 1-2, 2-4 and 4-8 hour intervals, only Ex-4 significantly reduced food intake (1-2 hr, 4-8 hr: p<0.001 vs. saline; 2-4 hr: p<0.05 vs. saline). GCG increased food intake during these time intervals, but this effect was not statistically significant. In the 8-24 hour interval, there was no difference in food intake among any of the groups receiving proglucagon peptides compared to control. Over the total 24 hour study period, Ex-4 administration significant decreased in food intake compared to saline controls (p<0.001, vs saline). There were no significant differences in food intake between groups at any other time points at which it was measured (Figure 3.5).
Figure 3.5. Interval food intake (expressed as percentage of saline controls on each study day) following subcutaneous administration of saline, Exendin 4 (50 nmol/kg), GLP-1 (500 nmol/kg), GCG (500 nmol/kg) in mice. The food intake of the saline group is shown as an average of 2 separate study days during which peptides were tested, n=5-7 mouse/group. Statistics: One way ANOVA with Dunnett’s post-hoc test performed on raw data (not shown) *p<0.05, **p<0.01, ***p<0.001, vs. saline.
3.4.3 The effect of Glucagon-Chimera analogue peptides on cAMP accumulation in hGLP-1R overexpressing HEK 293 cells

The chimeras GX1-GX6 stimulated the production of cAMP in HEK293 cells overexpressing the GLP-1 and GCG receptors at various concentrations (Figure 3.6). Table 3.2 summarises the EC50 values.

<table>
<thead>
<tr>
<th>peptide</th>
<th>hGLP-1R EC50 (nM)</th>
<th>fold activation x GLP 1</th>
<th>hGCGR EC50 (nM)</th>
<th>fold activation x GCG</th>
</tr>
</thead>
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<td>1.00</td>
<td>115.7</td>
<td>17893.60</td>
</tr>
<tr>
<td>Exendin 4</td>
<td>3.23</td>
<td>2.99</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GCG</td>
<td>--</td>
<td>--</td>
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</tr>
<tr>
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<td>0.31</td>
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</tr>
<tr>
<td>GX6*</td>
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<td>0.72</td>
<td>0.15</td>
<td>23.68</td>
</tr>
</tbody>
</table>

Table 3.2. Summary of EC50 values of Glucagon-Exendin4 chimeras with substitutions at positions 17, 20, 24 and 27 at the rat and human GLP-1R and GCGR.
Figure 3.6. cAMP accumulation in A) human GLP-1R and B) human GCGR expressing HEK 293 cells in response to incubation with GCG, Ex4, GLP-1 and analogues GX1-6. The EC$_{50}$ values were calculated from 3 separate experiments.
3.4.4 The effect of Glucagon-Exendin4 chimera on acute food intake in overnight fasted mice

All GX chimeras significantly reduced food intake 0-1 hours following injection (p < 0.001 vs. saline). In the 1-2 hour interval, all groups consumed less than the saline group but these differences were not statistically significant. Between 2-4 and 4-8 hours post injection, GX1, GX5 and GX6 significantly reduced food intake compared to the saline control (GX1, GX5 and GX6 p<0.001 vs saline). GX3, GX4, GX5 and GX6 significantly increased food intake compared to saline controls in the 8-24 hour interval (GX3, GX4, GX6 p<0.05 vs saline; GX5 p<0.01 vs saline). All GX chimeras significantly reduced food intake over the entire 24 hour period compared to saline controls (GX1, GX2, GX6 p<0.001; GX3 p<0.01; GX4, GX5 p<0.05 vs. saline). (Figure 3.7)
Figure 3.7 Interval food intake (expressed as percentage of saline controls on each study day) following subcutaneous administration of saline and GX1-6 (50 nmol/kg) in C57BL/6 mice. The food intake of the saline group is shown as an average of 4 separate study days during which peptides were tested, n=5-7 mouse/group. Statistics: One way ANOVA with Dunnett’s post-hoc test performed on raw data (not shown) *p<0.05, **p<0.01, ***p<0.001, vs. saline.
3.4.5 Effect of Glucagon-Exendin4 chimeras food intake in 24 hour fasted rats

All GX chimeras significantly reduced food intake 0-1 hours following injection (p < 0.001 vs. saline) and in the 1-2 hour interval (p<0.01 vs. saline.) In the 2-4 hour interval, the GX2 significantly increased food intake compared to saline controls (p<0.01) while all other groups significantly decreased food intake (p<0.001 vs. saline). During the 4-8 and 8-24 hour intervals post injection, all GX chimeras significantly decreased food intake compared to saline controls (p<0.001 vs. saline). Over the entire 24 hour period, all GX chimeras groups significantly reduced food intake compared to saline controls (p<0.001 vs. saline). (Figure 3.8)
Figure 3.8. Interval food intake (expressed as percentage of saline controls on each study day) following subcutaneous administration of saline and GX1-6 (50 nmol/kg) in Wistar rats. The food intake of the saline group is shown as an average of 4 separate study days during which peptides were tested, n=5-7 mouse/group. Statistics: One way ANOVA with Dunnett’s post-hoc test performed on raw data (not shown) *p<0.05, **p<0.01, ***p<0.001, vs. saline.
3.4.6 GX6 plasma peptide levels following subcutaneous administration at 10 mg/ml in male Wistar rats

