Engineering an oxyntomodulin analogue for weight reduction

A thesis submitted for the degree of Doctor of Philosophy at Imperial College London

Samantha Price
2015

Section of Investigative Medicine
Division of Diabetes, Endocrinology & Metabolism
Department of Medicine
Imperial College London
Abstract

There is currently a lack of safe and effective treatments for obesity. The administration of long acting analogues of satiety gut hormones has been suggested as a potential weight loss pharmacotherapy. The gut hormone oxyntomodulin is a natural dual agonist that generates weight loss by reducing food intake and increasing energy expenditure; effects which are believed to be mediated by the GLP-1 receptor and glucagon receptor respectively. Previously designed long acting oxyntomodulin analogues have limited potency compared to native glucagon and GLP-1, due to the addition of large molecules to enhance circulatory half-life. Previous research in this lab has developed a slow release formulation that facilitates depot formation, allowing gradual peptide release over an extended period of time.

The first half of this thesis details the development of a potent oxyntomodulin-like (OXL) dual agonist. A combination of conservative substitutions to the amino acid sequence of glucagon that improve in vitro GLP-1 receptor efficacy without diminishing glucagon receptor activity are identified. Addition of a histidine ‘tail’ enhances the in vivo pharmacokinetic profile of these OXL analogues, which is likely to be due to improved interaction with the slow release diluent. Feeding studies in mice and rats demonstrate significant food intake reduction and weight loss following acute and chronic analogue administration. Metabolic analysis demonstrates that the optimised analogue OXLT9 increases energy expenditure in rats. Reductions in fat mass and improvements in blood glucose homeostasis are observed following chronic administration of OXLT9 in DIO mice. The second half of this thesis investigates the increase in food intake following low doses of OXL analogues that was observed during analogue screening, and results suggest that this phenomenon is probably specific to rats. The novel effects of GLP-1 receptor selective Glu-3 OXL analogues on bodyweight are also investigated. Findings indicate they may act as antagonists at the human and rat glucagon receptors and could block endogenous glucagon activity; making them unsuitable tools to investigate the mechanisms of oxyntomodulin activity in rat models.

The studies described in this thesis identify a potent dual agonist at the glucagon receptor and GLP-1 receptors that has the potential for once monthly administration as a weight loss therapy in man. Investigation of the novel food intake effects of low dose OXL analogues provides a starting point for further examination of analogue effects on energy expenditure and may be of relevance for human studies using oxyntomodulin-like analogues.
Acknowledgements

I would like to thank my supervisor Professor Steve Bloom for giving me the opportunity to work in his lab and for his endless words of wisdom. I am immensely grateful to James Minnion for all his advice, encouragement, hard work and friendship. I am also very grateful to the late Mohammad Ghatei for encouraging me to take on this PhD and weighing out my never-ending lists of peptides.

I would like to thank all the members of the G-series team, past and present. Even the worst day in the lab doesn’t seem so bad when you’re working with your friends; thanks for making science fun. Thank you also to the co-inhabitants of fellows’ room 2 for your friendship and support. I wish you all the best of luck for the future.

Last but not least I would like to thank my friends and family for their endless love, support and encouragement, and the odd glass of wine. Thank you for keeping me going.
Copyright Declaration

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution-Non Commercial-No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or distribution, researchers must make clear to others the licence terms of this work.

Declaration of contributors

This thesis was composed entirely by the author and all of the work in this thesis was performed by the author with any collaborations and assistance described below.

Chapters 3 & 4

Acute mouse and rat feeding studies and pharmacokinetic studies were carried out with the assistance of various members of the G-series team. In particular, Claire Fives, Elina Akalestou, James Minnion, James Plumer, Joy Cuenco-Shillito, Meriel Vaal, Tanya Stezhka and Zainab Malik.

Chapter 4

Chronic OXLT9 administration in mice and rats was carried out in collaboration with Claire Fives. Claire Fives, James Minnion and Joy Cuenco-Shillito assisted with the mouse IPGTT and subsequent removal of the contents of the gastrointestinal tract.

Chapter 5

The initial chronic rat experiments were carried out with assistance from James Minnion or Joy Cuenco-Shillito.

The data relating to the increased food intake in rats is currently under review as part of a manuscript submitted to the journal 'Peptides'.
Abbreviations

ACTH Adrenocorticotrophic hormone
AgRP Agouti-related protein
ANOVA Analysis of variance
AP Area postrema
ARC Arcuate nucleus of the hypothalamus
BAT Brown adipose tissue
BMI Body mass index
BSA Bovine serum albumin
cAMP Cyclic adenosine monophosphate
CART Cocaine and amphetamine regulated transcript
CCK Cholecystokinin
CeA Central nucleus of the Amygdala
CNS Central nervous system
CRH Corticotrophin-releasing hormone
CTA Conditioned taste aversion
DA Dopamine
DIO Diet induced obese
DIT Diet induced thermogenesis
DMN Dorsomedial nucleus of the hypothalamus
DMX Dorsal motor nucleus of vagus
DVC Dorsal vagal complex
DPP-IV Dipeptidyl peptidase-IV
EDTA Ethylenediaminetetraacetic acid
EMA European Medicines Agency
ENS Enteric nervous system
EX-4 Exendin-4
EX(9-39) Exendin (9-39)
FDA Food and Drug Administration
FI Food intake
GABA γ-aminobutyric acid
GCGR Glucagon receptor
GHRH Growth hormone releasing hormone
GHSR Growth hormone secretagogue receptor
GIP Glucose-dependent insulinitropic peptide
GLP-1 Gluc agon-like peptide 1
GLP-2 Glucagon-like peptide 2
GLP-1R Glucagon-like peptide 1 receptor
GOAT Ghrelin O-Acetyl transferase
GPCR G-protein coupled receptor
GTT Glucose tolerance test
HPA Hypothalamic-pituitary-adrenal
ICV Intracerebroventricular
IP Intraperitoneal
KO Knockout
LHA Lateral hypothalamic area of the hypothalamus
MC3R Melanocortin 3 receptor
MC4R Melanocortin 4 receptor
MCH Melanocortin-concentrating hormone
ME Median eminence
MEMRI Manganese enhanced magnetic resonance imaging
mRNA Messenger ribonucleic acid
α-MSH α-melanocyte-stimulating hormone
NA Noradrenalin
NEAT Non-exercise activity thermogenesis
NPY Neuropeptide Y
NTS Nucleus tractus solitarius
OXM Oxyntomodulin
PACAP pituitary adenylate cyclase-activating poly-peptide
PBS Phosphate buffered saline
PBN Parabrachial nucleus
PC Prohormone convertase
PK Pharmacokinetic
PKA Protein kinase A
POMC Proopiomelanocortin
PP Pancreatic polypeptide
PPG Preproglucagon
PVN Paraventricular nucleus of the hypothalamus
PYY Peptide YY
RIA Radioimmunoassay
RNA Ribonucleic acid
SC Subcutaneous
SEM Standard error of the mean
SNS Sympathetic nervous system
SON Supraoptic nucleus of the hypothalamus
T2DM Type two diabetes mellitus
UCP1 Uncoupling protein-1
VIP Vasoactive intestinal peptide
VMN Ventromedial nucleus of the hypothalamus
VTA Ventral tegmental area
WAT White adipose tissue
WHO World Health Organisation
5HT 5-hydroxytryptamine/serotonin
Table of Contents

Abstract ............................................................................................................... 2
Acknowledgements ........................................................................................... 3
Copyright Declaration ....................................................................................... 4
Declaration of contributors .............................................................................. 4
Abbreviations ...................................................................................................... 5
Table of Contents .............................................................................................. 7
Index of Figures ................................................................................................ 12
Index of Tables .................................................................................................. 14

Chapter 1 .............................................................................................................. 15

General Introduction .......................................................................................... 15

1.1. Obesity ....................................................................................................... 16
  1.1.1. Current obesity therapies ................................................................. 17

1.2. Energy homeostasis .................................................................................... 20
  1.2.1. Energy expenditure .......................................................................... 20
    1.2.1.1. Brown adipose tissue (BAT) ..................................................... 21
  1.2.2. Food intake ....................................................................................... 22

1.3. Central regulation of energy homeostasis .................................................... 23
  1.3.1. The hypothalamus and homeostatic neuropeptides ......................... 23
    1.3.1.1. Alpha-melanocyte stimulating hormone (α-MSH) ..................... 24
    1.3.1.2. Cocaine and amphetamine regulated transcript (CART) .......... 25
    1.3.1.3. Neuropeptide Y (NPY) ........................................................... 26
    1.3.1.4. Agouti-gene related protein (AgRP) ......................................... 28
    1.3.1.5. Melanin-concentrating hormone (MCH) .................................. 29
    1.3.1.6. Orexins ................................................................................... 30
  1.3.2. Other neuropeptides influencing energy homeostasis ....................... 31
  1.3.3. Central non-peptidergic systems and energy homeostasis ............... 31
    1.3.3.1. Monoamines ........................................................................... 31
    1.3.3.2. Cannabinoids ......................................................................... 33
  1.3.4. The Brainstem .................................................................................. 34
  1.3.5. Corticolimbic reward systems ......................................................... 35

1.4. Peripheral regulation of energy homeostasis ............................................... 35
  1.4.1. Adiposity signals .............................................................................. 37
    1.4.1.1. Leptin ..................................................................................... 37
    1.4.1.2. Insulin ................................................................................... 38
  1.4.2. The enteric nervous system and vagus nerve ..................................... 39
2.2.5. Mouse IP glucose tolerance test

2.2.4. Chronic rat and chronic mouse studies

2.2.3. Acute rat studies

2.2.2. Acute mouse studies

2.2.1. Animal husbandry and conditions

2.1. Peptide Synthesis

1.5. Thesis overview & aims

1.4.3. Hormones of the gastrointestinal system

1.4.3.1. Ghrelin

1.4.3.2. Cholecystokinin (CCK)

1.4.3.3. Pancreatic Polypeptide (PP) - fold peptides

1.4.3.3.1. Peptide tyrosine-tyrosine (PYY)

1.4.3.3.2. Pancreatic polypeptide (PP)

1.4.3.4. Proglucagon derived hormones

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

Peptide

GLP-1 secretion & degradation

Receptors

Food intake

Energy expenditure

1.4.3.4.2. Glucagon

Peptide

Stimuli for glucagon secretion

Glucagon receptor

Regulation of food intake

Energy expenditure

Stress

1.4.3.4.3. Oxyntomodulin

Peptide

Receptors

Food intake

Energy expenditure

1.4.4. Family B G-protein coupled receptors (GPCRs)

1.5. Thesis overview & aims

Chapter 2

Materials and Methods

2.1. Peptide Synthesis

2.2. In Vivo Studies

2.2.1. Animal husbandry and conditions

2.2.2. Acute mouse studies

2.2.3. Acute rat studies

2.2.4. Chronic rat and chronic mouse studies

2.2.4.1. Pair-Feeding

2.2.5. Mouse IP glucose tolerance test
2.2.6. Mouse body composition analysis ......................................................... 64
  2.2.6.1. Carcass decomposition ......................................................................... 64
  2.2.6.2. Glycerol assay ...................................................................................... 64
  2.2.6.3. Protein assay ........................................................................................ 65
2.2.7. Metabolic measurements using CLAMS .................................................. 65
2.2.8. Peptide pharmacokinetics in rats ............................................................. 66
2.3. Radioimmunoassay ....................................................................................... 66
2.4. In Vitro Studies ............................................................................................. 67
  2.4.1. Cell maintenance ...................................................................................... 67
    2.4.1.1. cAMP accumulation bioactivity assay ................................................ 68
  2.4.2. Receptor binding affinity assay ............................................................... 69
    2.4.2.1. Preparation of membranes from tissues ............................................... 69
    2.4.2.2. Preparation of membranes from cells ................................................ 69
    2.4.2.3. Bradford assay .................................................................................... 70
    2.4.2.4. Iodination of peptides ........................................................................ 70
    2.4.2.5. Receptor binding affinity studies ......................................................... 71
2.5. Statistical analysis ....................................................................................... 72

Chapter 3 .......................................................................................................... 73
Investigation of amino acid substitutions to glucagon to produce a dual agonist at the glucagon
and GLP-1 receptors ............................................................................................ 73
3.1. Introduction .................................................................................................. 74
  3.1.1. Peptide structure-function studies .......................................................... 74
  3.1.2. Structure and properties of glucagon ...................................................... 74
    3.1.2.1. Peptide conformation ......................................................................... 74
    3.1.2.2. N-terminal residues .......................................................................... 75
    3.1.2.3. C-terminal residues .......................................................................... 76
  3.1.3. Structure and properties of GLP-1 and Exendin-4 ................................... 79
    3.1.3.1. Peptide conformation ......................................................................... 79
    3.1.3.2. Important residues ........................................................................... 80
  3.1.4. Dual agonists at the GLP-1 and glucagon receptors .................................. 81
  3.1.5. Aims ........................................................................................................ 83
3.2. Results ......................................................................................................... 84
  3.2.1. Effect of OXL analogues on human GLP-1 receptor mediated cAMP accumulation ......................................................... 84
  3.2.2. Effect of select OXL analogues on rat GLP-1 receptor mediated cAMP accumulation ......................................................... 86
  3.2.3. Affinity of select OXL analogues at the human, rat and mouse GLP-1 receptors ................................................................. 87
  3.2.4. Effect of OXL analogues on human glucagon receptor mediated cAMP accumulation ......................................................... 88
  3.2.5. Effect of select OXL analogues on rat glucagon receptor mediated cAMP accumulation ......................................................... 91
3.2.6. Affinity of select OXL analogues at the human, rat and mouse glucagon receptors

3.2.7. Effect of OXL analogues on acute food intake in fasted mice

3.2.8. The effect of amino acid substitutions on pharmacokinetic parameters of OXL analogues

3.2.9. Effect of chronic administration of select OXL analogues on energy homeostasis in rats

3.3. Discussion

3.3.1. Conclusion

Chapter 4

C-terminal amino acid extensions to OXL analogues: Assessment of an optimised dual agonist as a potential obesity therapy

4.1. Introduction

4.1.1. Slow release formulation

4.1.2. Rationale for second generation analogue design

4.1.3. Aims

4.2. Results

4.2.1. Effect of C-terminal amino acid extensions on the pharmacokinetic profiles of OXL-Tail analogues

4.2.2. Effect of OXL-Tail analogues on food intake and bodyweight in fasted DIO mice

4.2.3. Effect of OXL-Tail analogues on food intake and bodyweight in fasted rats

4.2.4. Effect of OXL-Tail analogues on human glucagon receptor and GLP-1 receptor mediated cAMP accumulation

4.2.5. Effects of OXLT9 on rat glucagon receptor and GLP-1 receptor mediated cAMP accumulation

4.2.6. Affinity of OXLT9 for the human, rat and mouse glucagon receptor and GLP-1 receptor

4.2.7. Effect of chronic OXLT9 administration on food intake, bodyweight, blood glucose and body composition in DIO mice

4.2.8. Effect of chronic OXLT9 administration on food intake and bodyweight in rats

4.2.9. Effect of acute OXLT9 administration on energy expenditure in rats

4.3. Discussion

4.3.1. Conclusion

Chapter 5

Increased food intake with OXL analogues

5.1. Introduction

5.1.1. Aims

5.2. Results

5.2.1. Chronic OXL analogue administration increases food intake in rats

5.2.2. Effects of OXL analogues on rat and human GLP-1 receptor and glucagon receptor mediated cAMP accumulation

5.2.3. Affinity of OXL analogues at the rat, human and mouse glucagon receptors and GLP-1 receptors

5.2.4. Chronic dose response effects of OXL analogues on food intake and bodyweight in rats
5.2.5. Chronic OXL analogue administration does not increase food intake in mice ........................................ 151
5.2.6. OXL analogues do not increase acute food intake in rats ................................................................. 152
5.2.7. Effect of chronic administration of Glu-3 OXL analogues on food intake and bodyweight in rats .... 153

5.3. Discussion ................................................................................................................................................. 155
5.3.1. Conclusion .............................................................................................................................................. 160

Chapter 6 .......................................................................................................................................................... 161

Glucagon receptor antagonism with Glu-3 oxyntomodulin-like analogues ................................................. 161

6.1. Introduction .............................................................................................................................................. 162
6.1.1. Aims ....................................................................................................................................................... 163

6.2. Methods ................................................................................................................................................... 165
6.2.1. In vitro glucagon receptor antagonism .............................................................................................. 165

6.3. Results ..................................................................................................................................................... 165
6.3.1. Chronic dose response effects of Glu-3 analogue administration on food intake and bodyweight in rats ............................. 165
6.3.2. Affinity and efficacy of select Glu-3 analogues at the rat glucagon receptor and rat GLP-1 receptor __ 167
6.3.3. Glu-3 analogue antagonism of the rat glucagon receptor in vitro ..................................................... 168
6.3.4. Affinity and efficacy of select Glu-3 analogues at the human glucagon receptor .......................... 170
6.3.5. Glu-3 analogue antagonism of the human glucagon receptor in vitro ........................................... 171

6.4. Discussion ................................................................................................................................................. 173
6.4.1. Conclusion .............................................................................................................................................. 175

Chapter 7 .......................................................................................................................................................... 176

General discussion ............................................................................................................................................ 176

References ......................................................................................................................................................... 186

Appendix A: Peptide structures .................................................................................................................... 215
Appendix B: Amino acid abbreviations ........................................................................................................ 218
Appendix C: General principle of a radioimmunoassay .............................................................................. 219
Index of Figures

Figure 1.1: Simplified schematic of the uncoupling of ATP synthesis in BAT mitochondria by UCP1. 22
Figure 1.2: Overview of the hypothalamic and brainstem expression of neuropeptides and neuropeptide receptors involved in energy homeostasis. 24
Figure 1.3: Schematic diagram illustrating the neuropeptides released, and some of the receptors expressed, by NPY/AgRP and POMC/CART neuronal populations in the ARC. 29
Figure 1.4: Peripheral signals regulating energy homeostasis. 36
Figure 1.5: Tissue-specific post-translational processing of proglucagon. 44
Figure 1.6: Summary the interaction between glucagon and the HPA axis. 55
Figure 1.7: Simpilified schematic of Gas coupled GPCR activation. 59
Figure 3.1: Amino acid sequences of the glucagon receptor family ligands. 78
Figure 3.2: Effect of native peptides on cAMP accumulation in CHO-K1 cells over-expressing the human GLP-1 receptor. 84
Figure 3.3: Effect of OXL analogues on cAMP accumulation in CHO-K1 cells over-expressing the human GLP-1 receptor. 85
Figure 3.4: Effect of native peptides and analogues OXL10 and OXL11 on cAMP accumulation in CHL cells over-expressing the rat GLP-1 receptor. 86
Figure 3.5: Competitive receptor binding affinity of the native peptides versus 125I-GLP-1 at the human GLP-1 receptor. 87
Figure 3.6: Effect of native peptides on cAMP accumulation in CHO-K1 cells over-expressing the human glucagon receptor. 89
Figure 3.7: Effect of OXL analogues on cAMP accumulation in CHO-K1 cells over-expressing the human glucagon receptor. 90
Figure 3.8: Effect of native peptides and analogues OXL10 and OXL11 on cAMP accumulation in CHO-K1 cells over-expressing the rat glucagon receptor. 92
Figure 3.9: Competitive receptor binding affinity for the native peptides and analogues OXL10 & OXL11 versus 125I-glucagon at the human glucagon receptor. 93
Figure 3.10: Effect of glucagon and oxyntomodulin on acute food intake in mice. 94
Figure 3.11: Effect of GLP-1 and exendin-4 on acute food intake in mice. 95
Figure 3.12: Effect of OXL1 and OXL2 on acute food intake in mice. 96
Figure 3.13: Effect of OXL analogues on acute food intake in mice. 96
Figure 3.14: Effect of OXL analogues on acute food intake in mice. 97
Figure 3.15: Line graphs illustrating the pharmacokinetic profile of four OXL analogues. 98
Figure 3.16: Effect of chronic OXL10 and OXL11 administration on food intake and bodyweight in Wistar rats. 100
Figure 4.1: Line graphs illustrating the pharmacokinetic profile of four OXL-Tail analogues. 113
Figure 4.2: Effect of acute administration of OXL-Tail analogues on food intake and bodyweight in mice. 115
Index of Tables

Table 1.1: Summary of the pharmacological treatments for obesity currently available in Europe and the USA. 19

Table 3.1: Summary of mean IC\textsubscript{50} values for the native peptides and OXL analogues at the human, rat and mouse GLP-1 receptors. 88

Table 3.2: Summary of the mean EC\textsubscript{50} values for the native peptides and OXL analogues at the human glucagon receptor and GLP-1 receptor. 91

Table 3.3: Summary of the mean EC\textsubscript{50} values for the native peptides and analogues OXL10 & OXL11 at the rat glucagon receptor and GLP-1 receptor. 92

Table 3.4: Summary of mean IC\textsubscript{50} values for native peptides and analogues OXL10 & OXL11 at the human, rat and mouse glucagon receptors. 94

Table 3.5: Summary of pharmacokinetic parameters of OXL analogues. 98

Table 4.1: Summary of pharmacokinetic parameters of OXL-Tail analogues. 113

Table 4.2: A summary of receptor efficacy for the native peptides and OXL-Tail analogues at the human glucagon receptor and GLP-1 receptor. 119

Table 4.3: A summary of receptor efficacy for the native peptides and OXL-Tail analogues at the rat glucagon receptor and GLP-1 receptor. 120

Table 4.4: A summary of receptor affinity for the native peptides and select OXL analogues at the human, rat and mouse glucagon receptors. 121

Table 4.5: A summary of receptor affinity for the native peptides and select OXL analogues at the human, rat and mouse GLP-1 receptors. 121

Table 4.6: A summary of receptor efficacy for OXLT9 and Glu-3 equivalent OXLT9E3 at the rat glucagon receptor and GLP-1 receptor. 127

Table 5.1: Summary of day 6 cumulative food intake and bodyweight change data compared to vehicle. 145

Table 5.2: Summary of day 6 cumulative food intake and bodyweight change data compared to vehicle. 145

Table 5.3: A summary of native peptide and OXL analogue receptor efficacy ratios. 146

Table 5.4: A summary of mean IC\textsubscript{50} values for native peptides and analogues OXL14 & OXL15 at the rat, human and mouse glucagon receptors. 147

Table 5.5: A summary of mean IC\textsubscript{50} values for native peptides and analogues OXL14 & OXL15 at the rat, human and mouse GLP-1 receptors. 148

Table 5.6: A summary of mean EC\textsubscript{50}’s for the native peptides, OXL analogues and Glu-3 analogues at the rat glucagon receptor and GLP-1 receptor. 153

Table 6.1: A summary of Glu-3 peptide efficacy and affinity at the rat glucagon receptor and rat GLP-1 receptor. 167

Table 6.2: A summary of Glu-3 peptide efficacy and affinity at the human glucagon receptor. 170
Chapter 1

General Introduction
1.1. Obesity

Obesity, once a disease commonly associated with affluence, is now pandemic across the world afflicting developed and developing countries alike. The World Health Organisation (WHO) estimated that in 2014 13% of the adult population (18≥) were obese and 39% were overweight (WHO 2014). This equates to more than half a billion overweight adults, indicating that the worldwide prevalence of obesity has almost doubled since 1980. If current trends continue, it is predicted that 51% of the world population will be obese by 2030 (Finkelstein et al. 2012). Worryingly, the incidence of obesity in the under 5’s is also increasing dramatically. It was estimated that 6.3% of under 5’s were obese in 2013, a rise of 1.3% since 2000, and this prevalence is predicted to rise to 11% by 2025 (WHO 2014).

Being overweight or obese significantly increases the risk of developing debilitating and life threatening co morbidities, including type 2 diabetes mellitus (T2DM), cardiovascular disease, stroke, hypertension, sleep apnoea, osteoarthritis, back pain and numerous cancers (Guh et al. 2009; Brown et al. 2009). The obvious burden upon health services is accompanied by the substantial economic cost of treating obesity related co morbidities and paying for the indirect cost of lost productivity (Finkelstein et al. 2009; Withrow & Alter 2011; Wang et al. 2011).

Obesity has a complex aetiology, but is ultimately a consequence of interaction between genes and environmental factors, resulting in energy intake exceeding energy expenditure for a prolonged period of time. The recent rapid increase in the prevalence of obesity has been attributed to the wide availability and low cost of calorie-dense foods, coupled with increasingly sedentary lifestyles (Swinburn et al. 2011). Humans are predisposed to adiposity and several theories have been postulated to explain the evolutionary selection of the obesity phenotype. The ‘thrifty gene’ hypothesis suggests that, as early man was subjected to periods of famine, natural selection would have retained genes that promote efficient fat storage (Prentice et al. 2008). On the other hand, the ‘drifty gene’ theory posits that obesity is the result of genetic drift that occurred once humans were free of predators. Yet another theory suggests that obesity may be a maladaptive by-product of the positive selection of other advantageous traits (Albuquerque et al. 2015). Common obesity is polygenic and there is clear evidence for ethnic heterogeneity in susceptibility to obesity. Several risk alleles relating to the common obesity phenotype have been identified, but they only explain a small percentage of susceptibility (Albuquerque et al. 2015).

Studies in monozygotic twins have indicated that adult BMI is highly heritable, but can be modified to some extent by an active lifestyle (Naukkarinen et al. 2012). In the general overweight population
it has been shown that although lifestyle changes that promote energy expenditure and reduce energy intake do result in significant short term weight loss, this is not sustained and weight loss is often not maintained long term (Wadden et al. 1989; Ayyad & Andersen 2000; Foster et al. 2003).

1.1.1. Current obesity therapies

At present the most successful treatment for obesity is bariatric surgery. Surgical treatments involve making the stomach pouch much smaller, often coupled with resection of the small intestine, resulting in reduced appetite, significant sustained weight loss and improvement or amelioration of comorbidities. However, due to the high cost and limited availability of specialist surgeons, as well as the invasive nature of the operation, bariatric surgery is only available to a small percentage of patients. Following particular types of bariatric surgery, for example gastric bypass and sleeve gastrectomy which involve resection of the small intestine, alterations in gut hormone levels have been observed and this has been postulated to contribute to the significant weight loss and rapid amelioration of comorbid diabetes in these patients (le Roux et al. 2006a; Laferrere 2011; Madsbad et al. 2014). In particular, levels of PYY, GLP-1 and oxyntomodulin are increased following surgery and the effects of these hormones on food intake and bodyweight have subsequently been extensively studied (Section 1.4.3), with the view to creating a ‘medical bypass’ by exogenously administering gut hormones to mimic post bypass levels (Holdstock et al. 2008; Laferrére et al. 2010; Falkén et al. 2011; Alexander et al. 2014).

Current pharmacological treatment options for obesity are limited and several act non-specifically on ubiquitous neurotransmitter systems, resulting in numerous side effects (see Table 1.1 for a summary of current anti-obesity drugs and section 1.3.3.1 for the central effects of Qsymia®, Belviq® and Contrave®). Ideally, pharmacological anti-obesity therapies would utilise specific endogenous satiety and metabolic factors to reduce the drive to eat and promote weight loss. In recent years, advances in the scientific understanding of the physiological mechanisms controlling appetite and energy expenditure have provided a number of potential therapeutic targets. Continued physiological and pharmacological investigation of these systems coupled with the changes in gut hormone profile that are observed following bariatric surgery, will aid the design and development of specific anti-obesity therapies.
<table>
<thead>
<tr>
<th>Trade name</th>
<th>Active ingredients</th>
<th>Other uses of active ingredients</th>
<th>Licence date</th>
<th>Reported weight loss efficacy</th>
<th>Potential side effects/warnings</th>
<th>Contraindications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenical®/ Alli®</td>
<td>Orlistat – lipase inhibitor</td>
<td></td>
<td>• EMA – 1998 (2009 for Alli) • FDA – 1999 (2007 for Alli)</td>
<td>Less than 30% of patients achieve &gt;5% placebo adjusted weight loss at 1 year (Powell et al. 2011)</td>
<td>- Gastrointestinal (oily stools, loose bowel movements)</td>
<td></td>
</tr>
<tr>
<td>Qsymia® (Qnexa/Qsiva)</td>
<td>Topiramate – anti convulsant • Phentermine – amphetamine derivative</td>
<td>• Topiramate – epilepsy &amp; migraine • Phentermine – previous weight loss drug</td>
<td>• FDA – 2012 • EMA – rejected</td>
<td>79% of subjects achieve &gt;5% placebo adjusted weight loss at 1 year (Garvey et al. 2012)</td>
<td>- Tachycardia • Eye problems • Teratogenicity/foetal toxicity • Depression/suicidal ideation • Anxiety • Sleep disorders • Cognitive/memory impairment • Increased heart rate</td>
<td>- Women of childbearing age • Glaucoma • Thyroid problems • Monoamine oxidase inhibitors</td>
</tr>
<tr>
<td>Belviq®</td>
<td>Lorcaserin – 5HT2c receptor selective agonist</td>
<td></td>
<td>• FDA – 2012 • EMA – rejected</td>
<td>3.6% weight loss vs placebo (Smith et al. 2010)</td>
<td>- Cognitive/memory impairment • Psychiatric disturbance (euphoria/dissociation) • Depression/suicidal ideation • Priapism • Valvulopathy</td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Pharmacological Action</td>
<td>FDA Approval</td>
<td>EMA Approval</td>
<td>Weight Loss Achieved</td>
<td>Additional Warnings</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>----------------------</td>
<td>--------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Contrave®/Mysimba®</td>
<td>Buproprion – aminoketone (Buproprion) – antidepressants/smoking cessation, Naltrexone – alcohol &amp; opioid dependence</td>
<td>FDA – 2014</td>
<td>EMA – 2015</td>
<td>50.5% of subjects achieve &gt;5% placebo adjusted weight loss in 1 year (Apovian et al. 2012)</td>
<td>BLACK BOX WARNING Potential neuropsychiatric effects including depression/suicidal ideation - Seizures - Increased blood pressure/tachycardia - Hepatotoxicity - Eye problems</td>
<td></td>
</tr>
<tr>
<td>Saxenda®</td>
<td>Liraglutide (As Victoza® for treatment of T2DM (1.2-1.8mg dose vs 3.0mg dose of Saxenda®))</td>
<td>FDA – 2014</td>
<td>EMA – 2015</td>
<td>63.2-76% of subjects achieve &gt;5% weight loss (Astrup et al. 2009; Pi-Sunyer et al. 2015)</td>
<td>BLACK BOX WARNING Risk of thyroid C-Cell tumours - Acute pancreatitis - Acute gallbladder disease - Hypoglycaemia - Tachycardia - Renal impairment - Hypersensitivity reactions - Suicidal ideation - Gastrointestinal reactions</td>
<td>- Personal or family history of medullary thyroid carcinoma - Multiple endocrine neoplasia syndrome</td>
</tr>
</tbody>
</table>

Table 1.1: Summary of the pharmacological treatments for obesity currently available in Europe and the USA.
1.2. Energy homeostasis

Individual bodyweight tends to remain relatively stable despite the daily and monthly variation in food intake due to environmental and social variables (de Castro 2000; Schwartz et al. 2000; Woods et al. 2000). Both energy intake and energy expenditure can be adjusted to maintain homeostatic regulation of bodyweight and a prolonged imbalance between the two can result in obesity (Janowitz & Hollander 1955; Fregly et al. 1957; Keesey & Powley 1986; Keesey & Corbett 1990).

1.2.1. Energy expenditure

The energy expended by the body can be broadly delineated into 3 categories: basal metabolism; activity related thermogenesis; and adaptive thermogenesis. Basal metabolism (or basal metabolic rate) is the minimum energy requirement to maintain vital metabolic functions in an organism at rest. This includes respiration and circulation as well as cellular integrity, ion gradients, protein turnover and enzyme activity. It is the largest single component of energy expended by the body, contributing around 60% to the body’s total daily energy use (Levine 2002).

Activity related energy expenditure can be further separated into two categories, exercise-related activity thermogenesis and non-exercise activity thermogenesis (NEAT). For the vast majority of people intentional voluntary sporting-like exercise is a minor or negligible component of activity related thermogenesis. NEAT encompasses all activities that are not eating, sleeping or sports-like exercise and includes fidgeting and posture maintenance as well as occupational activities, walking to the shops and playing guitar (Levine 2002). Even in regular exercisers NEAT is the main component of activity related thermogenesis and is the main contributor to the inter-personal variability in energy expenditure. It has been suggested that a general reduction in NEAT due to an increase in sedentary lifestyles has contributed to the current obesity epidemic (Levine et al. 2006).

Adaptive thermogenesis includes energy expended during the process of food digestion and absorption, termed diet induced thermogenesis (DIT) (Westtererp 2004), and energy consumed to maintain body temperature in response to a change in ambient temperature. The latter category can be further categorised as shivering and non-shivering thermogenesis (Griggio 1988). Non-shivering thermogenesis has also been suggested to play a role in the regulation of energy expenditure following feeding. Brown adipose tissue (BAT) is the major site of non-shivering thermogenesis in mammals (Himms-Hagen 1984).
1.2.1.1. Brown adipose tissue (BAT)

BAT is a thermogenic organ found in the interscapular, subscapular, axillary, intercostals, perirenal, periaortic regions of small mammals and the newborns of larger mammals, including humans (Cinti 1999; Sell et al. 2004). BAT utilises fatty acids produced by lipolysis to dissipate chemical energy as heat by uncoupling fuel oxidation from ATP synthesis. This process is orchestrated by uncoupling protein 1 (UCP1) which is exclusively expressed in the inner mitochondrial membranes of brown adipocytes and is subsequently a specific marker of BAT activation (Cinti 1999; Lowell & Spiegelman 2000) (Figure 1.1). UCP1-mediated thermogenesis in BAT is under sympathetic nervous system (SNS) control, governed by specific brain regions in the brainstem and hypothalamus including the nucleus tractus solitarius (NTS), medial preoptic area and paraventricular nucleus (PVN) (Bamshad et al. 1999; Tupone et al. 2014). Brown adipocytes express adrenergic receptors and BAT thermogenesis is activated by noradrenalin (NA) (Tupone et al. 2014). The hypothalamic-pituitary-thryoid (HPT) axis has also been suggested to play a role in the regulation of BAT activity (Silva 2001; Lowell & Spiegelman 2000).

It was initially believed that the majority of human BAT involuted during childhood and only vestigial amounts remained into adulthood. However, recent developments in imaging technology have revealed that large amounts of active BAT are retained in adult humans, particularly in the subclavicular region (Virtanen et al. 2009; Symonds et al. 2012). There appears to be an inverse relationship between BAT activity and BMI and also BAT activity and age, suggesting that reduced BAT activation could contribute to the development of obesity and weight gain during aging (Cypess et al. 2009; Saito et al. 2009; van Marken Lichtenbelt et al. 2009; Robinson et al. 2014). It is well known that feeding rodents high fat diet results in an increase in diet induced thermogenesis and BAT activation is thought to contribute to this increase (Rothwell & Stock 1979).

BAT activation in rodents and some humans can be induced by exposure to cold. Individuals with cold inducible BAT activation demonstrated increased resting energy expenditure and insulin sensitivity, and more efficient whole body glucose disposal (Chondronikola et al. 2014). Increasing energy expenditure via specific activation of BAT is being investigated as a potential treatment for obesity; however the mechanisms regulating BAT activity do not appear to be identical in rodents and humans (Roman et al. 2015). Also, current imaging methods can only identify metabolically active tissue; therefore it is unclear whether there is actually any BAT present in individuals who do not display cold inducible BAT (Izzi-Engbeaya et al. 2015). It has been estimated that as little as 40-50g of active BAT could contribute a 20-25% increase in metabolic rate in a sedentary human,
which could prevent weight gain of up to 20kg a year (Rothwell & Stock, 2012); making BAT an attractive therapeutic target for the treatment of obesity.

Figure 1.1: Simplified schematic of the uncoupling of ATP synthesis in BAT mitochondria by UCP1. The energy produced by shuttling donated electrons (e⁻) along the electron transport chain is used to pump H⁺ across the inner mitochondrial membrane, creating a proton electrochemical gradient. During coupled respiration, the energy resulting from the movement of H⁺ along the electrochemical gradient into the mitochondrial matrix is harnessed by ATP-synthase to produce ATP. UCP1 uncouples the movement of the H⁺ from ATP synthesis by providing an alternative route across the membrane and the energy is dissipated as heat instead. UCP: uncoupling protein 1.

1.2.2. Food intake

Daily food intake is determined by a complex array of signals relating to the size and frequency of meals as well as the nutritional composition, the duration of satiety and total body adiposity (Blundell 1996). In humans in particular, energy intake is not entirely dependent on energy requirements, increasingly the hedonic or rewarding aspects of food have become more salient (Berthoud 2007; Shin et al. 2009; Zheng et al. 2009).

Within the body the regulation of energy balance is tightly controlled via a complex network of central and peripheral systems, including hypothalamic satiety systems, limbic reward pathways, the gastrointestinal system, the liver and adipose tissue. Bi-directional communication between these systems is mediated via a multitude of neuroendocrine factors, hormones, metabolites and the autonomic nervous system.
1.3. Central regulation of energy homeostasis

A number of brain areas are involved in the regulation of energy homeostasis, the most important of which are thought to be the hypothalamus and the caudal brainstem along with parts of the cortex and limbic system. These interconnected regions integrate and interpret peripheral and central signals regarding an animal’s energy status and nutrient requirements and can feedback to adjust subsequent intake and expenditure accordingly.

1.3.1. The hypothalamus and homeostatic neuropeptides

One of the earliest brain areas identified as having a crucial role in the regulation of energy balance was the hypothalamus (Brobeck et al. 1943; Hetherington & Ranson 1983). It is situated at the base of the brain and surrounds the third ventricle, which enables direct access to peripheral hormones and nutrients. The hypothalamus is comprised of a number of nuclei, including the arcuate nucleus (ARC), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) (Figure 1.2), which collectively integrate and propagate central and peripheral orexigenic and anorexigenic signals (Berthoud 2002). The ARC is located in the periventricular zone at the base of hypothalamus and is in close proximity to the median eminence (ME), a circumventricular organ which has a partial blood brain barrier, thus allowing direct access of circulating hormones and nutrients. The ARC contains two functionally distinct populations of first order neurons involved in the regulation of energy homeostasis: orexigenic neurons co-expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP), which stimulate food intake; and anorexigenic proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) co-expressing neurons, which inhibit food intake (Figure 1.3) (Elias et al. 1998; Hahn et al. 1998). These neuronal populations project extensively to second order neurons within the hypothalamus, in particular the PVN and VMN, and also throughout the brain.

A number of neuropeptides have been identified as playing a role in the regulation of energy homeostasis and these neuropeptides and their receptors are expressed throughout the hypothalamus and brainstem (Figure 1.2).
Figure 1.2: Overview of the hypothalamic and brainstem expression of neuropeptides and neuropeptide receptors involved in energy homeostasis.


1.3.1.1. Alpha-melanocyte stimulating hormone (α-MSH)

The anorexigenic neuropeptide α-melanocyte-stimulating hormone (α-MSH) is a post-translational processing product of POMC. POMC is expressed in the ARC and NTS, whereas α-MSH, is expressed in the ARC, DMN and PVN, as well as the anterior hypothalamus and NTS (Jacobowitz & O’Donohue 1978; Palkovits et al. 1987). Central, ventricular administration of α-MSH analogues significantly decreases food intake (Grill et al. 1998; Brown et al. 1998), as does intranuclear administration in the hypothalamus and brainstem (Kim et al. 2000; Williams et al. 2000; Skibicka & Grill 2009). Consistent with a role in inhibiting food intake, hypothalamic POMC expression in mice is significantly decreased following fasting (Mizuno et al. 1998).

α-MSH acts via two receptors, MC3R and MC4R. MC4R are widely distributed throughout the brain (Gantz et al. 1993; Mountjoy et al. 1994; Kishi et al. 2003), whereas MC3R expression is more limited and mainly concentrated in the ARC and DMN, but lower levels are also present in the thalamus, hippocampus, amygdala, raphe nucleus and the ventral tegmental area (VTA) (Roselli-Rehfuss et al.
Transgenic MC4R embryonic knock-out mice are hyperphagic and display a mature onset obesity phenotype (Huszar et al. 1997) whereas MC3R knock-out mice display a milder phenotype that still results in increased fat mass but without hyperphagia (Chen et al. 2000). Mutations in the MC4R gene are the most common cause of monogenic obesity in humans (Farooqi et al. 2000) and the most severe phenotypes are associated with mutations that cause a complete loss of MC4R function (Farooqi et al. 2003). At present the downstream mechanisms by which α-MSH activity at the MC3/4R regulate energy homeostasis are not completely understood.