GX6 was undetectable in the plasma of rats injected with 500 nmol/kg of GX6, a maximum concentration was observed 4 hours following administration of the time points investigated. Circulating levels of GX6 were 934.7 pmol/L at 4 hours, 420.6 pmol/L at 24 hours and 167.0 pmol/L at 36 hours. There was also no GX6 immunoreactivity detected in the plasma 3 days following injection (Figure 3.9).

Figure 3.9. Plasma concentration-time curve following subcutaneous administration of GX6 at 500 nmol/kg. Plasma was collected from the tail vein of conscious male Wistar rats, n=5. GX6-Immunoreactivity (IR) was measured using RIA.
3.4.7 Effect of 10 day GX6 administration on food intake, body weight change and energy expenditure induced weight change as detected by pair feeding in Wistar Rats

Daily subcutaneous administration of 30 nmol/kg of GX6 significantly reduced cumulative food intake over the entire study period compared to saline controls. Food matched controls (pair-fed to GX6: GX6-PF) received daily allotments of food equivalent to the mean food intake of the GX6 groups, and as a result also ate significantly less than saline controls over 10 days. As expected, there was no difference in food intake between the GX6 treated rats and pair-fed controls (cumulative food intake on day 10: Saline (283.3 ± 7.1 g); GX6 (211.5 ± 9.2 g; p<0.001 vs. saline); GX6-PF (213.1 ± 0.1 g; p<0.001 vs. saline)) (Figure 3.10).

Both the GX6 and the GX6-PF groups exhibited a significant decrease in body weight over the 10 day period compared to saline controls, which showed an increase in body weight (cumulative body weight change on day 10: Saline: (+19.8 ± 3.7 g); GX6 (-22.6 ± 3.8 g; p<0.001, vs. saline); GX6-PF (-6.3 ± 2.1 g (p<0.001, vs. saline)). Notably, GX6 administration resulted in a greater reduction in body weight compared to GX6-PF from day 6 onward (p<0.01, GX6 vs GX6-PF) (Figure 3.11)
Figure 3.10. The effect of daily administration of saline, GX6 (30 nmol/kg) and pair-feeding controls (GX6-PF) on A) cumulative food intake (g) over 10 days in male Wistar rats and B) Final food intake (g) on day 10. Statistics: GEE followed by Mann-Whitney U test. ***p<0.001, GX6 vs. saline; $$$ p<0.001, GX6-PF vs. saline; GX6 vs. GX6-PF; n=8-10
Figure 3.11. The effect of daily administration of GX6 (30 nmol/kg) and pair-feeding controls (GX6-PF) body weight change over 10 days in male Wistar rats A) Cumulative body weight change (g) from initial (g) over 10 days and B) Final body weight change (g) on day 10. Statistics: GEE followed by Mann-Whitney U test. ***p<0.001, GX6 vs. saline; $$p<0.001, \text{GX6-PF vs. saline}; \dagger\dagger p<0.01, \text{GX6 vs. GX6-PF}; n=8-10.
3.4.8 Effect of 10 day GX6 and Exendin-4 administration and Pair Feeding on food intake, body weight change and energy expenditure induced weight change in Wistar Rats

GX6 (30 nmol/kg) and Ex-4 (30 nmol/kg) administration significantly decreased cumulative food intake over the 10 day study period compared to saline controls [cumulative food intake on day 10: Saline: (329.3 ± 10.0 g); GX6 (221.6 ± 12.6 g; p<0.001, vs. saline); Ex-4 (233.9 ± 11.1 g (p<0.001, vs. saline)]. GX6-PF and Ex-4-PF groups, receiving daily allotments of food equivalent to the mean food intake of their respective matched group, also showed significantly decreased food intake than saline controls over 10 days [cumulative food intake on day 10: GX6-PF (219.1 ± 0.3 g; p<0.001, vs saline); Ex-4-PF (230.2 ± 0.5 g (p<0.001, vs. saline)]. As intended, there was no difference in food intake between rats given GX6 and GX6-PF controls and between those given Ex-4 and Ex-4-PF controls (Figure 3.12).

GX6 administration and GX6-pair feeding decreased body weight over the 10 day period compared to saline controls. Saline controls increased in body weight [cumulative body weight change on day 10: Saline: (+19.8 ± 3.7 g); GX6 (-22.6 ± 3.8 g; p<0.001, vs. saline); GX6-PF (-6.3 ± 2.1 g (p<0.001, vs. saline)]. Interestingly, GX6 administration significantly decreased body weight compared to their pair-fed controls (p<0.01, GX6 vs GX6-PF) (Figure 3.13).

Ex-4 and Ex-4-pair feeding significantly reduced body weight during the course of the experiment compared to saline (Ex4: -3.3 ± 4.3 g (p<0.001, vs saline); Ex-4-PF: -21.3 ± 3.3 g (p<0.001, vs. saline). While Ex-4 administration reduced body weight on day 1 and remained stable throughout the study period, Ex-4-PF significantly decrease in body weight during this significantly greater reduction in body weight compared to Ex-4 from day 3 to day 10 (p<0.001).
Figure 3.12. The effect of daily administration of GX6 (30 nmol/kg) and Exendin-4 (30 nmol/kg) and pair-feeding (GX6-PF and Ex4-PF) in male Wistar rats on A) cumulative food intake (g) over 10 days and B) final food intake (g) on day 10. Statistics: GEE followed by Mann-Whitney U test. ***p<0.001, Ex4 and Ex4-PF vs. saline; $$$ p<0.001, GX6 and GX6-PF vs. saline
Figure 3.13. The effect of daily administration of GX6 (30 nmol/kg) and Exendin 4 (30 nmol/kg) and pair-fed controls (GX6-PF and Ex4-PF) body weight change over 10 days in male Wistar rats. A) Cumulative body weight change from initial (g) over 10 days; B) Final body weight change on day 10. Statistics: GEE followed by Mann-Whitney U test. ***p<0.001, GX6 vs. saline; $$$ p<0.001, GX6-PF vs. saline; †† p<0.01, GX6 vs. GX6-PF; n=8-10
3.4.9 Metabolic Gene expression

GCG and CL316 appeared to increase expression of Pck1α in BAT by 1.5 fold and 2.5 fold, respectively, however these were not significant. Ex-4 and GX6 decreased Pgc1α expression in BAT by 0.3 fold and 0.5 fold, respectively. CL316 administration appeared to increase Ucp1 expression in a 1.5 fold increase in and GCG and GX6 administration slightly increased expression by 1.2 fold. Exendin 4 decreased Ucp1 expression 0.7, although not significantly. Exendin-4 and GX6 appeared to increase expression of Pparα by following administration and a small decrease following GCG administration, however this was not statistically significant (Figure 3.14).

In the liver, there was no change in the expression levels of Pck1α in any experimental group. Ex-4 administration appeared to increased G6P expression 1.6 fold, while CL316 and GX6 increased G6P expression 1.2 and 1.3 fold, respectively. Exendin-4 and GX6 administration increased expression of Pparα 1.5 and 1.3 fold, respectively and CL316 decreased expression 0.5 fold, although not significantly. CL316 increased Fgf21 expression 3 fold increase GX6 increased expression 1.6 fold, however this was not significant. Ex-4, GCG and GX6 administration resulted in 3.9 fold, 7.5 fold and 15.3 fold increases, respectively, in liver Pgc1α, compared to saline (p<0.01 GX6 vs saline; p<0.05 GX6 vs Ex-4) (Figure 3.15).
Figure 3.14. Relative expression levels for Pgc1α, Ucp1, Pparγ compared to saline normalised to internal controls β-actin and β2-myoglobulin. mRNA was extracted from BAT 6 hours post-administration of subcutaneous CL316 (1mg/kg), Ex4 (30 nmol/kg), GCG (900 nmol/kg) and GX6 (30 nmol/kg) (n=8 rats per treatment group).
Figure 3.15. Relative expression levels of liver G6Pase, Pck1a, Pparα, FGF21, PGC1α compared to saline normalised to internal controls β-actin and β2-myoglobin. mRNA was extracted from liver 6 hours post-administration of subcutaneous CL316 (1mg/kg), Ex4 (30 nmol/kg), GCG (900 nmol/kg) and GX6 (30 nmol/kg) (n=8 rats per treatment group). *p<0.05 GX6 vs Saline; $p<0.05 GX6 vs Ex4.
3.5 DISCUSSION

Recent data suggests that dual agonists of the GLP-1 and glucagon receptors may be useful in the treatment of diabetes and obesity (Day et al., 2009; Pocai et al., 2009). The proglucagon hormone OXM activates both the GLP1R and GCGR in vitro, albeit with 10- to 100-fold reduced potency compared with the cognate ligands GLP-1 and glucagon (Baldissera et al., 1988). It has been proposed that OXM modulates glucose and energy homeostasis solely by GLP1R agonism, while its protective effects on murine islets and inhibition of gastric acid secretion appear to be independent of GLP1R signalling (Maida et al., 2008). In addition, pharmacological activation of GCGR by glucagon, a regulator of fasting metabolism, decreases food intake in rodents and humans. It is therefore possible that OXM mediates some of its effects via the GCGR.