Weight gain in MC4R knock-out animals is excessive relative to the increase in food intake, particularly when fed high fat diet, suggesting a concomitant reduction in energy expenditure (Huszar et al. 1997; Chen et al. 2000; Garza et al. 2008). Diet induced thermogenesis and physical activity are increased in response to dietary fat in wild type and MC3R knockout mice, but not MC4R deficient mice, (Butler et al. 2001). Fourth ventricle administration of an MC3/4R agonist and direct stimulation of the NTS and PVN increases core temperature, heart rate and spontaneous activity (Skibicka & Grill 2009; Zheng et al. 2005) indicating a possible effect of MC3R/4R activity on energy expenditure. However, re-expression of MC4R in the PVN and amygdala of knock-out mice prevents 60% of the obesity phenotype and rescues the associated hyperphagia without ameliorating the reduction in energy expenditure (Balthasar et al. 2005).

α-MSH plays a vital but complex role in the regulation of energy homeostasis. The wide expression of MC4R receptors makes it difficult to delineate exactly what that role involves and increases the likelihood of off-target effects of MC4R agonists in the treatment of obesity. Indeed, preclinical studies of MC4R agonists have also identified effects on penile function (Wessells et al. 2005), and psychological stress (Ryan et al. 2014).

1.3.1.2. Cocaine and amphetamine regulated transcript (CART)

The role of CART in energy homeostasis is unclear. In rodents, CART is expressed throughout the hypothalamic nuclei known to be important for the regulation of food intake, including the PVN, ARC, VMN, LHA and supraoptic nucleus (SON); as well as the pituitary and medulla. A similar pattern of expression is observed in the equivalent brain areas in humans (Koylu et al. 1997; Vrang et al. 1999; Menyhért et al. 2007). In the rodent ARC, CART is co-expressed in all anorexigenic POMC neurons (Vrang et al. 1999), whereas in the LHA CART is co-expressed with the orexigenic neuropeptide melanin concentrating hormone (MCH, section 1.3.1.5) (Vrang et al. 1999; Broberger 1999; Menyhért et al. 2007). Additionally, cell bodies of CART expressing neurons in the rodent ARC, LHA, DMN and the medial posterodorsal nucleus of the amygdala are in close apposition to nerve
terminals immunopositive for the orexigenic neuropeptide NPY (Lambert et al. 1998; Broberger 1999), suggesting a complex role for CART in the regulation of food intake.

Numerous studies have reported that ICV administration of CART has anorectic effects and reduces bodyweight (Lambert et al. 1998; Kristensen et al. 1998; Larsen et al. 2000; Stanley et al. 2001; Asakawa et al. 2001; Abbott et al. 2001; Rohner-Jeanrenaud et al. 2002; Wortley et al. 2004). However, the anorectic effects of ICV CART may be due to hindbrain mediated disturbance of motor function rather than effects on satiety (Kristensen et al. 1998; Larsen et al. 2000; Abbott et al. 2001; Aja et al. 2001; Stanley et al. 2001). Over-expression or direct injection of CART into the specific hypothalamic nuclei causes hyperphagia and weight gain (Abbott et al. 2001; Kong et al. 2003; Smith et al. 2008). However, ICV injection of CART anti-serum also increases food intake (Lambert et al. 1998), indicating endogenous CART may have both anorectic and orexigenic effects in different areas of the brain. The complex role of CART in the regulation of energy homeostasis is also reflected in the varying phenotypes reported for different strains of CART knockout mice (Asnicar et al. 2001; Wierup et al. 2005). To date a specific receptor for CART has not been identified, which makes it difficult to investigate its effects on energy homeostasis.

Over-expression of CART in the arcuate of rats is associated with greater weight loss following fasting, suggesting an increase in energy expenditure (Kong et al. 2003). CART mRNA levels in the arcuate are increased following cold exposure and a possible role of CART in the thermogenic response to cold has been suggested (Kong et al. 2003). Human genome studies have identified a mutation in the CART gene associated with reduced energy expenditure and early onset obesity (del Giudice et al. 2001).

CART appears to have both anorectic and orexigenic effects, probably related to its co-expression with MCH, α-MSH and close interaction with NPY neurons. The identification of a CART receptor/s may help to delineate its actions.

1.3.1.3. Neuropeptide Y (NPY)

NPY is a potent orexigenic neuropeptide widely expressed throughout the brain, with the highest concentrations found in the ARC, PVN and cerebral cortex (Chronwall et al. 1985; Gehlert et al. 1987; Morris 1989). Within the ARC, NPY neurons co-express AgRP (see section 1.3.1.4) and NPY expression is increased after acute food deprivation or chronic food restriction (Beck et al. 1990; Brady et al. 1990; Bi et al. 2003). NPY is expressed in the DMN under conditions involving a prolonged increase in energy demand (Malabu et al. 1994; Bi et al. 2003), or in some rodent models of obesity (Kesterson et al. 1997; Guan et al. 1998; Bi et al. 2001).
Evidence suggests a critical role for NPY in energy homeostasis. Central administration or over expression of NPY causes hyperphagia and obesity (Clark et al. 1984; Stanley & Leibowitz 1984; Billington et al. 1991a; Zarjevski et al. 1993; Billington et al. 1994; Yang et al. 2009). AAV mediated over-expression of NPY in the PVN increases meal frequency and in the LHA increases meal size (Tiesjema et al. 2007), indicating that the role of NPY in the regulation of feeding differs between hypothalamic areas. Reducing NPY expression in the ARC or DMN of adult rodents causes dramatic reductions in food intake and bodyweight (Gropp et al. 2005; Luquet et al. 2005; Gardiner et al. 2005; Yang et al. 2009). However, NPY knockout mice do not display an overt bodyweight or appetite phenotype, suggesting that compensation for NPY loss may occur in germline knock-outs. A possible role for NPY in the regulation of non-shivering thermogenesis has also been suggested; ICV NPY decreases BAT thermogenesis and increases lipid storage in WAT (Billington et al. 1991a) and intra-PVN NPY decreases BAT UCP1 expression (Billington et al. 1994) and suppresses sympathetic activation of BAT (Egawa et al. 1991).

There are 5 mammalian Y receptors which bind NPY and its related peptides with differing affinities (Larhammar 1996; Cabrele & Beck-Sickinger 2000). The orexigenic actions of NPY are mediated via Y1 and Y5 receptors (Kanatani et al. 1996; Wieland et al. 1998; Parker et al. 2000; Antal-Zimanyi et al. 2008). Y1 receptors are expressed throughout the central nervous system (CNS), but within the hypothalamus expression is specifically in the ARC, PVN and SON (Larsen et al. 1993; Kopp et al. 2002). Y5 receptors are less widely expressed but are present in the ARC, PVN, LH and SON of the hypothalamus, as well as the hippocampus, amygdala and brainstem (Gerald et al. 1996; Durkin et al. 2000; Morin & Gehlert 2006). The Y2 receptor is expressed on arcuate NPY neurons and is postulated to act as a presynaptic inhibitory autoreceptor to dampen NPY release (Broberger et al. 1997). Additionally, Y1 receptors are present on POMC neurons in the ARC (Broberger et al. 1997) enabling NPY to modulate the activity of these neurons (Roseberry et al. 2004). Energy expenditure is reduced following central administration of Y5 receptor agonists (Hwa et al. 1999; Mashiko et al. 2003; Henry et al. 2005) suggesting that the effects of NPY on energy expenditure may be mediated via this receptor.

Although studies suggest that Y1 and Y5 receptor antagonists may be useful as potential anti-obesity therapies, many fail to progress to clinical trials due to lack of selectivity and poor brain penetrability (MacNeil 2007). Those that have managed to progress demonstrated poor efficacy in long term randomised control trials (Erondu et al. 2006). Blocking both Y1 and Y5 receptors simultaneously may be more successful, although the wide expression of these receptors throughout the brain increases the likelihood of off target effects.
1.3.1.4. Agouti-gene related protein (AgRP)

AgRP expression is specifically limited to the ARC where it is co-expressed in approximately 95% of NPY neurons (Broberger et al. 1998). Central administration of AgRP in rodents is orexigenic (Rossi et al. 1998; Kim et al. 2000) and fasting increases AgRP expression in the ARC (Bi et al. 2003). AgRP is an antagonist of the MC3R (Ollmann et al. 1997) and an inverse agonist of the constitutively active MC4R (Nijenhuis et al. 2001), enabling AgRP to regulate melanocortin receptor activity independently of α-MSH. Thus, AgRP is orexigenic even in the absence of α-MSH and expression of AgRP under the control of a ubiquitous promoter results in obesity (Graham et al. 1997).

Similar to the findings with NPY, germline knock-out of AgRP does not result in an abnormal feeding or bodyweight phenotype (Qian et al. 2002). However, the reduced feeding and bodyweight effects of postnatal ablation of NPY/AgRP neurons are much greater than the effects seen in double germline knock-outs (Qian et al. 2002; Luquet et al. 2005), indicating a degree of developmental compensation occurs in the germline knock-out animals. It has been postulated that the role of AgRP in food intake regulation is more important under conditions of high energy demand, as AgRP expression is increased during pregnancy (Rocha et al. 2003) and in the ob/ob and db/db mouse model of obesity (Ollmann et al. 1997; Shutter et al. 1997). Conversely, AgRP expression in the hypothalamus of rats is increased following an acute fast but not after chronic food restriction (Bi et al. 2003).

POMC/AgRP double knock-out mice have the same phenotype as POMC knock-out mice, suggesting AgRP is not the mediator of the hyperphagia and increased bodyweight that results from the lack of POMC (Corander et al. 2011). Similarly, blocking the melanocortin receptors doesn’t prevent the increase in food intake observed following stimulation of NPY/AgRP neurons (Aponte et al. 2011). Post-natal ablation of NPY/AgRP neurons results in rapid starvation and germline NPY/AgRP knock-out mice are still susceptible to this (Phillips & Palmiter 2008), suggesting another factor relating to these neurons is responsible. NPY/AgRP neurons also express GABA, and the administration of a GABA_{A} receptor agonist in to the parabrachial nucleus (PBN) following ablation of NPY/AgRP neurons can maintain feeding. Thus, GABAergic signalling from the ARC to the PBN appears to be essential for maintenance of food intake and bodyweight (Wu et al. 2009).
Figure 1.3: Schematic diagram illustrating the neuropeptides released, and some of the receptors expressed, by NPY/AgRP and POMC/CART neuronal populations in the ARC.

Orexigenic NPY/AgRP neurons inhibit anorexigenic POMC/CART via GABA and NPY. AgRP is an inverse agonist at the MC4R and antagonist at the MC3R. NPY/AgRP neurons express autoinhibitory pre synaptic Y2 receptor. The MC3R is an inhibitory autoreceptor of POMC/CART neurons. α-MSH: alpha-melanocyte stimulating hormone, AgRP: agouti related peptide, CART: cocaine and amphetamine regulated transcript, GABA: gamma-aminobutyric acid, GHSR: growth hormone secretagogue receptor (ghrelin receptor), MC3R: melanocortin 3 receptor, MC4R: melanocortin 4 receptor, NPY: neuropeptide Y, Ob-Rb: leptin receptor, POMC: pro-opiomelanocortin, 5HT2cR: serotonin receptor 2C, Y1,Y5,Y2: NPY family receptors. (Cone 2005; Broberger et al. 1997)

1.3.1.5. Melanin-concentrating hormone (MCH)

The putative orexigenic neuropeptide melanin-concentrating hormone (MCH) is highly expressed in the LHAs and subthalamic zona incerta (Bittencourt et al. 1992). Central ICV administration of MCH stimulates food intake and increases bodyweight (Della-Zuana et al. 2002; Ito et al. 2003) and over-expression in the LHA results in obesity (Ludwig et al. 2000). Two MCH receptors have been identified, MCH-1R (Chambers et al. 1999) and mammal specific MCH-2R (Sailer et al. 2001). MCH-1R is widely expressed throughout rodent brains in feeding related hypothalamic and brainstem nuclei and also throughout the limbic system, including the hippocampus, amygdala and nucleus accumbens shell (Hervieu et al. 2000; Saito et al. 2001). Expression of MCH-1R in the limbic system supports findings suggesting a possible role for MCH in the motivation and reward aspects of feeding
The pattern of MHC-1R expression and MCH neuron projections in the brainstem suggests MCH may play a role in the functional aspects of feeding, for example mastication and saliva release. MCH activity in the LHA may be modified by reciprocal synaptic interaction with neurons expressing the neuropeptide orexin (Guan et al. 2002; Rao et al. 2008) (See section 1.3.1.6 for orexins). Transgenic mice with a targeted ablation of the MCH gene have lower bodyweights than controls due to a combination of hypophagia and increased energy expenditure (Shimada et al. 1998; Alon & Friedman 2006). However, the effects of MCH ablation on food intake and bodyweight seem to be dependent on diet composition and the genetic background of the animal (Kokkotou et al. 2005). MCH-1R knockout mice do not have lower bodyweights but are significantly leaner than controls, and this appears to be due to an increase in energy expenditure as the knockout animals are hyperactive and hyperphagic (Chen et al. 2002; Marsh et al. 2002). MCH-1R knockout mice also demonstrate an upregulation in mesolimbic dopamine receptors (Smith et al. 2005) and MCH-1R antagonists have anti-depressant and anxiolytic effects (Borowsky et al. 2002), indicating that MCH plays a number of complex roles within the brain.

1.3.1.6. Orexins

The putative orexigenic neuropeptides Orexin A and Orexin B are highly expressed in the LHA (Sakurai et al. 1998). The two orexin receptors, OX1R and OX2R are widely expressed throughout the brain; within the hypothalamus, expression of OX1R is highest in the VMN and OX2R in the PVN (Trivedi et al. 1998). The OX1R selectively binds orexin A with high affinity whereas OX2R binds non-selectively to both orexin A and orexin B (Sakurai et al. 1998). Food intake is increased by central administration of orexin A and, to a lesser extent, orexin B (Sakurai et al. 1998; Haynes et al. 1999). Although another study reported no effect at all following orexin B administration throughout the hypothalamus or brainstem (Dube et al. 1999). The differing affinity and expression of OX1R and OX2R suggest the orexins may have several different roles.

Orexin knockout mice do display hypophagia (Willie et al. 2001); however the main phenotype of these animals is narcolepsy, indicating that the effects on food intake may result from alterations in arousal or wakefulness (Chemelli et al. 1999). Administration of orexins directly into key hypothalamic and brainstem nuclei involved in the regulation of food intake help to identify effects not linked to arousal. Administration of orexin A directly in to the PVN, DMN, LHA and perifornical area increased food intake. However there was no effect of administration in to the ARC, VMN, preoptic area, CeA (central nucleus of the amygdala) or NTS (Dube et al. 1999). Orexin neurons originating in the LHA project to the VTA, an area known to be involved in learning, motivation and reward. Orexin A increases operant responding and conditioned place preference for palatable food
and drug rewards (Harris et al. 2005; Kay et al. 2014), which can be blocked by an OX1R antagonist (Kay et al. 2014; Zarepour et al. 2014). Current evidence suggests a role for orexin A and the OX1R in hindbrain mediated effects on reward and motivation relating to feeding.

1.3.2. Other neuropeptides influencing energy homeostasis

Arcuate NPY/AgRP and POMC/CART neurons project to second order neurons in the PVN which express corticotrophin-releasing hormone (CRH), thyrotrop releasing hormone (TRH) or oxytocin, all of which have been suggested to have central anorexigenic effects (Valassi et al. 2008; Sabatier et al. 2013). CRH and TRH are part of the hypothalamic-pituitary-adrenal (HPA) stress axis (Figure 1.6) and the hypothalamic-pituitary-thyroid (HPT) axis respectively. There are a number of other neuropeptides which have been suggested to play a role in the regulation of energy homeostasis, possibly via effects on the HPA or HPT axes, these include relaxin-3 (McGowan et al. 2014), urocortin (Spina et al. 1996), neuropeptide S (NPS) (Smith et al. 2006), galanin and galanin-like peptide (Seth et al. 2003).

1.3.3. Central non-peptidergic systems and energy homeostasis

1.3.3.1. Monoamines

The neurotransmitters serotonin (5-hydroxytryptamine, 5-HT), noradrenalin (NA) and dopamine (DA) are part of the monoaminergic system and play putative roles in feeding and appetite alongside more established roles in emotion and cognition. Early obesity drugs were derived from amphetamine which increases central monoamine concentrations. Several recently approved obesity treatments were originally developed for the treatment of central disorders such as depression, and decrease food intake as a side effect of their actions on monoamines. The effect of monoamines on feeding varies depending upon neurotransmitter type and the location of the target receptor within the brain (Valassi et al. 2008) and effects may be mediated via modulation of hypothalamic NPY and α-MSH neurons (Ramos et al. 2005).

The effects of NA on food intake depend upon which receptors are activated. Activation of the α1- and β2-adrenoceptors decreases food intake (Wellman et al. 1993; Ramos et al. 2005), whereas activation of α2-adrenoceptors in the PVN increases food intake (Leibowitz et al. 1984). The mechanisms behind the effects of NA on food intake are unclear and the relationship between NA and hypothalamic NPY and melanocortins has not been extensively investigated. NA may play a role in weight homeostasis independent of effects on food intake. β3-adrenoceptors have been
implicated in BAT thermogenesis in rodents; however the presence and metabolic relevance of BAT in adult humans remains to be confirmed. Current β3-adrenoceptor agonists and antagonists are ineffective in humans, suggesting either a lack of BAT inducible thermogenesis or the involvement of other receptors (Mund & Frishman 2013).

The effect of DA on food intake is also dependent upon which of the many receptor subtypes are activated and the location of the receptor within the brain. DA in the ARC and LHA is associated with inhibition of food intake, and in the VMH it is associated with stimulation of feeding (Ramos et al. 2005). Midbrain dopamine neurons have also been implicated in the motivation and reward aspects of feeding (Palmiter 2007).

Some of the earliest obesity drugs, for example phentermine and diethylpropion, were anorectic amphetamine-derived stimulants of the sympathetic nervous system (SNS) (Rodgers et al. 2012). Their general mechanism of action involved increasing central NA and DA concentrations by inhibiting re-uptake and stimulating release (Nelson & Gehlert 2006). However, current clinical use of amphetamine derivatives is only permitted in the USA and is limited to less than 12 weeks due to their stimulant properties and potential for addiction, as well as possible adverse cardiovascular effects (Kakkar & Dahiya 2015). Phentermine is currently licensed in the USA for long term treatment of obesity in combination with topiramate, an anticonvulsant commonly used in the treatment of epilepsy and migraine (Jones & Bloom 2015; Kakkar & Dahiya 2015) (combination marketed as Qysmia®, see section 1.1.1). Several pharmacological obesity treatments contain bupropion, an aminoketone that prevents re-uptake of NA and DA. Contrave® (marketed as Mysimba® in Europe) (see section 1.1.1) is currently licenced in the USA and Europe and a similar combination therapy containing bupropion and an anti-epileptic, zonisamide which enhances DA and 5-HT neurotransmission, has recently completed Phase II clinical trials. Tesofensine which inhibits the re-uptake of NA, DA and 5-HT is currently in Phase III clinical trials (Kakkar & Dahiya 2015).

Serotonin is well known to suppress food intake and bodyweight gain (Lam et al. 2010). Although the mechanism(s) behind this effect remains to be elucidated, it is postulated that serotonin indirectly reduces food intake via interaction with endogenous hypothalamic melanocortin and NPY systems (Ramos et al. 2005; Lam et al. 2010). The 5-HT1B and 5-HT2C receptors are expressed throughout the hypothalamus (Makarenko et al. 2002; Sharma et al. 1997) and are thought to be the main anorexigenic serotonin receptors. In particular, 5-HT2C receptors are present on POMC neurons (Ramos et al. 2005; Lam et al. 2010). 5-HT2C knockout mice are hyperphagic and develop late onset obesity and re-expression of 5-HT2C on POMC neurons alone rescues the knockout phenotype (Xu et
Chronic activation of 5-HT\textsubscript{2C} receptors in obese human subjects significantly reduces appetite and bodyweight (Sargent et al. 1997).

Serotonin releasing agents fenfluramine and dexfenfluramine became popular weight loss drugs during the 1970’s and 80’s. A highly publicised study in 1992 demonstrated the weight loss efficacy of fenfluramine combined with phentermine (Weintraub et al. 1992) resulting in these two drugs being widely prescribed together off label as Phen-Fen. However, in 1997 both fenfluramine and dexfenfluramine were withdrawn from the market after reports of young patients developing valvular abnormalities (Boughner 1997; Connolly et al. 1997).