The dual agonist chimera molecules discussed in this chapter are based on an amino acid sequence derived from an N terminal Glucagon with C terminal Exendin-4. The analogues tested had this basic structure modified at positions 17, 20, 24 and 27. All 6 peptides tested maintained affinity comparable to that of native GLP-1 and Exendin-4 at both human and rat GLP-1 receptors but varied widely in their affinities for the GCGR. All molecules were effective at stimulating cAMP release in human GLP-1R overexpressing cells and significantly reduced acute food intake in rats and mice. This suggests that the introduction of an N-terminal GCG sequence to an Exendin-4 peptide together with individual amino acid substitutions at 17, 20, 24 and 27, did not compromise GLP-1R bioactivity. The chimera analogue, GX6, incorporating substitutions at positions 17, 20, and 24, exhibited the highest affinity for both GLP-1R and GCGR in both human and rat species and activated both receptors. It was therefore selected as a leading candidate to investigate the effects of simultaneous activation of both systems in vivo.

The regulation of body weight is dependent on both energy intake and output. When an experimental group exhibits a simultaneous decrease in food intake and loss of body weight following administration of a substance, it is often unclear what the contribution the reduced food intake, as opposed to increased energy expenditure, has made to the reduction in body weight. This is a major
problem in the investigations of energy homeostasis, as the regulation of body weight is dependent on both energy intake and output. An established way of controlling the differences in food intake among experimental groups is to employ pair feeding techniques. In such a procedure, the food intake of a control group is reduced in parallel that of the experimental group (CASTER and ARMSTRONG, 1955). In the pair feeding experiments described in this chapter, treatment with the dual agonist GX6 resulted in a greater reduction in body weight than pair fed controls, while treatment with the GLP-1 agonist Exendin-4 resulted in a lesser reduction in body weight than pair fed controls. In support of my hypothesis, this suggests that the glucagon agonism inherent to GX6 may be involved in increasing energy expenditure in addition to reducing food intake. As Ex-4 does not have known effects on energy expenditure, it was expected that pair-fed controls would exhibit a decrease in bodyweight to the same degree as Ex-4 treated rats, however it appeared that Ex-4 decreased energy expenditure and therefore reduced body weight loss. This is in support of published data, which reported that Ex-4 decreased energy expenditure by decreasing the volume of oxygen consumption and respiratory exchange ratio in mice (Baggio et al., 2004b). This further supports the hypothesis of combining the anorectic effects of GLP-1R agonism with the energy expenditure enhancing effects of GCG agonism in a dual therapy.

There are many mechanisms involved in the modulation of energy expenditure. The effect of GX6, Glucagon, Exendin-4 and the β-3 adrenergic agonist, CL316, on expression levels of UCP1, Ppare and Pgc1α were measured in brown adipose tissue (BAT). GX6 and GCG slightly increased expression of Ucp1, while Exendin-4 slightly decreased expression. It is unclear whether these changes are of metabolic significance. However, the positive control CL316 did increase Ucp1 expression in the literature (Weyer et al., 1999). GX6 together with Ex-4 slightly decreased expression of Pgc1α while GCG slightly increased expression. As GX6 contains elements of both Ex-4 and GCG, it appears to have biological effects similar to Ex-4 on Pgc1α and similar to GCG on Ucp1. It remains to be confirmed if this effect mimics that which would be seen following administration of Ex-4 and GCG simultaneously.
In the liver, GCG administration failed to increase the expression of two major gluconeogenic enzymes, G6Pase and Pck1a. This is presumably due to the short acting nature of GCG and the 6 hour post-injection time point used. This time point was chosen based on an experiment described in Pocai and colleagues’ study in which a large increase in several gluconeogenic and glycogenolytic enzymes was reported 6 hours following administration of a long acting GLP-1/GCG receptor dual agonist (2009). This late time point may be sufficient when investigating long acting agonists, but not for short acting signals. A time course with multiple time points starting from 30 minutes onward to 6 hours is required to assess the changes in gene expression levels following GCG administration.

Interestingly, there was a remarkable change in liver Pgc1α expression in the liver following GX6 administration. GX6 stimulated a significantly greater increase than Ex-4 and greater increase than GCG (although not significantly), suggesting that there may be a synergistic effect elicited in the dual agonist. Pgc1α is a transcriptional regulator of gluconeogenesis during the fasting state (Yoon, et al, 2001). It has also been implicated in the response of adaptive thermogenesis to cold which is accompanied by increased mitochondrial biogenesis, increased fatty acid oxidation and activation of UCP1 (Wu, et al, 1999). Glucagon strongly stimulates Pgc1α in hepatocytes and hepatic gluconeogenesis in rodents (Puigserver and Spiegelman, 2003). Pgc1α regarded as a master regulator of mitochondrial function and oxidative metabolism and its activation may prove to be a useful therapy for metabolic disorders. While the isolated stimulation of gluconeogenesis in diabetic patients is undesirable it is suppressed by insulin and incretin hormones (Herzig et al., 2001) further implicating the benefits of a dual GLP-1R/GCGR agonist.