The dual monoamine (5-HT and NA) re-uptake inhibitor, sibutramine was originally developed as an anti-depressant but was approved as an anti-obesity therapy in 1997. In addition to reducing food intake by promoting satiety, sibutramine increased energy expenditure in both rodents and humans and studies indicated it may activate BAT in rodents. It was postulated that the increase in basal metabolic rate in humans following sibutramine administration prevented the decline in energy expenditure usually associated with weight loss (Nisoli & Carruba 2000). However sibutramine was withdrawn from the market in 2010 due to adverse cardiovascular side effects (James et al. 2010). Despite the previous adverse effects relating to monoamine targeted therapies, in 2012 the FDA approved the use of 5-HT agonist Lorcaserin (Belviq\textsuperscript{®}) as a long term obesity treatment (see section 1.1.1). Lorcaserin is a selective 5-HT\textsubscript{2C} receptor agonist (Thomsen et al. 2008) with minimal activity at the 5-HT\textsubscript{2A} receptor which mediates hallucinations (Nichols 2004), or the 5-HT\textsubscript{2B} receptors which have been implicated in the development of cardiac abnormalities (Fitzgerald et al. 2000). The anorectic effect of Lorcaserin is postulated to be mediated by stimulation of α-MSH release via activation of 5-HT\textsubscript{2C} receptors on POMC neurons (Lam et al. 2008; Redman & Ravussin 2010).

Although the exact mechanisms by which monoamines exert their effects on energy homeostasis are unclear, anti-obesity therapies that increase 5-HT, NA and DA neurotransmission are relatively successful at reducing food intake and body weight. However, the use of monoamines as obesity therapies may be limited due to their wide range of central and peripheral effects and long term monitoring for adverse effects is essential.

1.3.3.2. Cannabinoids

Stimulation of food intake by phytocannabinoids, such as those found in the cannabis plant, and endocannabinoids, a group of endogenous neuromodulatory lipids, have been well documented in animals and humans (Kirkham & Williams 2001; Pagotto & Pasquali 2006). Cannabinoids exert their
orexigenic effects via CB1 receptors (Williams & Kirkham 1999; Colombo et al. 1998) which are widely distributed throughout the brain, including the basal ganglia, hippocampus, cerebellum, olfactory bulb and to a lesser extent the hypothalamus (Herkenham et al. 1990; Herkenham et al. 1991); as well as peripheral tissues including the enteric nervous system, liver and testes (Mackie. 2005). CB1 receptor expression has also been identified in adipocytes and an increase in circulating endocannabinoid concentrations, coupled with decreased adipocyte CB1 receptor expression has been observed in obese patients (Engeli et al. 2005). It has been postulated that cannabinoids stimulate appetite via interaction with central incentive and reward systems (Kirkham & Williams 2001). The potential use of the CB1 receptor as a target for the pharmacological treatment of obesity has previously been investigated. In 2006 the CB1 receptor inverse agonist rimonabant was approved as an anti-obesity agent in Europe. However it was withdrawn from the market in 2008 following reports of serious psychiatric side-effects such as depression, anxiety and suicidal ideation (Christensen et al. 2007). Rimonabant inhibits the neural processing of rewarding food stimuli in humans and increases aversive responses, which could have contributed to the anhedonia and depression experienced by patients (Horder et al. 2010). Several pre-clinical trials with neutral and peripherally restricted CB1 receptor antagonists have shown good efficacy in reducing food intake and bodyweight in mice without antagonising central CB1 receptors (Pavón et al. 2008; LoVerme et al. 2009; Alonso et al. 2011).

1.3.4. The Brainstem

In addition to the hypothalamus, the brainstem is another important brain area involved in the regulation of energy homeostasis. In particular, the dorsal vagal complex (DVC) located in the caudal brainstem plays a critical role in integrating peripheral and central information regarding energy status. The DVC comprises the dorsal motor nucleus of the vagus (DMX), the NTS and the area postrema (AP).

The NTS receives input from the gastrointestinal tract and associated digestive viscera via vagal afferents, which relay mechano- and chemosensory information and also express receptors for various peripheral endocrine signalling molecules (Schwartz 2000) (discussed in greater detail in section 1.4). The glossopharyngeal nerve also projects to the NTS, relaying taste information from the gustatory system (Schneeberger et al. 2014). The NTS has reciprocal connections with the hypothalamus, in particular ARC POMC neurons project to the NTS where there is high expression of MC4R receptors (Kishi et al. 2003). Approximately 10% of total POMC neurons are expressed within the NTS (Palkovits et al. 1987) indicating a local α-MSH circuit. Orexigenic MCH and orexin neurons in the LHA and also neurons in the PVN project to the NTS. Ascending neurons from the NTS project
extensively throughout the brain to areas implicated in central control of energy homeostasis (Ter Horst & Streefland. 1993), including limbic forebrain areas involved in arousal, motivation and reward (Rinaman 2010), as well as throughout the hypothalamus (Ito & Seki 1998).

The DMX is a major target of NTS projections (Ito & Seki 1998); it also receives afferent projections from the hypothalamic paraventricular nucleus and olfactory system (Roges et al. 1980). The preganglionic visceromotor neurons that innervate the gut, stomach and pancreas arise from the DMX (Powley et al. 1991).

The AP has an incomplete blood brain barrier and is therefore a circumventricular organ allowing CNS access to peripheral humoral signals that convey information about adiposity and nutritional status (see Section 1.4). The AP is also responsible for detecting toxins in the periphery and is involved in the emetic response (Miller & Leslie 1994). Projections from the AP extend to the NTS, DMX and parabrachial nucleus (Ito & Seki 1998).

1.3.5. Corticolimbic reward systems

Lower bodyweight limits are vigorously defended by various homeostatic mechanisms, but there appears to be little or no defence of an upper bodyweight limit. The lack of over-eating prevention has been attributed to environmental enhancement of the hedonic aspects of eating, which override physiological satiety signals (Shin et al. 2009; Berthoud 2007; Zheng & Berthoud 2007). Environmental cues have been postulated to affect the corticolimbic brain areas associated with learning, memory, reward, mood and emotion to alter ‘liking’ and ‘wanting’ responses to food (Zheng et al. 2009). In particular, ‘hedonic hotspots’ have been identified in the nucleus accumbens, which is part of the mesocorticolimbic dopaminergic pathway, and the ventral pallidum which receives projections from the lateral hypothalamus (Castro & Berridge 2014).

1.4. Peripheral regulation of energy homeostasis

Peripheral signals arising from the GI tract, pancreas and adipose tissue convey information about the body’s nutritional status to central homeostatic pathways via the hypothalamus and brainstem. With the exception of the gastric hormone ghrelin, all known peripheral signalling factors relating to energy homeostasis are anorexigenic. They can be short-acting signals released episodically, for example gut hormones released in response to nutrient ingestion, or tonic signals like leptin and insulin that convey information about long-term fat stores. (Figure 1.4)
Energy homeostasis is influenced by peripheral signals from adipose tissue, the pancreas and the gastrointestinal tract acting on central circuits in the hypothalamus and brainstem, either directly or via the vagus nerve. Blue arrows = anorexigenic actions, green arrows = orexigenic actions. CCK: cholecystokinin, GCG: glucagon, OXM: oxyntomodulin, PP: pancreatic polypeptide, PYY: peptide tyrosine-tyrosine.

Figure 1.4: Peripheral signals regulating energy homeostasis.
1.4.1. Adiposity signals

1.4.1.1. Leptin

The discovery of leptin in 1994 was a major breakthrough in obesity research. It is the protein product of the ob gene, derived in the adipocytes of white adipose tissue (WAT) (Zhang et al. 1994). The primary role of leptin is to inform the brain about the long term status of WAT energy stores. In general, circulating leptin levels correlate with body adiposity (Maffei et al. 1995; Considine et al. 1996), although plasma leptin levels can also reflect short term perturbations in energy intake, for example fasting (Boden et al. 1996; Harris et al. 1996; Kolaczynski et al. 1996b; Weigle et al. 1997; Dubuc et al. 1998). Increased leptin signalling reduces food intake and increases energy expenditure (Weigle et al. 1995; Halaas et al. 1997; Rosenbaum et al. 2005). Conversely, decreased leptin signalling due to gene or receptor defects results in obesity due to hyperphagia and increased fat deposition (Coleman 1973; Cleary et al. 1980; Zhang et al. 1994; Farooqi & O’Rahilly 2014). Reduced leptin signalling also has a profound negative effect on numerous other regulatory systems, including reproductive and stress response axes and the immune system (Ahima & Flier 2000; Park & Ahima 2015). The magnitude of the physiological response to a small reduction in leptin levels is significantly greater than that seen in response to an increase in leptin levels, suggesting the primary role of leptin is to prevent starvation.

Leptin signalling is mediated via the long form of the leptin receptor (Ob-Rb or Lep-Rb) (Chen et al. 1996) which is highly expressed within the hypothalamic ARC, VMN and DMN and to a lesser extent in the PVN and LHA (Elmquist et al. 1998). Within the ARC, the Ob-Rb receptor is expressed on the orexigenic NPY/AGRP neurons (Baskin et al. 1999) as well as the anorexigenic POMC/CART neurons (Cheung et al. 1997). Circulating leptin crosses the blood brain barrier via an unidentified, saturable transport system (Banks et al. 1996), subsequently inhibiting orexigenic neuron activity and stimulating the release of the anorectic peptides α-MSH and CART (Cowley et al. 2001; Pinto et al. 2004; Morton et al. 2006; Baver et al. 2014).

After the initial discovery of leptin and its success in treating leptin deficient ob/ob mice (Pelleymounter et al. 1995), exogenous administration was pursued as a treatment for obesity. However, the phenotype of polygenic obesity is associated with leptin resistance; therefore exogenous leptin has little or no effect on bodyweight or food intake in these patients (Heymsfield et al. 1999; Hukshorn et al. 2000). Resistance to leptin has been postulated to result from a combination of factors including reduced transport across the blood brain barrier (Caro et al. 1996; Schwartz et al. 1996) and impaired leptin signalling in hypothalamic leptin-sensitive neurons (Halaas et al. 1997; El-Haschimi et al. 2000; Münzberg et al. 2004).
The use of leptin in the treatment of obesity has not been ruled out completely. The maintenance of a lower bodyweight following initial weight loss is one of the most difficult aspects of combating the obesity epidemic. Weight loss is associated with a disproportionate reduction in energy expenditure, greater feelings of hunger and delayed satiation (Leibel et al. 1995; Rosenbaum et al. 2008; Kissileff et al. 2011), which are reminiscent of reduced leptin signalling. Leptin administration following moderate weight loss has been shown to prevent these effects and to assist in weight maintenance in the post-obese state (Rosenbaum et al. 2005; Kissileff et al. 2011). Therefore, leptin therapy post weight loss may be a useful strategy for weight maintenance.

1.4.1.2. Insulin

Insulin is a protein secreted by pancreatic β-cells which plays a critical role in the acute regulation of blood glucose. Following nutrient ingestion, insulin is rapidly secreted and stimulates tissues such as the liver, adipose tissue and skeletal muscle to take up excess glucose from the circulation. During fasting and times of negative energy balance plasma insulin levels decrease (Owen et al. 1974). Circulating insulin levels are much higher in obese patients and correlate with levels of adiposity, suggesting a role for insulin as a signal of long term energy stores (Bagdade et al. 1967; Polonsky et al. 1988). Despite the increase in basal circulating insulin levels, the acute pulsatile response of insulin following nutrient ingestion is maintained in obesity (Polonsky et al. 1988).

Peripheral insulin crosses the blood brain barrier in proportion to circulating concentrations via a saturable receptor-mediated transport system (Baura et al. 1993). The tyrosine kinase insulin receptor (IR) is distributed throughout the brain but most notably in the ARC, where it is expressed on POMC neurons (Benoit et al. 2002), and also the DMN and PVN (Corp et al. 1986; Marks et al. 1990). Central administration of insulin reduces food intake and bodyweight in rats (Woods et al. 1979; McGowan et al. 1992; Air et al. 2002). Neuron specific disruption of the IR results in diet induced obesity and sex-specific hyperphagia (Bruning et al. 2000).

Insulin’s effects on food intake appear to be mediated partly by effects on hypothalamic neuropeptides. Central administration of insulin increases POMC mRNA expression and blocking central melanocortin receptors prevents the insulin mediated decrease in food intake, suggesting interaction between insulin and anorexigenic α-MSH (Benoit et al. 2000). Animal models of obesity and diabetes display increased hypothalamic expression of NPY and AgRP (Williams et al. 1989; Abe et al. 1991; Marks et al. 1993; Havel et al. 2000), while expression of POMC is decreased (Havel et al. 2000). Hypothalamic NPY levels are normalised following insulin administration in insulin deficient models (Abe et al. 1991; Marks et al. 1993; Sipols et al. 1995; Havel et al. 2000). Central insulin administration fails to inhibit food intake (Ikeda et al. 1986) or alter NPY levels (Abe et al. 1991;
Schwartz et al. 1992) in insulin resistant diabetic models. These findings could explain the hyperphagia experienced in uncontrolled diabetes and indicate that insulin may contribute to the control of NPY expression under physiological conditions.

The effects of peripheral insulin administration on food intake are more difficult to gauge due to the ensuing hypoglycaemia. However, sub-hypoglycaemic peripheral doses of insulin decrease food intake (Vanderweele et al. 1982) and a reduction in food intake has also been reported during hyperinsulinaemic euglycaemic clamps in both rats (Nicolaidis & Rowland 1976) and primates (Woods et al. 1984). Collectively, the data demonstrates a role for insulin in the regulation of feeding.

1.4.2. The enteric nervous system and vagus nerve

The gastrointestinal tract is the only organ to possess an extensive intrinsic nervous system, termed the enteric nervous system (ENS). The primary role of the ENS is to co-ordinate local activity within the digestive tract via modulation of various processes, including gut movement patterns; local blood flow; gastric and pancreatic secretions and the secretion of gut hormones (Furness 2012). Although the ENS is implicated in every aspect of gut function and is capable of maintaining intestinal function when completely cut off from the CNS, it does not function autonomously under normal physiological conditions.

The vagus nerve is the major extrinsic nerve responsible for communication along the gut-brain axis. Sensory vagal afferents originating in the gastrointestinal mucosa respond to mechanical and chemical stimuli (Phillips & Powley 2000; Berthoud et al. 2001; Page et al. 2002) and relay information to the NTS in the brainstem (Schwartz 2000). Although there is some evidence that gut hormones can cross the blood brain barrier and act directly within the brain, studies using surgical or chemical subdiaphragmatic vagotomy have suggested that indirect signalling via the vagus nerve is the major communication route between gut hormones and the brain (Date et al. 2002; Koda et al. 2005). Vagal afferents are sensitive to a number of hormones produced by the enteroendocrine cells of the gastrointestinal tract including cholecystokinin (CCK), peptide tyrosine-tyrosine (PYY), glucagon-like peptide 1 (GLP-1) and ghrelin (MacLean 1985; I˙meryüz et al. 1997; Date et al. 2002; Koda et al. 2005) (Section 1.4.3).

Vagal efferent fibres originate in the DMX of the brainstem but the terminating postganglionic efferent fibres are considered part of the ENS. These efferent fibres influence esophageal propulsion; gastric and pancreatic exocrine secretion; and secretion of gut hormones (Furness et al. 2014).
1.4.3. Hormones of the gastrointestinal system

1.4.3.1. Ghrelin

Ghrelin is the only known orexigenic gut hormone. The 28 amino acid hormone is present in the circulation as an acylated and a non-acylated form. The bioactive acylated form possesses octanoate attached at Ser-3 by the enzyme ghrelin O-Acyltransferase (GOAT) (Yang et al. 2008) and it is this form that is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al. 1999; Hosoda et al. 2000). Ghrelin is released from X/A-like cells mainly found in the fundus of the stomach, but also present in lower numbers throughout the gut (Date et al. 2000; Hosoda et al. 2000; Sakata et al. 2002). In addition to stimulating food intake, ghrelin also stimulates the release of growth hormone from the pituitary (Kojima et al. 1999), helps to maintain fasting blood glucose (Scott et al. 2012) and increases gastric emptying (Masuda et al. 2000; Levin et al. 2006).

Ghrelin levels rise sharply immediately before a meal and fall to trough levels post prandially suggesting a role for ghrelin in signalling meal initiation (Cummings et al. 2001). The post prandial suppression of ghrelin is blunted in obese patients which may influence satiety and contribute to the maintenance of obesity (le Roux et al. 2005a). Circulating ghrelin levels increase during fasting and normalise following re-feeding (Tschop et al. 2000; Wren et al. 2001a). Plasma ghrelin levels are inversely correlated with BMI which may indicate ghrelin is a signal of long term energy stores (Cummings et al. 2002; Shiiya et al. 2002).

Food intake is increased following peripheral ghrelin administration in humans (Wren et al. 2001b; Druce et al. 2005) and peripheral and central administration in rodents (Tschop et al. 2000; Wren et al. 2001a; Date et al. 2002). Vagotomy prevents the increase in food intake indicating that the vagus nerve is the major signalling pathway of ghrelin (Date et al. 2002; le Roux et al. 2005b). GHS-Rs are expressed on the vagus nerve (Date et al. 2002) and in the hypothalamic ARC (Mondal et al. 2005).

Interestingly, ghrelin mRNA is also expressed in neurons within the hypothalamus (Kojima et al. 1999; Mondal et al. 2005; Sato et al. 2005; Menyhért et al. 2006). The orexigenic effects of ghrelin appear to be mediated via altered signalling of arcuate NPY/AgRP and POMC neurons (Nakazato et al. 2001; Date et al. 2002; Tamura et al. 2002; Chen et al. 2004).

The increase in bodyweight following ghrelin administration may not be solely due to increased food intake. In rodents, ghrelin directly promotes adiposity by reducing fat utilisation and upregulating expression of fat storage enzymes. In addition it reduces energy expenditure by suppressing sympathetic nervous activity and decreasing BAT UCP1 expression (Tschop et al. 2000; Thompson et al. 2004; Tsubone et al. 2005; Theander-Carrillo et al. 2006; Mano-Otagiri et al. 2009).
Obese subjects remain responsive to the appetite stimulating effects of ghrelin; therefore supressing ghrelin may be a possible therapeutic approach. Ghrelin antibodies supress feeding and reduce fat mass in rodents (Nakazato et al. 2001; Shearman et al. 2006) and pigs (Vizcarra et al. 2007), however results from the only clinical trial using ghrelin antibodies reported a lack of weight reduction (Patterson et al. 2011b). GOAT antagonists are another possible target to reduce levels of active acyl ghrelin and studies in rodents have shown promising reductions in food intake and fat mass (Barnett et al. 2010).

1.4.3.2. Cholecystokinin (CCK)

CCK is primarily released from I cells in the upper small intestine (Polak et al. 1975; Buchan et al. 1978; Larsson & Rehfeld 1978) in response to nutrient ingestion (Liddle et al. 1985). Plasma levels of CCK rise around 15 minutes after eating and remain elevated for up to 2 hours (Liddle et al. 1985). CCK is also expressed in the majority of GLP-1 containing L-cells in the upper small intestine (Habib et al. 2012), suggesting that enteroendocrine cell populations may not be as discrete as initially thought (Egerod et al. 2012).

CCK is postulated to act as a satiety signal via activation of vagal CCK$_{A}$ (CCK$_{1}$) receptors (MacLean 1985; Moran et al. 1997; Moran et al. 1998). Rats lacking CCK$_{A}$ receptors are hyperphagic and obese, demonstrating an increase in meal size resulting from an apparent lack of satiation (Moran et al. 1998; Schwartz et al. 1999; Covasa & Ritter 2001). Administration of CCK antibodies or CCK$_{A}$ receptor antagonists increases food intake and bodyweight (McLaughlin et al. 1985; Brenner & Ritter 1995). Interestingly, neither CCK nor CCK$_{A}$ receptor knockout mice are obese and CCK knockout mice appear to be resistant to high fat diet induced obesity (Bi et al. 2007; Lo et al. 2010).