In addition to isolating BAT and liver from treated rats, WAT and skeletal muscle were also taken. These will be assayed by collaborators in the department for the expression levels of PRDM16, UCP3 and various markers of thermogenesis. Particularly in humans, the site of adaptive thermogenesis might not be restricted to brown adipose tissue. Adult humans have no large deposits of brown adipose tissue, although brown adipose fat cells may be present in small numbers within the white adipose tissue (Lean et al, 1986). As a result of the low content of brown adipose tissue from adult humans, its contribution to adrenaline-induced thermogenesis was estimated to be maximally 25%, so
other tissues must therefore be involved (Astrup et al, 1985). The same study showed that, in humans, skeletal muscle is the most important tissue for adaptive thermogenesis, accounting for up to 50% of adrenaline-induced thermogenesis. Comparable results were found by Simonsen et al., who estimated that 40% of the increase in thermogenesis after adrenaline administration could be attributed to skeletal muscle (1993).

One parameter yet to be investigated is the effect of our dual agonist on blood glucose, an important consideration of glucagon agonism. GCGR-selective agonism is typically associated with the risk of hyperglycemia because elevation of endogenous glucagon levels and simultaneous reduction in insulin levels/action are accepted as key players in the pathogenesis of diabetic hyperglycemia. However, Pocai and colleagues found that daily administration with a dual agonist reversed diet-induced obesity and normalised basal blood glucose levels and improved glucose tolerance as detected using and i.p glucose tolerance test (IP-GTT) and similar results were reported by another group (Day et al., 2009). It has been reported that simultaneous GLP-1R in rodents mitigates the metabolic risks associated with GCGR activation while leveraging the beneficial pharmacological effects of activating each receptor, including enhanced weight loss efficacy, antihyperglycemic activity, and lipid-lowering effects (Day et al., 2009). My hypothesis is that pharmacological GLP-1R agonism results in enhanced glucose-dependent insulin secretion, which enhances glucose utilisation to balance the glucoregulatory and catabolic effects of simultaneous GCGR agonism. Hence, the GLP-1R/GCGR dual agonist may mediate effective weight loss and glucose homeostasis.

Currently, there is work underway in our department investigating the ideal ratio of GLP-1R/GCGR agonism. One proposed ratio is that GLP-1R agonism should be four-fold higher than that of GCGR agonism, and at this point maximum weight loss is achieved without detrimental effects on blood glucose (Figure 3.16). It has been proposed that the intrinsic GLP-1R agonism of these molecules opposes and potentially neutralizes any glucagon receptor-mediated diabetogenic effects; the considerable decrease in fat mass provides strong metabolic benefits that synergize to dominate any hyperglycemic drive; and sustained pharmacological glucagon receptor activation is an understudied phenomenon and is worthy of additional research in the absence of body weight
lowering with GLP-1 co-agonism (Figure 3.16) (Day et al., 2009). It has yet to be determined if the effects of a dual agonist shown in rodents is of clinical significance, although the weight loss obtained with native OXM in overweight subjects is encouraging (Wynne et al., 2005a). In conclusion, the data presented in this chapter, together with the results reported by other research laboratories suggest that a long-acting GLP-1R/GCGR dual agonist, such as GX6, represents a novel pharmaceutical agent for the treatment of obesity.

![Diagram of glucagon and GLP-1 receptor agonism ratios of compounds](image)

**Figure 3.16 Schematic of glucagon and GLP-1 receptor agonism ratios of compounds.** The effect of varying ratios of GLP-1R and GCGR agonism by a single dual agonist on body weight without sacrificing the beneficial impact on glucose homeostasis. This diagram suggests a preferred ratio 1:4 of GCGR:GLP-1R co-agonism for optimizing effects on blood glucose and body weight.

Taken together, the results described in this chapter suggest that a dual GLP-1 and GCG receptor agonist may be beneficial in the treatment of obesity and diabetes by reducing food intake and increasing energy expenditure.
Chapter 4:
General Discussion
The increasing prevalence of obesity results from an interaction between environmental factors (such as widely available, calorie-dense foods and a reduction in physical activity) and genetic factors which predispose to weight gain. The understanding and manipulation of systems which regulate energy homeostasis may lead to the development of novel anti-obesity therapies. This thesis has explored two hormonal systems involved in the regulation of appetite: PYY and the Proglucagon family. The results, together with those from related work, raise the possibility of their use as agents in the treatment of obesity.

Targeting endogenous gut satiety signalling hormones for the use as anti-obesity therapies may be more useful than previously investigated appetite suppressing agents with ubiquitously expressed receptors. For example, the anti-obesity drug rimonabant is an antagonist at the CB1 receptor of the endocannabinoid system and has an appetite suppressing effect (Padwal and Majumdar, 2007b). The CB1 receptor is the most abundant receptor expressed in the brain and is also expressed at lower concentrations in peripheral tissues. The endocannabinoid system plays important roles in neuroprotection, neurodegenerative diseases, perception of pain, mood disorders, gut functioning and immunity and inflammation (Pacher et al., 2006). The role of the CB1 receptor in multiple systems suggests that antagonising the endocannabinoid system to suppress appetite may be associated with a number of side effects. Indeed, rimonabant was withdrawn in October 2008 by the EMEA due to the increased incidence of psychiatric disorders in patients (Kulkarni, 2008).