Short term peripheral CCK administration in humans reduces food intake by increasing satiation (Kissileff et al. 1981; Muurahainen et al. 1988; Geary et al. 1992). However in rodents, peripheral infusion of CCK decreases meal size but increases meal number to compensate, resulting in normal cumulative food intake and weight gain (West et al. 1984; West et al. 1987). During chronic administration in rodents tolerance to CCK induced satiety has been shown to develop in as little as 4 hours (Crawley & Beinfeld 1983a), indicating that the therapeutic potential of exogenous CCK may be limited. There is no evidence that CCK production and function is altered in obese individuals. However co-administration of CCK with leptin results in a greater amount of weight loss for a small reduction in food intake (Matson et al. 2000). Therefore CCK may be useful to enhance the anorexigenic and energy expenditure effects of leptin in leptin resistant obese patients.
1.4.3.3. Pancreatic Polypeptide (PP) -fold peptides

The PP-fold family of proteins consists of NPY and the gastrointestinal hormones pancreatic polypeptide (PP) and peptide tyrosine-tyrosine (PYY). All three peptides share the same structural characteristic of a hair-pin turn within the amino acid backbone that is essential for their biological activity (Germain et al. 2013). All three PP-fold proteins play distinct roles in appetite regulation and their effects are mediated via a superfamily of 5 G-protein-coupled receptors termed Y1, Y2, Y4, Y5 and Y6, which are classified according to their differing affinities for NPY, PP and PYY (Larhammar 1996; Cabrele & Beck-Sickingner 2000).

1.4.3.3.1. Peptide tyrosine-tyrosine (PYY)

Following nutrient ingestion, PYY is released from enteroendocrine L-cells in the terminal ileum and colon in proportion to caloric content, macronutrient composition and consistency of the meal (Adrian et al. 1985). Two biologically active forms of PYY are present in the circulation; PYY$_{1-36}$ and the predominant truncated form PYY$_{3-36}$, produced by N-terminal cleavage of PYY$_{1-36}$ by the endopeptidase dipeptidyl peptidase-IV (DPPIV) (Mentlein et al. 1993b). PYY$_{1-36}$ binds with differing affinities to all 5 Y-receptors whereas PYY$_{3-36}$ is a Y2r selective agonist which also has low affinity for the Y5r (Cabrele & Beck-Sickingner 2000; Kanatani et al. 2000; Keire et al. 2000). Peripheral PYY$_{1-36}$ does not appear to have a role in energy homeostasis (Sloth et al. 2007), whereas PYY$_{3-36}$ is widely accepted as an anorexigenic hormone that reduces food intake in lean and obese humans and animals via the Y2r (Batterham et al. 2002; Batterham et al. 2003b; Talsania et al. 2005; le Roux et al. 2006b).

There is some evidence that PYY$_{3-36}$ exerts its anorectic effects by modulating the activity of hypothalamic neurons in the ARC via the Y2r, which is expressed on over 80% of NPY/AgRP neurons where it is believed to function as a presynaptic inhibitory receptor (Broberger et al. 1997; Batterham et al. 2002). Another study found that PYY$_{3-36}$ inhibits firing of both NPY and POMC ARC neurons (Acuna-Goycolea & van den Pol 2005) and the anorectic response to PYY$_{3-36}$ is maintained in POMC-null mice (Challis et al. 2004), suggesting melanocortin signalling is not necessary. PYY$_{3-36}$ may generally dampen ARC activity. There is also substantial evidence that vagal afferent signalling is required for the anorectic effects of PYY$_{3-36}$ and alterations in hypothalamic neuronal firing (Zhang et al. 1996; Abbott et al. 2005a; Koda et al. 2005).

A limited number of studies have also suggested PYY$_{3-36}$ may be involved in the modulation of neuronal activity in higher cortical and corticolimbic areas, in particular the orbitofrontal cortex which is involved in reward, emotion and decision (Batterham et al. 2007; De Silva et al. 2011). It has
been postulated that the presence of a post prandial satiety factor like PYY\textsubscript{3-36} may switch the regulation of food intake from homeostatic-hypothalamic control to hedonic-corticolimbic control (Batterham et al. 2007).

Obese humans and rodents have lower plasma levels of PYY and a blunted post prandial rise, but remain responsive to the anorectic effects of exogenous PYY\textsubscript{3-36} (le Roux et al. 2006b; Batterham et al. 2003b; Sloth et al. 2007). The use of exogenous PYY\textsubscript{3-36} as a treatment for obesity is limited as it has a very short circulatory half-life due to rapid degradation and clearance (Addison et al. 2011). Additionally, the supraphysiological doses of PYY\textsubscript{3-36} that are likely to be required to prolong satiety and food intake reduction in obesity treatment are associated with nausea (le Roux et al. 2008), and Y receptor promiscuity is more likely at these higher doses. Therefore the development of potent long-acting, Y2r specific PYY\textsubscript{3-36} analogues is necessary.

1.4.3.3.2. Pancreatic polypeptide (PP)

PP is an amidated 36 amino acid peptide secreted from a small distinct population of cells in the islets of Langerhans in the pancreas (Larsson et al. 1975; Adrian et al. 1976). Circulating levels of PP rise rapidly following nutrient ingestion and remain elevated for up to 6 hours post prandially (Adrian et al. 1976). Similar to PYY, PP levels are lower in obese subjects and display a much smaller postprandial increase (Lassmann et al. 1980; Marco et al. 1980). PP administration reduces food intake in rodents (Malaisse-Lagae et al. 1977; Asakawa et al. 2003) and normal weight humans (Batterham et al. 2003a; Jesudason et al. 2007). PP has a high affinity for the Y4 receptor (Y4r) (Larhammar 1996) which is widely expressed throughout the hypothalamus and brainstem, including the ARC, PVN, DMN, VMN, AP and NTS (Parker & Herzog 1999). The mechanism by which PP mediates its anorectic effect is unclear. IP PP administration increases POMC mRNA expression in the ARC and activates \( \alpha \)-MSH expressing neurons, both of which are abolished in mice with ARC specific knockout of the Y4r (Lin et al. 2009). Peripheral PP administration also increases neuronal activity in the VMN, PVN, and LHA as well as the AP and NTS in the brainstem (Lin et al. 2009). This suggests PP may directly activate central Y4 receptors. However, food intake inhibition by PP is abolished following vagotomy, suggesting signalling via the vagus nerve is essential (Asakawa et al. 2003). PP may also decrease gastric emptying and reduce expression of the orexigenic hormone ghrelin, which could contribute to its effects on satiety (Asakawa et al. 2003; Ueno et al. 1999).

Energy intake was reduced for up to 24 hours following a 90 minute infusion of PP, highlighting its potential as a pharmacological treatment for obesity (Batterham et al. 2003a). However, PP has a short \textit{in vivo} half-life of around 7 minutes in humans (Adrian et al. 1976), thus long acting analogues
of PP have been developed. Results from an initial human trial suggest PP analogues are well tolerated but further studies are necessary to confirm weight loss efficacy (Tan et al. 2011).

1.4.3.4. Proglucagon derived hormones

The preproglucagon (PPG) gene is expressed in α-cells in the pancreas, L-cells in the intestine and a population of neurons in the caudal NTS (Mojsov et al. 1986; Orskov et al. 1986; Larsen et al. 1997a). The gene is transcribed to produce the PPG protein and processed to proglucagon by the cleavage of an N-terminal signal sequence. In mammals, tissue-specific post-translational processing of proglucagon by either prohormone convertase (PC) 2 or 1/3 produces glucagon in the pancreas and GLP-1, GLP-2 and oxyntomodulin in the intestine and NTS (Rouille et al. 1994; Tucker et al. 1996; Rouille et al. 1997) (Figure 1.5).

Figure 1.5: Tissue-specific post-translational processing of proglucagon.

IP1: Intervening peptide 1.
1.4.3.4.1. Glucagon-like peptide-1 (GLP-1)

**Peptide**

Active GLP-1 is secreted from L-cells in the distal small intestine (Eissele et al. 1992) as two equipotent, truncated peptides, GLP-1(7-37) and GLP-1(7-36amide), with the latter being the dominant circulating form in humans (henceforth GLP-1) (Mojsov et al. 1990; Orskov et al. 1993; Orskov et al. 1994). Low levels of GLP-1 are released during fasting conditions and secretion increases rapidly following nutrient ingestion (Vilsboll et al. 2003b; Orskov et al. 1994). GLP-1 plays a well characterised role in glucose homeostasis; it is a potent incretin which also inhibits glucagon release (Holst et al. 1987; Mojsov et al. 1987; Ørskov et al. 1996; Vilsboll et al. 2003b). Additionally GLP-1 delays gastric emptying which is postulated to contribute to its satiety inducing effects (Holst et al. 1987; Naslund et al. 1998).

**GLP-1 secretion & degradation**

Plasma levels of GLP-1 rise within 15 minutes of meal initiation and peak at around 30 minutes (Elliott et al. 1993; Herrmann et al. 1995a; Ørskov et al. 1996). Secretion of GLP-1 is partly stimulated by the direct action of nutrients at the luminal surface of the gut (Ezcurra et al. 2013); however the initial rise of GLP-1 occurs prior to ingested nutrients reaching the lumen of the distal small intestine (Herrmann et al. 1995a). Initial release of GLP-1 may be stimulated by humoral factors, like glucose-dependent insulinotropic peptide (GIP), and gastrin releasing peptide (GRP) released from the upper small intestine and also via vagal impulses (Herrmann-Rinke et al. 1995b; Rocca & Brubaker 1999).

Once in the circulation GLP-1 has a very short half-life of less than 5 minutes which is mainly due to rapid degradation, although renal filtration also contributes (Orskov et al. 1993; Deacon et al. 1996; Meier et al. 2004). Degradation is mostly due to cleavage of the two N-terminal amino acid residues by the enzyme DPPIV (Deacon et al. 1995a; Deacon et al. 1995b; Kieffer et al. 1995). The resulting cleavage product GLP-1(9-36) has been suggested to act as an antagonist at the GLP-1 receptor, despite it’s very weak binding (Knudsen & Pridal 1996), and also appears to have GLP-1r independent properties (Ban et al. 2008; Ban et al. 2010).

Several long acting GLP-1 receptor agonists are available. Exendin-4 is a naturally occurring peptide isolated from the venom of the lizard *Heloderma suspectum* or “Gila monster” (Eng et al. 1992), and shares 50% homology with GLP-1 (Pohl & Wank 1998). It is a potent agonist of the GLP-1 receptor, whereas the truncated peptide exendin(9-39) is a potent antagonist (Goke et al. 1993; Thorens et al. 1993). Exendin-4 exhibits enhanced resistance to degradation by DPPIV and neprilysin (Bongers et al. 1992; Hupe-Sodmann et al. 1995; Deacon et al. 1998) resulting in a circulatory half-life which is
substantially greater than GLP-1 (Edwards et al. 2001; Parkes et al. 2001b). As a result synthetic analogues of exendin-4 have been used extensively to successfully treat patients with T2DM (Tella & Rendell 2015).

Liraglutide is an acylated GLP-1 analogue originally developed as treatment for T2DM, but a higher dose has recently been approved for use as an anti-obesity therapy (Section 1.1.1). A fatty acid attached via Lys$^{26}$ increases the circulatory half-life of liraglutide compared to GLP-1 by enabling it to transiently bind to albumin and subsequently be administered once a day (Knudsen et al. 2000).

**Receptors**

The actions of GLP-1 are mediated via a specific receptor which is a member of the Family B class of G-protein coupled receptors (GPCRs) (Family B GPCRs discussed in greater detail in section 1.4.4). Receptor activation stimulates cAMP production and increases intracellular calcium levels (Figure 1.7), which facilitates insulin release in pancreatic islets (Dillon et al. 1993; Wheeler et al. 1993). Increased cAMP production also activates signalling molecules including PKA and a superfamily of proteins known as Epac, which mediate the upregulation of insulin biosynthesis and release and also the pro-mitotic effects of GLP-1 on β-cells (Doyle & Egan 2007).

Consistent with GLP-1’s role as an incretin the GLP-1r is expressed in the α- and β-cells of the pancreatic islets (Thorens 1992), but expression is also seen in other organs including the lungs (not in humans), heart, kidneys, stomach, intestines and brain (Richter et al. 1990; Schepp et al. 1994; Schmidtler et al. 1994; Wei & Mojsov 1995; Bullock et al. 1996). GLP-1 receptors are expressed throughout the brain with the densest expression seen in the ARC, PVN, DMN and SON in the hypothalamus (Shughrue et al. 1996; Merchenthaler et al. 1999) and the NTS, AP and raphe nuclei in the brainstem (Merchenthaler et al. 1999). The widespread presence of GLP-1 receptors within the brain indicates a possible role for GLP-1 within the CNS.

**Food intake**

Administration of GLP-1 and its analogues potently reduce food intake, however the specific sites of action and the mechanisms mediating the anorectic effects of GLP-1 are unclear and remain under investigation.

In both lean and obese rodents, peripheral or central administration of GLP-1 dose dependently reduces acute food intake (Turton et al. 1996; Rodríguez de Fonseca et al. 2000). Peripheral infusion of GLP-1 also dose dependently inhibits food intake in lean and obese humans (Gutzwiller et al. 1999; Verdich et al. 2001). Several studies have found that obese subjects have a reduced post prandial rise in GLP-1 following oral carbohydrate intake (Ranganath et al. 1996) or a mixed meal
This suggests a reduction in GLP-1 induced satiety could contribute to the development or maintenance of obesity. Food intake is reduced in obese subjects following an infusion of normal postprandial levels of GLP-1, indicating that sensitivity to GLP-1 is maintained (Flint et al. 2001).

A similar pattern of neuronal activation is observed in the hypothalamus (ARC, PVN and SON) brainstem (AP, NTS, parabrachial nucleus (PBN)) and CeA following either central or peripheral administration of GLP-1 in rodents (Van Dijk et al. 1996; Larsen et al. 1997b; Baggio et al. 2004b; Abbott et al. 2005b). The GLP-1r is expressed in these areas suggesting peripheral GLP-1 may cross the blood brain barrier and act directly within the brain. In particular, current evidence suggests that GLP-1 can directly inhibit orexigenic NPY neurons and activate anorexigenic POMC neurons in the hypothalamic ARC (Heppner & Perez-Tilve 2015a). However, a similar pattern of neuronal activation is also observed following peripheral administration of albumin-bound GLP-1 (Albugon) which cannot cross the blood brain barrier, indicating other mechanism(s) are involved (Baggio et al. 2004b).

Due to its short circulatory half-life the CNS effects of peripherally released or administered GLP-1 are likely to be primarily mediated via vagal signalling. Consistent with this hypothesis the anorectic effects of peripheral GLP-1 are abolished in vagotomised human subjects (Plamboeck et al. 2013) and in rodents following subdiaphragmatic total truncal vagotomy (Abbott et al. 2005b). Also, blockade of central GLP-1 receptors with antagonist Exendin(9-39) fails to prevent the anorectic effect of peripherally administered GLP-1 (Williams et al. 2009). Peripherally administered Albugon is unable to cross the blood brain barrier but still reduces food intake (Baggio et al. 2004b). These findings suggest that peripheral GLP-1 does not act directly in the brain to reduced food intake. However, mice with specific genetic knockdown of vagal afferent GLP-1 receptors maintain similar food intake and bodyweight as control mice when exposed to both normal chow and HFD (Sisley et al. 2014a). This may suggest that GLP-1’s effects on satiety are not vagally mediated. Interestingly, global germline GLP-1 receptor knockout mice also do not display an altered food intake or bodyweight phenotype on regular chow diet (Scrocchi et al. 1996) and are protected against diet induced obesity (Hansotia et al. 2007; Wilson-Pérez et al. 2013). These findings appear to be at odds with a role for GLP-1 in the control of food intake and bodyweight. However, developmental compensation for receptor loss could occur in germline knockouts. The GLP-1 receptor has been implicated in peripheral adipogenesis, which could account for the lack of bodyweight increase on HFD (Challa et al. 2012). Peripheral administration of a long acting GLP-1 receptor antagonist to DIO
mice increases food intake and bodyweight, indicating that endogenous GLP-1 does play an important role in energy homeostasis (Patterson et al. 2011a).

Unlike with native GLP-1, both the acylated GLP-1 analogue liraglutide and the long acting GLP-1 receptor agonist exendin-4 continue to reduce food intake in rats that have undergone subdiaphragmatic vagal deafferentation; although higher doses are required (Kanoski et al. 2011). This indicates vagal afferent signalling may play a role in GLP-1 analogue mediated hypophagia but it is not an essential mechanism by which food intake is reduced. Similar to findings with GLP-1, food intake reduction is also maintained following liraglutide administration in mice with specific deletion of vagal afferent GLP-1 receptors (Sisley et al. 2014a). It is possible that although liraglutide and exendin-4 are GLP-1 analogues, their effects on food intake may be mediated by a different mechanism to GLP-1 as a result of their enhanced potency and/or half-life, or differences in GLP-1 receptor binding (Barrera et al. 2009). Indeed, peripherally administered liraglutide has been demonstrated to enter the brain via a GLP-1 receptor mediated mechanism and act directly on POMC neurons in the ARC (Secher et al. 2014). Consistent with these findings, specific knockout of CNS GLP-1 receptors abolishes the anorectic effects of peripherally administered liraglutide (Sisley et al. 2014a).

There is some evidence GLP-1 acts as a neurotransmitter within appetite regulating circuits. A population of non-catecholaminergic, GLP-1 expressing cells in the NTS project heavily to the PVN and DMN with fewer projections to the ARC, LHA and CeA (Larsen et al. 1997a; Larsen & Holst 2005). A large satiating meal activates GLP-1 producing neurons in the brainstem suggesting they may relay satiety signals to other brain areas (Kreisler et al. 2014). Central GLP-1 receptor antagonism with Exendin9-39 increases food intake, suggesting a central role for endogenous GLP-1 in the tonic inhibition of food intake (Turton et al. 1996; Meeran et al. 1999). As seen with other germline GLP-1 receptor knockout models, central GLP-1 receptor knockout mice do not demonstrate altered food intake or bodyweight compared to controls (Scrocchi et al. 1996; Sisley et al. 2014a).

It has been postulated that GLP-1 may reduce food intake because it causes nausea. Central infusion of an anorectic dose of GLP-1 can generate conditioned taste aversion (CTA) (Thiele et al. 1997) and endogenous neuronal GLP-1 and GLP-1 receptor stimulation have been implicated in the anorexic response to LPS and LiCl induced illness and malaise (Rinaman 1999; Grill et al. 2004). Peripheral GLP-1 produces a similar pattern of neuronal activation to that which is seen following LiCl administration, including reduced PVN and increased VMH activity (Parkinson et al. 2009). However, CTA can be induced independently of anorexia following injection of GLP-1 directly in to the CeA (Kinzig et al. 2002). Similarly, GLP-1 administration directly in to the PVN or into the hindbrain via the
fourth ventricle results in reduced food intake without the development of CTA (McMahon & Wellman 1998). This suggests that central GLP-1 interacts with one or more separate brain systems to produce anorexia independently of nausea.

Nausea and vomiting are commonly reported gastrointestinal side effects of long acting GLP-1 analogues used in the treatment of T2DM and are often dose limiting factors. High levels of nausea are associated with the greatest levels of weight loss following chronic treatment with liraglutide (Lean et al. 2014), suggesting both the nausea and satiety systems may contribute to the food intake reduction and weight loss observed with peripheral GLP-1 analogue treatment. Interestingly, treatment with DPP4 inhibitors results in elevated endogenous GLP-1 levels due to diminished degradation, but is not associated with increased satiety or nausea (Vella et al. 2007; Vella et al. 2008). It has been postulated that in humans endogenous peripheral GLP-1 may not activate nausea systems but the therapeutic GLP-1 analogues may do so as a result of their enhanced potency, duration of action and altered GLP-1 receptor binding (Tong & Sandoval 2011).

The role of centrally released GLP-1 and whether central GLP-1 receptors are involved in mediating the effects of peripheral GLP-1 remains to be established. Similarly, the contribution of nausea to the anorectic actions of peripheral GLP-1 and long acting analogues is unclear, but nausea remains a major side effect and possible dose limiting factor of this drug class (Vrang & Larsen 2010; Trapp & Hisadome 2011).