PYY and GLP-1 are potent peripheral satiety signals, released from the L-cells of the GI tract following a meal in proportion to the calorie intake (Morinigo et al., 2008; Rodieux et al., 2008; Holdstock et al., 2008; Vidal et al., 2009). The reduced appetite and weight loss observed in patients who have undergone gastric bypass surgery has been suggested, at least in part, to be due to the significant rise in the concentration of circulating satiety signals including PYY and GLP-1 (Degen et al., 2005b; Sloth et al., 2007e; Sloth et al., 2007i; Le Roux et al., 2008b; Gantz et al., 2007a). The peripheral administration of PYY3-36 and GLP-1, significantly decreases food intake in lean and obese human volunteers (Batterham et al., 2002a; Batterham et al., 2003a; Degen et al., 2005a; Le Roux et al., 2008c).
However, the most effective treatment for obesity is bariatric surgery, but it is impractical to apply to the general obese population due to its cost and risk of mortality. Recent research has suggested that increased circulating PYY and GLP-1 contribute, at least in part, to the reduced appetite and body weight gain observed after bariatric surgery (Korner et al., 2005; Le Roux et al., 2006). This suggests that mimicking the post-bariatric state by peripherally administering PYY3-36 and GLP-1 to the obese may be a logical, cost effective therapeutic approach. However, the exogenous administration of gut hormones to promote weight loss in the obese has its own challenges. One major disadvantage is that gut hormones are extremely short-lived in the circulation, which limits their efficacy and ease of administration. The development of long-acting analogues of gut hormones is therefore an approach used by an increasing number of research groups, including ours. GLP-1, for example, has already been utilised as the basis for the development of Liraglutide, a once-daily therapy for type 2 diabetes treatment (Croom and McCormack, 2009).

Both PYY and GLP-1 are rapidly degraded in the circulation, as they are physiologically short acting signals. Therefore, several research groups, including ours, are investigating the design and development of long acting agonists of the Y2 receptor and the GLP-1 receptor in the form of peptide analogues. This thesis explores the effect of prolonged agonism of the Y2 receptor by PYY3-36 infusion in order to elucidate the limitations that may be inherent to development of such pharmacotherapies. To date, there has been little research in the area of Y2 receptor downregulation and the homeostatic defence of body weight following exogenous gut hormone administration, however it is an area that requires more understanding. The proglucagon system has been more extensively researched in the context of the inherent incretin effect of GLP-1 and Exendin-4 and its use as a therapy for type 2 diabetes. However, until recently, agonism of the related glucagon receptor has been avoided due to its role in enhancing blood glucose levels. This thesis also explores the development of GLP-1 and glucagon receptor agonists which are hypothesized to decrease food intake and increase energy expenditure while normalising blood glucose. However, the effects of glucagon on energy expenditure have been brought to light in recent publications (Day et al., 2009; Pocai et al., 2009).
Strong appetite inhibition stimulates counter regulatory mechanisms which act to prevent starvation, by increasing food seeking behaviour and decreasing metabolic rate. Thus, by combining a therapy such as PYY or GLP-1 with the effects of glucagon, which may prevent the resulting hypoglycaemia from periods of low food intake and may increase energy expenditure, these mechanisms may be overridden. Perhaps, a combination pharmacotherapy in which Y2R, GLP-1R and GCGR agonism is administered together may be useful in the development of an anti-obesity treatment.

This thesis is divided into 2 sections. In the first section, I aimed to elucidate the biphasic anorectic profile observed during Y2 receptor agonism by a PYY3-36 infusion. I aimed to distinguish between the homeostatic defence of body weight resulting from rapid weight loss and between Y2R desensitisation due to chronic PYY3-36 administration. In the second section, I examined the development of GLP-1 and GCG co-agonism using synthetic Glucagon-Exendin-4 chimera analogues. Overall, this thesis investigated the role of gut hormones in energy homeostasis by elucidating the mechanisms involved in food intake, energy expenditure and the homeostasis defence of body weight.

As the Y2R is a GPCR, is susceptible to downregulation, internalisation and desensitisation (Parker et al., 2007b). From the view of homeostatic control mechanisms, the brain is charged with maintaining body weight (or fat mass or energy content) at a given level, opposing change, and restoring it following any significant deviation. Hence, if body weight dropped, the brain would sense the change, oppose it, such as by slowing metabolic rate, and attempt to restore body weight to the set point value, perhaps by stimulating hunger and food-seeking behaviours (Keesey and Powley, 1986; Levin and Keesey, 1998). In this thesis, I have shown that while there is a homeostatic drive to restore body weight and food intake to baseline levels, the administration of the pharmacological anorectic peptide hormone PYY3-36 results in overriding this physiological control mechanism.