Energy expenditure

Weight loss observed following administration of GLP-1 or long acting analogues was purported to be due to food intake inhibition. However more recently several studies in mice have suggested that there may be a small food-intake-independent, reduction in bodyweight following GLP-1 receptor activation (Beiroa et al. 2014; Yang et al. 2014; Heppner et al. 2015b). However, these studies used the long acting GLP-1 analogues liraglutide or exendin-4 and it remains unclear whether endogenous GLP-1 also generates weight loss independent of food intake reduction. GLP-1 receptor agonism was postulated to generate weight loss by increasing energy expenditure, possibly by inducing BAT thermogenesis, however results are conflicting (refer to section 1.2.1.1 for a detailed explanation of BAT thermogenesis). Central liraglutide or GLP-1 administration is reported to increase energy expenditure and BAT activation in lean chow fed mice, via activation of the sympathetic nervous system (Lockie et al. 2012; Beiroa et al. 2014). Conversely, peripheral liraglutide administration does not appear to affect energy expenditure or BAT activity in DIO mice or lean rats (Larsen et al. 2001; Heppner et al. 2015b). Both peripheral and central administration of exendin-4 was shown to reduce energy expenditure in lean chow fed mice (Baggio et al. 2004a). GLP-1 receptor KO mice
demonstrate a normal thermogenic response to cold exposure on regular chow (Lockie et al. 2012; Heppner et al. 2015b), but display reduced energy expenditure and impaired BAT thermogenesis during cold exposure following high fat feeding (Heppner et al. 2015b). This suggests that GLP-1 receptor signalling is not essential for BAT activation but may play a role in diet induced thermogenesis. BAT is an essential and highly active thermogenic organ in rodents and is therefore likely to contribute a large percentage to their total energy expenditure. The presence of BAT in humans and its contribution to energy expenditure remains to be confirmed.

The effect of GLP-1 and its long lasting analogues on energy expenditure in humans is unclear. Fasting plasma GLP-1 levels were found to be positively correlated with resting energy expenditure independent of age, sex and body composition (Pannacciulli et al. 2006). GLP-1 infusion in healthy normal weight subjects increased resting energy expenditure, however this was abolished when insulin levels were clamped suggesting GLP-1 may indirectly affect energy expenditure via its incretin effect (Shalev et al. 1997). Several other studies have found that infusing approximately physiological levels of GLP-1 in normal weight, overweight or obese subjects has either no effect on energy expenditure, or causes a reduction (Flint et al. 2000; Flint et al. 2001; Schmidt et al. 2014). Liraglutide treatment in obese or overweight subjects with or without diabetes has been shown to increase (Horowitz et al. 2012; Beiroa et al. 2014), decrease (van Can et al. 2014), or have no effect (Harder et al. 2004) on energy expenditure. However the different subject populations, doses and treatment periods make direct comparison between these studies difficult.

Whether or not GLP-1 and GLP-1 receptor signalling plays a role in energy expenditure and contributes to BAT mediated thermogenesis remains under investigation.

1.4.3.4.2. Glucagon

Peptide
Glucagon is a 29 amino acid peptide hormone produced in pancreatic α-cells by PC2 cleavage of proglucagon. The best characterised role of glucagon is its glycogenolytic and gluconeogenic effects in response to low blood glucose (Levine 1965; Curnow et al. 1975; Band & Jones 1980; Ikezawa et al. 1998).

Stimuli for glucagon secretion
Glucagon is secreted in response to hypoglycaemia, however at present the mechanisms involved in the regulation of glucagon release are not fully understood. Low blood glucose concentrations prompt the closure of α-cell membrane K⁺ATP channels, preventing the efflux of K⁺ and resulting in depolarisation of the α-cell membrane (Olsen et al. 2005; MacDonald et al. 2007). This elicits the
influx of Ca\textsuperscript{2+} which triggers the release of glucagon from intracellular granules (Gromada et al. 1997). It is postulated that impaired control of α-cell K\textsuperscript{+} ATP channels may underlie the disordered glucagon regulation observed in T2DM patients (Walker et al. 2011; Zhang et al. 2013).

Glucagon secretion from α-cells is influenced by intrinsic mechanisms and paracrine signalling and the overall cellular response appears to be dependent upon the physiological context of the signals. Thus hyperglycaemia (Rorsman et al. 1989; Greenbaum et al. 1991; Ravier & Rutter 2005), insulin (Maruyama et al. 1984; Ravier & Rutter 2005), GLP-1 (Gromada & Rorsman 2004; De Marinis et al. 2010) and somatostatin (Cejvan et al. 2003) have all been shown to directly or indirectly inhibit glucagon secretion. However, under certain conditions hyperinsulinemia and hyperglycaemia have also been shown to paradoxically augment glucagon release (Walker et al. 2011).

Central brain mechanisms are also involved in blood glucose sensing and the control of glucagon secretion (Frizzell et al. 1993). The VMN and LHA are thought to be two of the main glucose sensing centres in the brain (Borg et al. 1995; Borg et al. 1997; Wu et al. 2004). Brainstem activity is also essential for glucagon secretion in response to glucoprivation (Andrew et al. 2007). The autonomic nervous system (ANS) has been postulated to be responsible for approximately 75-80% of glucagon release in response to insulin induced hypoglycaemia (Havel & Ahren 1997).

Catecholamine release in the brainstem (Hudson & Ritter 2004) and VMH (Szepietowska et al. 2011) has been suggested to play a role in the stimulation of glucagon secretion during systemic hypoglycaemia. The early release of glucagon at meal onset is thought to be mediated by noradrenergic signalling from the VMN (de Jong et al. 1977) and activation of hypothalamic noradrenergic projections has been postulated to be responsible for glucagon secretion in response to stress (Jones et al. 2012).

Glucagon receptor

Similar to the GLP-1 receptor, with which it shares 47% homology (Graziano et al. 1996), the glucagon receptor is also a member of the family B class of GPCRs. Ligand binding activates adenylate cyclase via the G\textsubscript{α}s G-protein subunit, triggering increases in intracellular levels of the second messenger cAMP and Ca\textsuperscript{2+} (Jelinek et al. 1993) (Figure 1.7). Glucagon receptors are primarily expressed in the liver and kidney with relatively high levels also present in the adrenals, heart and adipose and lower expression identified in the pancreas, thymus, spleen and brain (Svoboda et al. 1994; Hansen et al. 1995; Dunphy et al. 1998). A single study utilising \textsuperscript{125}I-labelled glucagon identified glucagon receptor binding sites in isolated membranes from a number of brain areas including the hypothalamus, hippocampus, amygdala, anterior pituitary and olfactory bulb (Hoosein
& Gurd 1984). However the central distribution of the glucagon receptor has not been extensively studied.

**Regulation of food intake**

Peripherally administered glucagon induces postprandial satiety in rodents (Martin & Novin 1977; Geary & Smith 1982a; Le Sauter & Geary 1991a; Geary et al. 1993; Parker et al. 2013) and humans (Schulman et al. 1957; Geary et al. 1992) and portal infusion of glucagon antibodies during a meal increases food intake (Le Sauter et al. 1991b). However the mechanisms behind the satiating effects of glucagon are currently unknown. It has been postulated that food intake may be inhibited indirectly as a result of glucagon induced hyperglycaemia or insulin secretion (Martin & Novin 1977; Vanderweele et al. 1979). However, a hyperglycaemic dose of glucagon does not induce satiety in sham feeding rats (Geary & Smith 1982b) and the anorectic effects of glucagon are not prevented by co-infusion with insulin anti-bodies (Geary et al. 1997).

Glucagon may exert its anorectic actions via vagal signalling to the brain, as vagotomy or lesioning of brainstem vagal afferent terminals prevents exogenous glucagon mediated satiety (Geary et al. 1993; Martin et al. 1978; Weatherford & Ritter 1988). However glucagon receptors have not currently been identified on these nerve fibres. There is some evidence that glucagon may act directly in the brain; ICV administration inhibits food intake in rats (Inokuchi et al. 1984) and intra-nuclear administration of glucagon inhibits electrical activity in glucose-sensitive neurons in the LHA and also neurons of the DMN and VMN (Inokuchi et al. 1986). Peripheral glucagon administration into the carotid artery or hepatic portal vein also results in suppression of neuronal firing in the LHA, although to a lesser extent than intra-nuclear injection (Inokuchi et al. 1986). The similarities between the effects of centrally and peripherally administered glucagon may suggest direct effects within the brain rather than a vagal mediated mechanism.

It is unclear whether the anorectic effects of glucagon are mediated via the same receptor as its glucoregulatory actions. IP administration of glucagon receptor antagonists restores glucose tolerance and improves insulin sensitivity in obese, diabetic mouse models without altering food intake or bodyweight (O’Harte et al. 2014). A peripheral anorectic dose of glucagon has been shown to induce a similar pattern of neuronal activation in the brainstem and amygdala as an anorectic dose of GLP-1 (Parker et al. 2013). Given the evolutionary relationship and high level of homology between the glucagon and GLP-1 receptors it is possible glucagon exerts its anorectic actions via the GLP-1 receptor. Indeed, glucagon does cross-react to some extent with the GLP-1 receptor but with much lower affinity (Thorens 1992; Druce et al. 2009) and efficacy (Jorgensen et al. 2007; Kosinski et al. 2012) than GLP-1. Investigating the effects of glucagon administration on feeding behaviour
glucagon receptor knock-out mice or in conjunction with a glucagon receptor antagonist may help to assess the contribution of the glucagon receptor to glucagon induced satiety.

**Energy expenditure**
Consistent with its effects on food intake, peripheral glucagon administration is associated with decreased bodyweight or reduced weight gain in humans (Schulman et al. 1957) and rodents (Chan et al. 1984; Billington et al. 1991b). Weight loss has also been demonstrated to occur independently of effects on food intake, indicating glucagon may play a separate role in bodyweight regulation (Salter et al. 1957; Chan et al. 1984). Energy expenditure is increased following glucagon administration in rodents and humans (Davidson et al. 1957; Davidson et al. 1960; Calles-Escandón 1994; Tan et al. 2013) and this is postulated to be due to increased thermogenesis resulting from the activation of BAT (see section 1.2.1.1 for a more detailed discussion of BAT).

BAT is activated in rodents exposed to cold and produces heat via non-shivering thermogenesis. The glucagon receptor is expressed by brown adipocytes (Iwaniw et al. 1994) and plasma glucagon levels are increased in cold acclimatised rats (Helman et al. 1984; Edwards & Howland 1986; Yahata & Kuroshima 1987). Glucagon administration enhances BAT activation in cold exposed rats (Doi & Kuroshima 1982; Yahata et al. 1983; Billington et al. 1991b) and chronic glucagon administration increases BAT mass in non-cold exposed rats (Billington et al. 1987). However in a more recent study, glucagon failed to augment thermogenesis in cold acclimated animals leading the authors to suggest BAT might not be the main mediator of glucagon induced thermogenesis (Dicker et al. 1998).

It is unclear whether glucagon stimulates BAT cells directly. In vitro, glucagon increases oxygen consumption and heat production in isolated rat BAT (Joel 1966; Kuroshima & Yahata 1979). However glucagon receptor mRNA expression was found to be decreased in the BAT of cold exposed animals (Morales et al. 2000). ICV glucagon administration increases BAT thermogenesis in mice via activation of the sympathetic nervous system (SNS) (Lockie et al. 2012). Several other studies have implicated SNS catecholamine signalling in glucagon induced BAT activation. Increases in oxygen consumption and BAT blood flow following glucagon infusion in new born rabbits can be blocked by injection of a β-adrenergic antagonist (Heim & Hull 1966). Noradrenaline levels are increased in ducklings following glucagon injection and preventing catecholamine release in these animals with chemical sympathectomy decreases metabolic rate (Filali-Zegzouti et al. 2005). Additionally, denervation of BAT blunts the thermogenic response to glucagon in rats (Billington et al. 1987) and glucagon injection directly in to the LHA increases sympathetic nerve activity to interscapular BAT (Shimizu et al. 1993).
Glucagon administration also stimulates lipolysis in white adipose tissue (WAT) (Hagen 1961; Vaughan 1961; Vaughan & Steinberg 1963; Lefebvre 1975; Richter et al. 1989), possibly via a SNS dependent mechanism (Lefebvre et al. 1973); indicating increased oxygen consumption and bodyweight reduction may partly be due to increased fat metabolism.

It is difficult to ascertain whether glucagon plays a physiological role in thermogenesis and weight regulation. Although the early studies used supraphysiological doses of glucagon, Billington et al. (1991b) demonstrated BAT activation with more physiologically representative levels. A mutation in the leptin receptor gene causes obesity in Zucker rats, but these animals also have reduced blood glucagon levels. Bodyweight is significantly decreased in these animals following chronic glucagon administration without an effect on food intake, suggesting reduced glucagon levels may contribute to the obese phenotype of this model (Chan et al. 1984). Although glucagon receptor knockout mice exhibit impaired responses to fasting, in that they do not increase their fatty acid utilisation, they unexpectedly display a lean phenotype with reduced adiposity but normal bodyweight, food intake and energy expenditure (Gelling et al. 2002). Interestingly these animals also display supraphysiological levels of glucagon and 3- to 10-fold increased GLP-1 levels. However the relevance of findings from studies utilising genetically abnormal animals may be limited.

**Stress**

It has been hypothesised that glucagon’s characteristic response to hypoglycaemia is part of a more complex role as a stress hormone. In addition to increasing substrate availability, glucagon also affects the rate and force of cardiac contractions (Regan et al. 1964) and both of these actions would contribute to a fight or flight response. Similarly, feeding would be disadvantageous during fight or flight and thermogenesis would be a beneficial stress response to extreme cold or infection. Therefore a role as a stress hormone could explain the seemingly paradoxical effects of glucagon on food intake and energy expenditure in relation to its widely purported role in glucose homeostasis.

Hyperglucagonaemia in the absence of hypoglycaemia is observed in a number of situations involving a physiological stress response (Bloom et al. 1973; Jones et al. 2012). Adrenalin or noradrenalin stimulation of α-1 and β-adrenergic receptors expressed on pancreatic α-cells induces glucagon secretion (Vieira et al. 2004) at plasma glucose concentrations that would ordinarily inhibit glucagon release (Iversen 1973). Glucagon in turn can stimulate catecholamine release (Lenders et al. 2009) and may affect the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.6). The effects of glucagon on the HPA axis are unclear and appear to be species dependent. Adrenocorticotropic hormone (ACTH) and cortisol are elevated in humans following glucagon administration, however in rats glucagon supresses ACTH release (Waldhausl et al. 1976; Rao 1995;
Arvat et al. 2000). There is some evidence that glucagon may stimulate the release of corticotrophin-releasing hormone (CRH) from the hypothalamus; studies in chicks demonstrated an upregulation of CRH mRNA in the hypothalamus of glucagon treated animals (Honda et al. 2012). However this has not been investigated in mammals. Central administration of CRH inhibits feeding in rats (Benoit et al. 2000), whereas peripheral administration of CRH in humans stimulates feeding (George et al. 2009). Part of glucagon’s anorectic actions may be mediated by effects on the HPA axis but further investigation is necessary.

![Diagram of the interaction between glucagon and the HPA axis](image)

**Figure 1.6:** Summary the interaction between glucagon and the HPA axis. The green arrow indicates stimulation and the red arrow indicates inhibition. ACTH: adrenocorticotrophic hormone, CRH: corticotrophin releasing hormone.
1.4.3.4.3. Oxyntomodulin

**Peptide**

Structurally, oxyntomodulin is the 29 amino acids of glucagon with an N-terminal octapeptide tail (Bataille et al. 1981; Bataille et al. 1982; Holst 1982). Following nutrient ingestion, oxyntomodulin is co-secreted with GLP-1 from L-cells in the small intestine in proportion to calorie intake (Ghatei et al. 1983; Le Quellec et al. 1992). Oxyntomodulin has also been identified in the caudal NTS in the brain where proglucagon is expressed and processed in a similar manner to intestinal processing, suggesting a possible role for oxyntomodulin as a neurotransmitter (Larsen et al. 1997a). In the circulation oxyntomodulin is rapidly degraded by the enzymes DPPIV and neprilysin (NEP) (Zhu et al. 2003; Druce et al. 2009), contributing to its short half-life of approximately 6 minutes in rodents (Kervran et al. 1990) and 12 minutes in humans (Schjoldager et al. 1988).

**Receptors**

A specific oxyntomodulin receptor has not been identified. Rather it is a dual agonist of the glucagon receptor and GLP-1 receptor, albeit with reduced affinity and potency compared to the native peptides (Baldissera et al. 1988; Schepp et al. 1996; Dakin et al. 2001; Jorgensen et al. 2007; Druce et al. 2009; Kerr et al. 2010; Santoprete et al. 2011; Kosinski et al. 2012). The physiological role of oxyntomodulin is unclear and the lack of a specific oxyntomodulin receptor coupled with activity at both the glucagon and GLP-1 receptors makes it difficult to delineate oxyntomodulin specific effects. Consistent with its ability to agonise both the glucagon and GLP-1 receptors the effects of oxyntomodulin administration are a combination of the effects observed following administration of glucagon or GLP-1. Oxyntomodulin stimulates insulin release via the GLP-1 receptor, although to a lesser degree than GLP-1 itself (Du et al. 2012). Chronic administration improves glucose tolerance in insulin resistant DIO mice, probably due to a combination of weight loss and GLP-1 receptor mediated stimulation of insulin release (Kosinski et al. 2012). Similar to glucagon and GLP-1, oxyntomodulin has also been suggested to play a role in the regulation food intake and energy expenditure.

**Food intake**

The postprandial rise in oxyntomodulin suggests it may play a role in satiety (Le Quellec et al. 1992). Food intake is reduced following peripheral and central administration of oxyntomodulin in rodents (Dakin et al. 2001; Dakin et al. 2002; Dakin et al. 2004; Maida et al. 2008; Kosinski et al. 2012) and peripheral administration in humans (Wynne et al. 2005; Wynne et al. 2006a; Cohen et al. 2003). The anorectic effects of oxyntomodulin are abolished in GLP-1 receptor knockout mice but not glucagon receptor null mice, indicating the feeding effects of oxyntomodulin are mediated solely via GLP-1.
receptor activity (Baggio et al. 2004a). However food intake and bodyweight are normal in GLP-1 receptor knockout mice (Scrocchi et al. 1996), suggesting neither GLP-1 nor oxyntomodulin plays a physiological role in the regulation of satiety; although it is possible that compensation occurs in these germline knockouts. Blocking the GLP-1 receptor using the antagonist Ex(9-39) also prevents the anorectic effects of oxyntomodulin (Dakin et al. 2001; Baggio et al. 2004b; Dakin et al. 2004). However, despite the reduced GLP-1 receptor efficacy of oxyntomodulin, exogenous administration appears to reduce food intake to the same extent and for a similar duration as GLP-1 (Dakin et al. 2001), suggesting other mechanism(s) may play a role in the anorectic effects of oxyntomodulin.

There are conflicting results from studies comparing the brain areas activated by peripheral oxyntomodulin and GLP-1. One study, which utilised c-Fos staining as a marker of neuronal activation, showed that peripheral oxyntomodulin produced a similar pattern of activation in the PVN, AP and NTS of mice as the GLP-1 receptor agonist exendin-4 (Baggio et al. 2004a). However manganese enhanced magnetic resonance imaging (MEMRI) in mice demonstrates differential patterns of hypothalamic neuronal activation following IP oxyntomodulin or GLP-1 (Chaudhri et al. 2006; Parkinson et al. 2009). In a separate study in rats, neuronal activation was seen in the ARC following peripheral oxyntomodulin. Intra-arcuate injection of GLP-1 receptor antagonist Ex(9-39) only blocked the effects of IP oxyntomodulin and not GLP-1, suggesting differences in their anorectic mechanisms (Dakin et al. 2004). GLP-1 reduces gastric emptying which is postulated to contribute to its satiety inducing effects; however a reduction in gastric emptying is not seen following oxyntomodulin administration (Cohen et al. 2003; Dakin et al. 2004; Maida et al. 2008). It has been suggested that the anorectic actions of oxyntomodulin may be partially mediated by suppression of the levels of orexigenic hormone ghrelin (Cohen et al. 2003; Dakin et al. 2004). However this is in contrast to several other studies which found ghrelin levels were unchanged following peripheral oxyntomodulin administration in human volunteers (Wynne et al. 2005; Wynne et al. 2006a).

Current evidence suggests that the anorectic effects of oxyntomodulin are partly mediated via the GLP-1 receptor. However, whether the mechanisms are the same as those postulated to be involved in GLP-1 induced satiety is unclear.

Energy expenditure

Consistent with a reduction in food intake, weight loss or reduced weight gain is observed following chronic oxyntomodulin administration in both rodents and humans (Dakin et al. 2001; Dakin et al. 2002; Dakin et al. 2004; Wynne et al. 2005; Wynne et al. 2006a). However the magnitude of weight loss following oxyntomodulin administration is much greater than expected for the reduction in food
intake (Dakin et al. 2002; Dakin et al. 2004), suggesting a concomitant increase in energy expenditure. Indeed an increase in energy expenditure following administration of exogenous oxyntomodulin has been demonstrated in rodents (Dakin et al. 2001; Dakin et al. 2002) and human volunteers (Wynne et al. 2006a). Glucagon has been demonstrated to increase energy expenditure (Davidson et al. 1957; Davidson et al. 1960; Tan et al. 2013), whereas the effects of GLP-1 receptor activity on energy expenditure are less clear and has often been observed to result in a reduction (Flint et al. 2000; Baggio et al. 2004a). Therefore oxyntomodulin is postulated to increase energy expenditure via the glucagon receptor; although there are currently no studies which have investigated energy expenditure in glucagon receptor knockout mice following oxyntomodulin administration. A study by Kosinski et al (2012) demonstrated greater weight loss in mice subcutaneously infused with oxyntomodulin compared to mice given an analogue with equivalent GLP-1 receptor potency but diminished glucagon receptor activity. This suggests a proportion of the weight loss following oxyntomodulin administration is due to increased energy expenditure mediated by glucagon receptor activity. However, Lockie et al (2012) reported that energy expenditure was not increased in GLP-1 receptor knockout mice following chronic ICV oxyntomodulin administration. Comparison of the central and peripheral effects of oxyntomodulin and investigation of energy expenditure following oxyntomodulin administration in glucagon receptor knockout mice is necessary.

GLP-1 administration results in minor weight loss and high doses are associated with nausea and the risk of hypoglycaemia. Glucagon administration can increase energy expenditure and reduce food intake but may result in hyperglycaemia. Therefore there has been considerable investigation of synthetic dual agonists of the glucagon receptor and GLP-1 receptor as potential obesity treatments and the results demonstrate significant reductions in bodyweight coupled with improvements in glycaemic control (Day et al. 2009; Druce et al. 2009; Pocai et al. 2009; Liu et al. 2010; Santoprete et al. 2011; Lynch et al. 2014). The balance of agonism at the glucagon and GLP-1 receptor will be vital for the control of blood glucose (Day et al. 2012).

1.4.4. Family B G-protein coupled receptors (GPCRs)

The superfamily of GPCRs all contain a core 7-transmembrane region consisting of alternating intra- and extracellular loops, with an extracellular N-terminal region and an intracellular carboxy terminus. GPCRs bind to a wide variety of structurally diverse ligands including photons, odorants, glycoproteins and peptides (Ulrich et al. 1998). Within this superfamily, receptors can be further categorised into 5 subfamilies based on their conserved structural features (Alexander et al. 2013).
Family B GPCRs (also known as class B or Secretin family receptors) are characterised by a relatively long N-terminal domain of around 100-200 residues which also contains several cysteines that are presumed to form a network of disulphide bridges. Family B GPCRs can be further separated into 4 subfamilies, of which the glucagon receptor family is one. The glucagon family consists of the receptors for glucagon, GLP-1, GLP-2, GIP, growth hormone releasing hormone (GHRH), pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal polypeptide (VIP) and secretin receptors (Gether 2000) (see Figure 3.1 for the amino acid sequences of all glucagon receptor family ligands). All members of the glucagon receptor family couple to GαS, activating adenylyl cyclase and consequently elevating intracellular levels of cAMP and calcium (Unson 2002) (Figure 1.7).

---

**Figure 1.7: Simplified schematic of GαS coupled GPCR activation.**

The ligand binds to the receptor which results in GαS releasing GDP and binding GTP which activates the protein. The α, β and γ G-protein subunits dissociate and GαS activates adenylyl cyclase which produces the second messenger cAMP. cAMP activates other signalling molecules, for example PKA, which facilitates the influx of Ca^{2+}. ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, GDP: guanosine diphosphate, GTP: guanosine triphosphate  GPCR: G-protein coupled receptor, PKA: protein kinase A.
1.5. Thesis overview & aims

Obesity poses a major healthcare problem worldwide; however there are limited treatment options available. In particular, current pharmacological treatments have off target effects resulting in a number of undesirable cognitive side-effects. Administration of anorectic gut hormones has been suggested as alternative obesity pharmacotherapy. The gut hormone oxyntomodulin has been shown to decrease bodyweight following peripheral administration in rodents and humans, by reducing food intake and increasing energy expenditure. Gut hormones have short half-lives and whilst previous attempts to produce a long acting oxyntomodulin analogue have been moderately successful, the analogues had much lower efficacy at both receptors compared to the native hormones.

The overall aim of this thesis was to develop an oxyntomodulin-like dual analogue of the glucagon and GLP-1 receptors with similar receptor efficacy to native glucagon and GLP-1 and the potential for once monthly administration in man as a treatment for obesity.

Chapter 3 aims

- To substitute single amino acid residues in the glucagon sequence with the corresponding residues from GLP-1 and Exendin-4 to increase the GLP-1 receptor activity of glucagon, resulting in a potent dual agonist with a similar ratio of receptor activity as native oxyntomodulin. In vitro receptor activity and in vivo effects on food intake, bodyweight and pharmacokinetic profile are investigated.

Chapter 4 aims

- To investigate the development of a C-terminal histidine ‘tail’ to enhance the compatibility of the oxyntomodulin-like dual analogues designed in Chapter 3 with a ZnCl₂ slow release formulation previously developed in the lab.
- To evaluate the effects of an optimised oxyntomodulin-like analogue on energy intake, energy expenditure and glucose homeostasis are evaluated to assesses its potential for use as an anti-obesity therapy.

Chapter 5 and Chapter 6 aims

- To investigate novel analogue effects on food intake and bodyweight observed during initial analogue development and assess the impact this may have on the development and use of oxyntomodulin-like analogues.
Chapter 2

Materials and Methods
2.1. Peptide Synthesis

Human glucagon, human oxyntomodulin, human GLP-1 and exendin-4 were purchased from Bachem, Ltd. (Merseyside, UK). Where mentioned, the synthetic human sequence of GLP-1, glucagon and oxyntomodulin were used in all experiments. GLP-1(7-36)NH₂ was the form used in all experiments, and will henceforth be referred to as GLP-1.

Peptide analogues were custom synthesised and purchased from Bachem, Ltd (Merseyside, UK) or Insight Biotechnology Limited (Middlesex, UK). Peptides were synthesised using an automated fluorenlymethyloxycarbonyl (FMOC) solid phase peptide synthesis method with each amino acid sequentially added from the C to the N terminus.

Peptides were cleaved from the resin and purified using reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. Peptide purity was determined by reverse-phase HPLC and by Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). All peptides supplied had a purity of >95%.

The amino acid sequences of all the peptides discussed in this thesis are shown in Appendix A.

2.2. In Vivo Studies

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/7236).

2.2.1. Animal husbandry and conditions

Adult male Wistar rats weighing 400-600g (Charles River, Margate, UK) and adult male C57BL/6 mice weighing 30-60g (Harlan, Bicester, UK) were housed in individual cages under controlled temperature (21-23°C) and lighting (12:12 hr light-dark cycle, lights on at 0700) conditions. Animals were allowed ad libitum access to water and RM1 diet (Special Diet Services, Witham, UK) unless otherwise stated. DIO mice were allowed ad libitum access to high fat diet containing 60% calories from fat from 4 weeks of age, unless otherwise stated. Animals were handled regularly and acclimatised to intra-peritoneal (IP) or subcutaneous (SC) injections to reduce any non-specific stress effects at the beginning of the study. Saline was used for vehicle injections.

2.2.2. Acute mouse studies

Animals were weighed and fasted at 1600 hours the day before the study started and randomised to treatment groups, ensuring that average bodyweight was consistent across all groups. Experiments commenced during the early light phase (0800-0900) in animals fasted from 1600 hours the previous
day. Mice received a single SC injection of either saline or a bodyweight adjusted dose of peptide (maximum volume 1000µlkg\(^{-1}\)) reconstituted in ZnCl\(_2\) slow release diluent, using a 0.5ml insulin syringe and 29 gauge needle. After injection mice were returned to their home cage and given free access to a weighed amount of food. Food was subsequently weighed at regular pre-determined intervals for up to 24 or 48 hours post injection using a balance accurate to 0.01g and including any small pieces of food found in the cage. In acute studies detailed in Chapter 4, food was also re-weighed at 6 days post injection and animals were weighed at 48hrs and 6 days post-injection. External disturbances, for example bedding changes, were avoided during the 48hrs immediately following injection. Animals were allowed continual access to water throughout the studies.

2.2.3. Acute rat studies

Animals were weighed and fasted at 0900 hours the day before the study started and randomised to treatment groups, ensuring that average bodyweight was consistent across all groups. Experiments commenced during the early light phase (0800-0900) in animals fasted from 0900 hours the previous day. Rats received a single SC injection of either saline or a bodyweight adjusted dose of peptide (maximum volume 100µlkg\(^{-1}\)) reconstituted in ZnCl\(_2\) slow release diluent, using a 0.5ml insulin syringe and 29 gauge needle. After injection rats were returned to their home cage and given free access to a weighed amount of food. Food was subsequently weighed at regular pre-determined intervals up to 72hrs post injection using a balance accurate to 0.1g and including any small pieces of food found in the cage. In acute studies detailed in Chapter 4, food was also re-weighed at 6 days post injection and animals were weighed at 72hrs and 6 days post-injection. External disturbances, for example bedding changes, were avoided during the 72hrs post injection. Animals were allowed continual access to water throughout the studies.

2.2.4. Chronic rat and chronic mouse studies

Animals were weighed at 1100 hours on the study day and randomised into treatment groups, ensuring that average bodyweight was consistent across all groups. Experiments commenced in the late light phase (1500-1700 hours) in fed animals. Animals were given SC injections of either saline or peptide (rat maximum volume 100µlkg\(^{-1}\); mouse maximum volume 1000µlkg\(^{-1}\)) reconstituted in ZnCl\(_2\) slow release diluent, using a 0.5ml insulin syringe and 29 gauge needle. Dosing frequency varied between studies but within a single study injections were given either daily or 3 times weekly. Animals were allowed to feed \textit{ad libitum} throughout the study; food and bodyweight measurements were taken at the time of injection for all studies. Animals were allowed continual access to water throughout the studies.
2.2.4.1. Pair-Feeding

Chronic studies involving groups of pair-fed animals were carried out as above, with pair-fed groups administered injections of saline. Each day rats in the pair fed groups were given a weighed amount of food equivalent to the mean food intake consumed by their corresponding peptide treatment group over the previous 24 hours. Food hoppers were inspected each day before feeding to ensure that all the food from the previous day had been consumed.

2.2.5. Mouse IP glucose tolerance test

A glucose tolerance test (GTT) was conducted in mice who had received 3x weekly SC injections of saline or various doses of OXLT9 over a period of 24 days. Mice were given a final SC vehicle or peptide injection and fasted from 0700 hours on the morning of the GTT. At 1300 hours mice had the very tip of their tail removed using a sharp scalpel and a T =-30 minutes a blood glucose measurement was obtained using a hand held glucometer (Contour, Bayer, Berkshire, UK). After 30 minutes a T= 0 blood glucose measurement was obtained and each animal received an IP injection of 2gkg\(^{-1}\) glucose solution (maximum volume 10mlkg\(^{-1}\)). Further blood glucose measurements were obtained at T= 30, 60 and 90 minutes following IP glucose injection.

2.2.6. Mouse body composition analysis

Following the IPGTT at the end of the 24 day study, mice were sacrificed using cervical dislocation and the contents of their gastrointestinal tract removed. Carcasses were weighed and stored at -20°C until required.

2.2.6.1. Carcass decomposition

Animal carcasses were placed in to separate containers and a solution of 3M KOH in 65% ethanol in equal volume to carcass weight was added. The carcass was incubated at 70°C for 3 days until dissolved. The resultant liquid was made up to 100ml with absolute ethanol and a sample taken for measurement of protein and fat content.

2.2.6.2. Glycerol assay

A glycerol assay (Assay GY105, Randox Laboratories, London, UK) was used to determine the fat content in the samples and subsequently the percentage fat mass of the animals. The assay is a direct colorimetric method for the measurement of glycerol, which utilises a quinoneimine chromagen system in the presence of glycerol kinase, peroxidase and glycerol phosphate oxidase. All reagents were prepared according to the manufacturer’s guidelines. An 11-point standard curve ranging from 0-10mM glycerol was created. Samples were diluted 1:100 with dH\(_2\)O and 30µl of
sample or standard was used in the assay. The absorbance of the reaction was read at a wavelength of 520nM using a spectrophotometer (Biotek ELx808, wolf Laboratories, York, UK). The fat content of each sample was calculated from the standard curve and the carcass fat content was then calculated by assuming that each molecule of triglyceride has a molecular weight of 885g (Bergmeyer, 1974).

2.2.6.3. Protein assay

A modified Lowry Assay (Pierce, Assay number 23240, Thermoscientific) was used to determine the protein content of the samples and subsequently the percentage protein content of the animals. The assay is a colorimetric method, using a cupric sulphate-tartrate system utilizing Folin-ciocalteu phenol reagent. All reagents were prepared according to the manufacturer’s guidelines. A 10-point standard curve ranging from 0-1500µg/ml bovine serum albumin was created. Samples were diluted 1:100 with dH₂O and 40µl of sample or standard was used in the assay. The absorbance of the reaction was read at a wavelength of 750nM using a spectrophotometer (Biotek ELx808, wolf Laboratories, York, UK). The protein content of each sample was calculated from the standard curve and the carcass protein mass calculated accordingly. Lean mass was calculated as total carcass mass – calculated fat mass.

2.2.7. Metabolic measurements using CLAMS

The Comprehensive Laboratory Animal Monitoring System (CLAMS) (Oxymax, Columbus instruments, OH, USA) is an automated cage system that enables continuous measurement of food intake, activity and other metabolic parameters. The rat CLAMS consisted of 16 open-circuit Plexiglas cages through which air was passed at a flow rate of 2.5 litres/minute. Food intake was measured by end-feeders resting on balances directly linked to a computer and measurements were recorded from each cage at 20 minute intervals. Volumes of oxygen consumption (VO₂) and carbon dioxide (VCO₂) production were measured by indirect calorimetry. Sample air from each cage was sequentially passed through sensors to determine O₂ and CO₂ content. VO₂ and VCO₂ values were determined by comparing percentage differences in the O₂ (ΔO₂) and CO₂ (ΔCO₂) content of each rat cage to a reference cage over 20 minute intervals. VO₂ and VCO₂ parameters were normalised with respect to body weight and corrected to the power of an effective mass value of 0.75. Respiratory exchange ratio (RER) values were calculated as a ratio of CO₂ production to O₂ consumption prior to normalisation and correction. Activity was measured using an optical beam technique (Opto M3, Columbus Instruments). Two sets of infra-red photo-beams were located along side each cage and recorded the number of beam breaks in 20 minute intervals. The XAMB beams, located close to the
bottom of the cage, measured all ambulatory movements along the X axis. The ZTOT beams, located above and parallel to the XAMB beams, measured all rearing movements along the Z axis.

Due to the design of the feeding hoppers, animals were fed powdered RM1 diet during studies in the CLAMS; therefore animals were given powdered RM1 diet in their home cages for 7 days prior to study commencement to allow acclimatisation.

Singly housed animals were allowed to acclimatise for 24 hours in the CLAMS cages prior to study commencement. The study commenced with an 8nmolkg SC injection of peptide or saline at 1500 hours and metabolic measurements recorded every 20 minutes for 48 hours thereafter. Bodyweight was measured manually at 1500 hours daily. Area under the curve data (AUC) was analysed by separating the 48hour time period into 2 light phases and 2 dark phases. One light phase = 1500 to 1900hrs + 0700 to 1500hrs. One dark phase = 1900hrs-0700hrs.

2.2.8. Peptide pharmacokinetics in rats

Rats were weighed and randomised into groups, ensuring that average bodyweight was equal across all groups. Each animal was administered a single SC injection of 1mg of peptide reconstituted in 20µl ZnCl₂ slow release diluent, using a 0.5ml insulin syringe and 29 gauge needle. Blood samples were collected before injection at time 0 and then at a range of time points including 0.5, 3, 24, 48, 72, 96, 168 and 240 hours post-injection. After superficial venesection of the lateral tail vein, blood samples were collected in to microtubes flushed with heparin and containing 4µl of 25x protease inhibitor cocktail (Sigma-Aldritch, Dorset, UK). Approximately 200µl of blood was collected and the microtubes placed on ice. Samples were centrifuged at 4°C for 8 minutes at 10,000 x g (Sigma lab centrifuges 3 K18, Rotor No. 19777-H) and the plasma layer removed and stored at -20°C. Plasma peptide concentration was determined by radioimmunoassay.

2.3. Radioimmunoassay

A sensitive in house radioimmunoassay (Appendix C) was used to specifically measure glucagon N-terminal-like immunoreactivity in order to quantify levels of peptide present in rat plasma at different post-injection time points. The assay was performed in a total volume of 0.7ml of 0.06M phosphate EDTA buffer (0.05M Na₂HPO₄·2H₂O, 0.0006M KH₂PO₄, 0.01M disodium-EDTA.2H₂O, 0.008M NaN₃), at pH 7.4, containing 0.3% bovine serum albumin (BSA). The purified glucagon(1-16) fragment was used to produce the antiserum (R6241) in rabbits, and this was then coupled to BSA by glutaraldehyde and used at a final dilution of 1:1000. The antibody specifically cross-reacted with the N-terminal glucagon sequence common in the glucagon analogues. $^{125}$I glucagon was produced by
direct iodination and purified by reverse phase high-pressure liquid chromatography (HPLC). All samples were assayed in duplicate. Standard curves were prepared using purified peptide diluted in assay buffer at a concentration of 2pmol/ml and added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl. The assay was incubated for 3 days at 4°C and then separated into free and antibody bound label using charcoal. Both the pellet and the supernatant were counted for 180 seconds in a γ-counter (model NE1600, NE Technology Ltd, Reading, UK) and the ratio of free to bound label calculated. Plasma peptide concentrations were calculated in terms of the individual peptide standard curves using two phase exponential decay (Prism, V5, GraphPad Software Inc)

2.4. In Vitro Studies

2.4.1. Cell maintenance

CHO-K1 cells stably over expressing the human glucagon receptor (hGCGr) were purchased from Invitrogen Life Technologies (Paisley, UK) as were all cell culture reagents unless otherwise stated. CHL cells stably over expressing the rat GLP-1 receptor were a gift from Professor Bernard Thorens (University of Lausanne, Switzerland). CHO cells over expressing the human GLP-1 receptor or the rat glucagon receptor were previously produced in house. All cells were maintained at 37°C with 5% CO₂ in T175cm² flasks with the following basal media supplemented as specified.

Basal media

- Dulbecco’s modified medium (DMEM)
- sodium pyruvate
- 4.5g/l glucose
- supplemented with 1% antibiotic (100U/ml Penicillin and 100µg/ml Streptomycin).

**CHO-K1 cells over-expressing the human glucagon receptor**
- 10% dialyzed foetal bovine serum (DFBS)
- 0.1mM non-essential amino acids (NEAA) (Sigma-Aldrich)
- 25mM HEPES (pH 7.3) (Sigma-Aldrich)
- Blasticidin 5µg/ml
- Hygromycin 600µg/ml

**CHO cells over-expressing the human GLP-1 receptor or rat glucagon receptor**
- 10% foetal bovine serum (DFBS)
- 0.1mM non-essential amino acids (NEAA) (Sigma-Aldrich)
- 25mM HEPES (pH 7.3) (Sigma-Aldrich)
- genetin (G418) 200µg/ml

**CHL cells over-expressing the rat GLP-1 receptor**
- 10% foetal bovine serum (FBS)
Media was changed every 2-3 days and cells were passaged when they reached 70% confluence. Briefly, after aspirating the medium from the flask 5ml of 0.05% Trypsin EDTA was added and incubated at room temperature for approximately 5 minutes until all cells were detached after gentle agitation. To recover the cells 5mls of fresh medium was added and the cell suspension centrifuged at 800 x g for 5 minutes. Cells were resuspended in 5mls of fresh medium and seeded in a new flask at a dilution of 1:5.

2.4.1.1. cAMP accumulation bioactivity assay

The following method was followed for all over-expressing cell lines used to assess cAMP production throughout this thesis. One day prior to assay, cells were seeded in 48 well plates at a density of 37 500 cells per well (150 000 cells/ml) in 250µl of standard culture medium (DMEM supplemented as above according to the cell line). On the following day the medium was aspirated and cells were incubated for 1 hour in 250µl of FBS free DMEM with 1% antibiotic (100U/ml Penicillin and 100µg/ml Streptomycin). An appropriate concentration range of the peptide to be tested was reconstituted in FBS free DMEM containing 1mM of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methFBS free media containing the test peptide. The cells were incubated for exactly 30 minutes from this point. After the incubation, media was aspirated and replaced with 120µl lysis buffer (0.1M HCl with 0.5% Triton-X) and incubated for 10 minutes. The lysate containing accumulated cAMP was stored at -20°C before being assayed by enzyme-linked immunosorbent assay (ELISA).