The dual agonist chimera molecules discussed in this chapter are based on an amino acid sequence derived from an N terminal Glucagon with C terminal Exendin-4. All analogues bound and activated the human and rat GLP-1 and GCG receptors, and the leading peptide, GX6, was investigated further in vivo.
The increased weight loss efficacy observed with GX6 compared with Ex-4 is consistent with previous research on the effect of glucagon on energy expenditure. The hormone has also been reported to decrease total cholesterol in rats and to cause a greater reduction in body weight compared with a pair-fed group of animals because of both reduced food intake and increased energy expenditure (SALTER et al., 1957). However, GCGR-selective agonism is typically associated with the risk of hyperglycemia because elevation of endogenous glucagon levels and concomitant reduction in insulin levels/action are accepted as key players in the pathogenesis of diabetic hyperglycemia (Unger and Orci, 1975). According to this bihormonal hypothesis for diabetes, hyperglucagonemia results in excessive hepatic glucose production, which is not balanced by glucose utilization under conditions of hypoinsulinemia and insulin resistance.

It has been reported that simultaneous GLP-1R in rodents counteracts the metabolic risks that may be associated with GCGR activation while leveraging the beneficial pharmacological effects of activating each receptor, including enhanced weight loss efficacy, antihyperglycemic activity, and lipid-lowering effects (Day et al., 2009). My hypothesis is that pharmacological GLP-1R agonism results in enhanced glucose-dependent insulin secretion, which enhances glucose disposal to balance the glucoregulatory and catabolic effects of concomitant GCGR agonism. Hence, the GLP-1R/GCGR dual agonist may mediate effect weight loss and glucose homeostasis.

Currently, there is work underway in our department investigating the ideal ratio of GLP-1R/GCGR agonism. One proposed ratio is that GLP-1R agonism should be four-fold higher than that of GCGR agonism, and at this point maximum weight loss is achieved without detrimental effects on blood glucose. It has been proposed that the intrinsic GLP-1R agonism of these molecules opposes and potentially neutralizes any glucagon receptor-mediated diabetogenic effects; the considerable decrease in fat mass provides strong metabolic benefits that synergize to dominate any hyperglycemic drive; and sustained pharmacological glucagon receptor activation is an understudied phenomenon and is worthy of additional research in the absence of body weight lowering with GLP-1 co-agonism (Day et al., 2009). It has yet to be determined if the effects of a dual agonist shown in
rodents is of clinical significance, although the weight loss obtained with native OXM in overweight subjects is encouraging (Wynne et al., 2005a).

In conclusion, the data presented in this thesis, together with the results reported by other research laboratories suggest that a long-acting GLP-1R/GCGR dual agonist, such as GX6, represents a novel pharmaceutical agent for the treatment of obesity. Overall, the results presented in this thesis have elucidated the mechanisms involved the gut hormone regulation of metabolism and energy homeostasis, in particular the effects on food intake, energy expenditure and the homeostatic maintenance of body weight.
Appendices
APPENDIX A: Three letter and single letter codes for proteinogenic amino acids

<table>
<thead>
<tr>
<th>Three Letter Code</th>
<th>Single Letter Code</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Cys</td>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Asp</td>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Glu</td>
<td>E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Phe</td>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Gly</td>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>His</td>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>Ile</td>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Lys</td>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>Leu</td>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>Met</td>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>Asn</td>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Pro</td>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>Gln</td>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Arg</td>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>Ser</td>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>Thr</td>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>Val</td>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>Trp</td>
<td>W</td>
<td>Tryptophan</td>
</tr>
</tbody>
</table>
APPENDIX B: Principles of Radioimmunoassay

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

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

\[ *\text{Ag} + \text{Ab} + \text{Ag} \rightleftharpoons *\text{AgAb} + \text{AgAb} \]

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

Separation of the bound from the free antigen is achieved by addition of either dextran-coated charcoal (free label is contained in the charcoal pellet following centrifugation) or using a primary-secondary antibody complex (free label is contained in the supernatant following centrifugation).
The secondary antibody is derived from an animal species different from that used to generate the primary antibody. After incubation and separation, the bound and free label are counted in a γ-counter (NE 1600, NE Technology Ltd.). The data are used to construct a standard curve from which the values of the unknowns can be obtained by interpolation.

Inter-assay variation can be calculated by assaying aliquots of the same sample in each assay performed and comparing the concentrations obtained in each. To measure and correct for baseline drift, tubes with no sample (‘zero’ tubes) are placed at regular intervals throughout the assay and standard curves are performed at the beginning and end of each assay. The general structure of the RIA is outlined in table 9.1, which shows the content of the tubes according to their designation.

The following tubes are important for the assessment of the quality for the label, antibody and overall performance of the assay:

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

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

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

Zero tubes: allows assessment of assay drift.

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

**Quality Controls:** includes previously aliquoted samples containing high and low levels of the antigen. These tubes allow the assays to be standardised.
<table>
<thead>
<tr>
<th>Tube number</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>3-4</td>
<td>$\frac{1}{2} \times$</td>
</tr>
<tr>
<td>5-6</td>
<td>$2 \times$</td>
</tr>
<tr>
<td>7-8</td>
<td>Zero</td>
</tr>
<tr>
<td>9-10</td>
<td>Zero</td>
</tr>
<tr>
<td>11-12</td>
<td>Standard 1</td>
</tr>
<tr>
<td>13-14</td>
<td>Standard 2</td>
</tr>
<tr>
<td>15-16</td>
<td>Standard 3</td>
</tr>
<tr>
<td>17-18</td>
<td>Standard 4</td>
</tr>
<tr>
<td>19-20</td>
<td>Standard 5</td>
</tr>
<tr>
<td>21-22</td>
<td>Standard 6</td>
</tr>
<tr>
<td>23-24</td>
<td>Standard 7</td>
</tr>
<tr>
<td>25-26</td>
<td>Standard 8</td>
</tr>
<tr>
<td>27-28</td>
<td>Standard 9</td>
</tr>
<tr>
<td>29-30</td>
<td>Standard 10</td>
</tr>
<tr>
<td>31-34</td>
<td>Zero</td>
</tr>
<tr>
<td>35-....</td>
<td>Samples</td>
</tr>
<tr>
<td>Zeros</td>
<td>Two zeros every 50 samples</td>
</tr>
<tr>
<td>Standard curve</td>
<td></td>
</tr>
<tr>
<td>Final two tubes</td>
<td>Excess</td>
</tr>
</tbody>
</table>

**Table 6.1:** Table outlining the general structure of an RIA and tubes used to assess of performance of the assay
APPENDIX C: Solutions used in this thesis

20 mM HEPES pH 11

4.76 g HEPES in 900 ml water. Adjust to pH 11 with 0.5 M NaOH and made up to 1 L with di-H2O.

GLP-1R RBA Buffer

20 ml 1M HEPES pH 7.4 (0.02M), 0.736g CaCl2.2H2O (5mM), 0.204g MgCl2.8H2O (1mM), made up to 1 L total volume with di-H2O. 1.7 ml 30% BSA added to 48.3 mL buffer (final concentration 1%) on day of assay.

GCGR RBA Buffer

25 ml 1 mM HEPES pH 7.4 (0.025M), 0.406g MgCl2 (2mM), 25 ml 10% Tween 20 (0.05%), 0.038 diprotin A (0.1mM). Made up to 1 L total volume with di-H2O. 1.7 ml 30% BSA added to 48.3 mL buffer (final concentration 1%) on day of assay.

Phosphate buffer (RIA buffer)

48 g of Na3HPO4.2H2O, 4.13 g KH2PO4, 18.6 g (HO2CCH2)2NCH2CH2N(CH3CO2H)2, 2.5 NaN3 dissolved in 5 L of water. pH is 7.6 ± 0.1 and stored at 4°C.
APPENDIX D: RNA Electrophorisis Summary (Agilent Bioanalyser)
Figure 5.1. Electropherogram and gel-like images generated during the analysis of total RNA extracted from BAT and liver from Wistar rats. RNA integrity numbers (RIN) is an indicator of overall RNA degradation. Samples analysed using the Agilent Bioanalyzer system.
Presentations and Publications:

*Presentation:*

**Klara Hostomska**, Victoria Salem, Benjamin CT Field, Kevin G Murphy, Mohammad A Ghafei, Stephen R Bloom. Resistance to the anorectic effect of PYY(3-36) during continuous infusion is independent of the stimulation of appetite resulting from weight loss. (Poster) *Endo 2010: The 92nd Annual Meeting and Expo* (June 2010)

*Publications:*

Amir H. Sam, David J Gunner, Aileen King, Shanta J Persaud, **Klara Hostomska**, Heather E Ford, Bo Liu, Mohammad A Ghafei, Steve R. Bloom, Gavin A Bewick. Selective ablation of Peptide YY cells in adult mice reveals a role in beta-cell survival. (under review at *Gastroenterology*)


**Klara Hostomska**, Victoria Salem, James Minnion, Joyceline Cuenco-Shillito, James S. Minnion, Jordan E. Baxter, Tricia Tan, Benjamin CT Field, Kevin G Murphy, Mohammad A Ghafei, Stephen R Bloom. Resistance to the anorectic effect of PYY(3-36) during continuous infusion is independent of the stimulation of appetite resulting from weight loss. (in preparation)
References


Ref Type: Abstract


Hetherington,A.W. and Ranson,S.W. (1940). Hypothalamic lesions and adiposity in the rat. The Anatomical Record 78, 149-172.


Ref Type: Electronic Citation