ELISA

The lysate was diluted 1:4 in lysis buffer and assayed using a direct cAMP ELISA kit (ADI-900-066, Enzo Life Sciences, UK) according to the manufacturer’s instructions. All reagents except samples were provided in the assay kit. Briefly, 50µl ‘neutralising reagent’ was added to each well. A 6 point standard curve from 0-200pmol/ml was created by performing a serial dilution of the provided cAMP standard. 100µl of cAMP standard and sample lysates were added to appropriate wells before adding 50µl of alkaline phosphatase conjugated cAMP and 50µl of anti-cAMP antibody into each well. The plate was sealed and left to incubate on a plate shaker (~450rpm) at room temperature for 2 hours. Contents were emptied from the wells and washed 3 times with 300µl of wash buffer. 100µl ‘substrate solution’ was added to each well and incubated at room temperature for 1 hour. Subsequently 50µl of ‘stop solution’ was added to each well and the optical density of the reaction read at a wavelength of 405nM using a spectrophotometer (Biotek ELx808, wolf Laboratories, York, UK). Concentrations of cAMP were calculated from a standard curve and plotted using Prism V5 (GraphPad Software Inc, San Diego, USA). The half maximal effective concentration (EC₅₀) was calculated using the following non-linear regression equation:
2.4.2. Receptor binding affinity assay

Cell membrane suspensions were prepared from cells over-expressing the human or rat GLP-1 receptor or glucagon receptor. Mouse liver and lung tissue was used to isolate membranes expressing the mouse glucagon receptors or GLP-1 receptors respectively. Membranes expressing the rat GLP-1 receptor were isolated from rat lung tissue. The competitive binding studies detailed below measure the specific binding affinity of peptides to the receptors present on these membranes.

2.4.2.1. Preparation of membranes from tissues

Membranes were prepared according to the homogenisation and differential centrifugation method described previously (O’Shea et al. 1997). Tissues were collected from rats and mice maintained under the conditions described in section 2.2.1, except they were housed in pairs (rats) or groups of 5 (mice). Lung tissue from a minimum of 20 rats was required and lung and liver tissue from 40 mice was required for a membrane prep of each tissue. Immediately after death tissues were harvested, snap frozen in liquid nitrogen and stored at -80°C.

Tissues were homogenised (Ultra-turrax T25, IKA Labortechnik, staufen, Germany) in ice-cold homogenisation buffer (50mM HEPES buffer pH 7.4 containing 0.25M sucrose, 10 µg/ml soybean trypsin inhibitor (Sigma), 0.5µg/ml pepstatin (Sigma), 0.1mg/ml benzamidine (Sigma), 1ml 100KIU/ml aprotinin, 10µl/ml Trasylol (Bayer, Haywards heath, UK)) at 4°C.

The homogenates were centrifuged at 1000 Xg (Beckman J2-21, rotor JS-13.1) for 15 minutes at 4°C and resulting supernatants were then centrifuged at 100000 Xg (Sorvall Ultracentrifuge OTD55B, rotor A-841, DuPont, ST.Netos, Cambridgeshire, UK) for 60 minutes at 4°C. Supernatant was removed and pellets re-suspended in the above buffer without sucrose using hand-held glass/Teflon homogenisers (Jencons, East Grinstead, West Sussex, UK), and centrifuged at 100000 Xg at 4°C as before. The supernatant was removed and the final pellet re-suspended to a final protein concentration of 2-3mg/ml and stored at -80°C. Protein content was measured by Bradford assay (section 2.4.3.3)

2.4.2.2. Preparation of membranes from cells

Cells over expressing the human or rat glucagon receptor or GLP-1 receptor were maintained as previously described (section 2.4.1).
Culture medium was removed and cells detached from the flasks using ice-cold 0.01M PBS and gentle scraping, a minimum of 30 T175 cm\(^2\) flasks were required for one membrane prep. Cells were pooled and centrifuged at 200 Xg for 5 minutes (Sigma Laboratory Centrifuges 3, K18, rotor No. 19777-H), the supernatant discarded and the pellets put on ice. Pellets were re-suspended drop wise in 20ml of ice-cold 1mM HEPES homogenisation buffer (1mM HEPES buffer pH 7.4 containing 0.25M sucrose, 10 µg/ml soybean trypsin inhibitor, 0.5µg/ml pepstatin, 0.1mg/ml benzamidine, 1ml 100KIU/ml aprotinin, 10µl/ml Trasylol) and homogenised for 1 minute with the Ultra Turrax homogeniser. Homogenates were centrifuged at 1000 Xg (Beckman J2-21, rotor JS-13.1) for 20 minutes and the resulting supernatant discarded. Pellets were re-suspended in a small amount of 5mM HEPES homogenisation buffer (same as previous homogenisation buffer but containing 50mM HEPES), homogenised with the Ultra Turrax and re-suspended in a total of 30ml 50mM HEPES homogenisation buffer. After centrifugation at 950 Xg (Beckman J2-21, rotor JS-13.1) the supernatant was removed and re-centrifuged at 100000 Xg (Sorvall Ultracentrifuge OTD55B, rotor A-841) at 4°C for 60 minutes. The supernatant was removed and the pellets re-suspended in 50mM HEPES homogenisation buffer at a final protein concentration of 1-2mg/ml and stored at -80°C. Protein concentration was measured by Bradford assay (section 2.4.3.3).

2.4.2.3. Bradford assay

0.1ml cell membrane solution (purified from either over-expressing cell lines or animal tissue as described previously) was added to 3ml of Bradford reagent (Sigma). A standard curve was also determined using known concentrations of bovine serum albumin (BSA) Sigma), 0.1ml of 0, 0.25, 0.5, 1 or 1.5mg/ml solutions were added to 3ml of Bradford reagent. All solutions were incubated for 45 minutes at room temperature. A sample of each solution was added to a cuvette and the absorbance was measured at a wavelength of 595nm using a spectrophotometer (WPA UV 1101). The standard curve was used to determine the unknown concentration of protein in the membrane samples.

2.4.2.4. Iodination of peptides

The direct iodogen method previously described by Owji et al was used to attach \(^{125}\)I molecules to glucagon and GLP-1. 3nM (15µg) of peptide in 10µl of 0.2M phosphate buffer pH 7.2 was reacted with 37MBq of Na\(^{125}\)I (Amersham) and 23nM (10µg) of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (iodogen reagent, Pierce Chemical Co, Perbio Science UK Ltd., Cramlington, UK) for 4 minutes at 22°C. The \(^{125}\)I-peptide was purified by reverse-phase high performance liquid chromatography (HPLC) using an/H\(_2\)O/0.05% TFA gradient.
2.4.2.5. Receptor binding affinity studies

The binding affinity of a peptide can be measured by its ability to displace the radio-labelled endogenous ligand from its cognate receptor. To investigate binding at the human or rat glucagon receptor or GLP-1 receptor cell membranes prepared from over expressing cell lines were used. Membranes prepared from rat liver tissue were also used to investigate binding at the rat glucagon receptor and membranes prepared from mouse liver and lung were used to investigate binding at the mouse glucagon receptor and GLP-1 receptor respectively. The specific buffers used for GLP-1 receptor binding and glucagon receptor binding studies were as follows:

**Buffer for GLP-1 receptor studies**
- 20mM HEPES (pH 7.4)
- 5mM CaCl₂
- 1mM MgCl₂
- 1% BSA
- 0.01% Tween20
- 0.1mM diprotin A
- 0.2mM PMSF

**Buffer for glucagon receptor studies**
- 25mM HEPES (pH 7.4)
- 2mM MgCl₂
- 1% BSA
- 0.05% Tween20
- 0.1mM diprotin A
- 0.2mM PMSF

A range of concentrations of unlabelled peptide were made up in the relevant assay buffer and added to siliconised polypropylene microtubes (Sigma). All peptide concentrations were assayed in duplicate or triplicate. 50µl of ¹²⁵I labelled glucagon or ¹²⁵I labelled GLP-1 in assay buffer at 1000 counts (Bq)/sec was added to each tube, followed by 10µl of relevant membrane suspension (protein concentration 1-2µg/ml). Tubes were incubated at room temperature for 90 minutes and then centrifuged at 15700 Xg (Sigma lab centrifuges 3 K18, Rotor No. 19777-H) at 4°C for 3 minutes. The supernatant was discarded and the pellet washed with 500µl of the relevant assay buffer and re-centrifuged as before. The supernatant was discarded and the γ radiation of the pellets counted using a γ-counter (model NE1600, NE Technology Ltd, Reading, UK) for a total of 240 seconds. The half maximal inhibition coefficient (IC₅₀) was calculated for each peptide using Prism V5 (GraphPad Software Inc) and the following non-linear regression equation

\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{((\log_{10}\text{IC}_{50} - X) \times \text{HillSlope})})} \]
2.5. **Statistical analysis**

All data are expressed as mean ± SEM.

Food intake and bodyweight data were analysed using either one-way ANOVA or two-way repeated measures ANOVA, as stated in figure legends, with post hoc tests employing either Dunnett or Bonferroni correction. Where values are expressed as a percentage of the vehicle group, statistical analysis was carried out on the actual data not the percentages. $P < 0.05$ was considered statistically significant. All statistical analysis was conducted using Prism V5 (GraphPad Software Inc).
Chapter 3

Investigation of amino acid substitutions to glucagon to produce a dual agonist at the glucagon and GLP-1 receptors
3.1. Introduction

3.1.1. Peptide structure-function studies

As discussed previously, obesity is a major health problem worldwide with limited treatment options available. A potentially highly effective pharmaceutical treatment for obesity would combine the actions of the homologous peptides glucagon and GLP-1 to both increase energy expenditure and reduce food intake, so as to create a negative energy balance. In order to produce an effective dual agonist it is necessary to understand how the structures of glucagon and GLP-1 are related to their functions.

Structure function relationships can be investigated using several different experimental approaches. Circular dichroism (CD) spectroscopy, nuclear magnetic resonance imaging (NMR) and X-ray crystallography are employed to investigate substitution effects on secondary and tertiary peptide structure, to establish possible structural reasons for alterations in receptor binding or efficacy (Berg et al. 2002; Kelly et al. 2005). Crystallography can also be utilised to model the interaction between ligand and receptor, highlighting areas or conformations that may be key to this event (Turnbull & Emsley 2013). Investigating the receptor affinity and bioactivity of truncated peptides and peptide fragments can identify domains of the peptide responsible for properties such as receptor affinity. Alanine scanning, the sequential substitution of amino acid residues with alanine, enables investigation of the contribution of single amino acids to receptor binding affinity and activation or susceptibility to degradation (Adelhorst et al. 1994). Due to its non-reactive side chain alanine is rarely directly involved in protein function and therefore acts as a neutral spacer amino acid, making it a suitable substitute in these studies (Gallwitz et al. 1994). Chimeric analogues of homologous peptides are useful for delineating regions that confer receptor selectivity (Hjorth et al. 1994; Unson et al. 2002).

3.1.2. Structure and properties of glucagon

3.1.2.1. Peptide conformation

Several different tertiary structures have been postulated for the conformation of glucagon. Initial X-ray analysis of the crystal structure of glucagon indicated a helical region between residues 10-25 (Sasaki et al. 1975). The 2D NMR visualisation of glucagon in a pseudo membrane environment suggested a C-terminal helical region between residues 17-29 and possibly another between 10-14 (Braun et al. 1983). However the N-terminal was not visible using this method and it was suggested this was due to the region being highly labile. Glucagon’s secondary and tertiary structure has been predicted based on a model that estimates the probability that individual amino acids will form
various secondary structures. This model predicts a high probability of three $\beta$-turns in the N-terminal region between residues 2-5, 10-13 and 15-18 (Chou & Fasman 1975). Interestingly the region between residues 19-27 has an almost equal propensity for $\beta$-sheet and $\alpha$-helix formation. It has been suggested that unbound glucagon at low concentrations adopts a helical formation in this region, whereas at higher concentrations, when it aggregates, and in the bound state it adopts a $\beta$-sheet structure. These predictions were investigated using the natural propensity of cysteines to form disulphide bridges to induce and stabilise turn conformations in glucagon; along with the formation of lactam bridges between lysine and glutamic acid residues to stabilise helix formation. An $\alpha$-helix formation between residues 17-21 significantly increased both receptor binding and activity (Ahn et al. 2001c). This is supported by the findings of Unson et al (1989) who suggested a reduction in helical potential leads to a decrease in receptor binding affinity of glucagon antagonists.

Several studies have demonstrated that attempts to enhance or stabilise N-terminal $\beta$-turns causes reductions in both receptor binding and activation, suggesting this is not the active conformation of glucagon (Krstenansky et al. 1986; Unson et al. 1989; Ahn et al. 2001c). Similarly, stabilisation of helices between residues 1-13 in the N-terminal of glucagon also causes significant reductions in receptor activity and/or binding, despite critical residues being conserved (Ahn et al. 2001c). This suggests that the N-terminal of glucagon needs to be labile to enable optimal receptor binding and/or activation.

Due to the inherent difficulties involved in the expression and solubilisation of family B GPCR's the receptor bound, active conformation of glucagon is yet to be determined (Unson 2002). Glucagon shares a high degree of homology with other family B GPCR ligands, particularly in the N-terminal region (Figure 3.1). It has been postulated that glucagon follows a two-step receptor binding model similar to that which has been shown for the homologous peptide PACAP; whereby the initial interaction between the C-terminal $\alpha$-helix of the peptide and the N-terminal of the receptor generates a change in conformation at the N-terminal of the peptide, which facilitates more specific interactions with the receptor core domain, subsequently leading to activation (Inooka et al. 2001; Unson et al. 2002).

3.1.2.2. N-terminal residues

It was initially postulated that the C-terminal of glucagon determined receptor recognition and binding and the N-terminal was responsible for receptor activation (Hruby 1982). However, further structure-function analysis has revealed that while this simplistic view is generally true, a number of residues and regions throughout the molecule are important for receptor binding and/or activation (Unson 2002). In particular the N-terminal residues His$^1$ and Asp$^9$ have been shown to be critical for
glucagon receptor activation. A substitution at either of these positions produces partial agonists and analogues with substitutions at both residues are potent antagonists at the glucagon receptor (Unson et al. 1987; Unson et al. 1989; Ahn et al. 2001b). Interestingly these residues are also conserved in GLP-1 and exendin-4. Out of the first 15 residues, 10 are conserved between glucagon and GLP-1 and 9 are also present in exendin-4. A large number of N-terminal residues are also conserved in other family B GPCR ligands, indicating they are likely to be critical for receptor binding or activation (Figure 3.1). Joint substitution of the four non-conserved glucagon residues with the corresponding residues in GLP-1 results in a greater than 650 fold reduction in glucagon receptor binding and a 1.5 fold increase in GLP-1r affinity. A similar substitution of the four glucagon residues in to the N-terminal sequence of GLP-1 increases glucagon receptor affinity more than 21 fold but only decreases GLP-1 receptor binding by 2.5 fold (Hjorth et al. 1994). These findings suggest that the N-terminal glucagon sequence is essential for selective identification and binding by the glucagon receptor. Individual substitution of the four non-conserved glucagon residues with the corresponding residues in GLP-1 also results in significant reductions in both binding and activity at the glucagon receptor. In particular substitutions at residues 3 and 12 have the largest detrimental effects which can be abrogated by switching the glucagon receptor core domain with that of the GLP-1 receptor, indicating that the glucagon receptor core domain is involved in recognising the N-terminal of glucagon (Runge et al. 2003a). Several other studies have also identified Gln3 as essential for glucagon receptor binding and activation (Gysin et al. 1986; Murphy et al. 1986; Robberect et al. 1988; Unson et al. 1989). The conservation of positive charge at position 12 is important for optimal glucagon receptor binding (Hruby 1982; Unson et al. 1998) and a negative charge at position 15 is critical (Unson et al. 1994). The sidechains of Phe6, Tyr10, Tyr13 and Leu14 are thought to form a hydrophobic patch which interacts with the receptor and substitution of Phe6 or alteration of the Tyr side chains significantly decreases receptor affinity (Sasaki et al. 1975; Braun et al. 1983).

3.1.2.3. C-terminal residues

The C-terminals of glucagon and GLP-1 are more divergent with only 4 common residues, which supports the theory that this region is mainly responsible for receptor recognition and binding rather than activation. However, because previous glucagon research was focussed on developing glucagon receptor antagonists for the treatment of diabetes, investigation has mainly focussed on the N-terminal residues that are responsible for receptor activation; therefore the C-terminal residues involved in receptor binding have been less well characterised. Conservation of charge at positions 17 and 18 is important for effective binding to the glucagon receptor (Hruby 1982; Unson et al.
Substituting arginine for lysine at these residues and also substituting glutamic acid at position 21 produces a super agonist at the glucagon receptor, with both enhanced affinity and efficacy (Krstenansky et al. 1986). Glucagon analogues with lactam bridges between Lys$^{18}$ and Glu$^{21}$ (Sturm et al. 1998) or Lys$^{17}$ and Glu$^{21}$ (Ahn et al. 2001c) demonstrate substantially increased glucagon receptor affinity and efficacy, indicating an α-helix in this region is beneficial for receptor interaction. Residue 28 has been identified as a critical residue for glucagon receptor binding (England et al. 1982) and an amino acid with an aromatic side chain is necessary at residue 25 for full biological activity (Murphy et al. 1986). The terminal residues 27-29 have been suggested to be important in maintaining maximal binding efficacy, but don’t appear to directly affect activity (Frandsen et al. 1981; England et al. 1982). Both GLP-1 and exendin-4 are amidated at the C-terminal along with numerous other biologically active peptides (Kim & Seong 2001). The addition of a C-terminal amide group has been suggested to improve the GLP-1 receptor binding affinity of several glucagon antagonists (Krstenansky et al. 1986; Unson et al. 1987), possibly due enhancement of the C-terminal helix dipole (Unson et al. 1987). The addition of a C-terminal amide to glucagon results in a 6 fold increase in activity at the GLP-1 receptor (Day et al. 2009). The contribution of the C-terminal NH$_2$ to the binding and activity of GLP-1 has not been investigated, although GLP-1$^{7-36}$amide and GLP-1$^{7-37}$ produce similar biological effects in vivo (Orskov et al. 1993). Therefore it is somewhat curious that C-terminal amidation of glucagon increases GLP-1 receptor activity; however it may be that other C-terminal additions at residue 30 of glucagon would have a similar effect on GLP-1 receptor activity.
Figure 3.1: Amino acid sequences of the glucagon receptor family ligands.
See Appendix B for the 3 letter amino acid abbreviations.
3.1.3. Structure and properties of GLP-1 and Exendin-4

3.1.3.1. Peptide conformation

Similar to glucagon, the structure of GLP-1 appears to be labile and forms different structures in different environments (Neidigh et al. 2001). NMR and CD analysis of GLP-1 in micelles and solution have consistently identified a well converged helical region between residues 18-27. The N-terminal of GLP-1 is also reported to form a helix between residues 7-14 which links with the C-terminal helix via a disordered region at residues 15-17, centred around the helix destabilising Gly\(^{16}\) (Thornton & Gorenstein 1994; Neidigh et al. 2001; Andersen et al. 2002). Restricting the structure of GLP-1 using lactam bridges demonstrates that this two helix structure linked by the destabilising Gly\(^{16}\) is likely to be the active receptor bound conformation (Murage et al. 2008). The crystal structure of receptor bound GLP-1 suggests that in the active conformation GLP-1 actually forms a continuous helix from residues 7 to 27 but with a kink around Gly\(^{16}\) (Underwood et al. 2010). Exendin-4 has a greater helical propensity in this region than GLP-1, which is postulated to be due to the absence of the helix disrupting Gly at position 16 (Neidigh et al. 2001; Andersen et al. 2002). Interestingly, extending the helix in GLP-1 to include residues 15-17 reduces affinity and potency at the GLP-1 receptor (Murage et al. 2008), indicating that flexibility in this region is essential for full bio-efficacy of GLP-1. However, although substituting Gly at position 16 of the truncated exendin\(^{9-30}\) analogue disrupts the helical structure, it doesn’t significantly reduce GLP-1 receptor binding affinity (Al-Sabah & Donnelly 2003), indicating that exendin-4 binding may be subtly different to that of GLP-1. In both GLP-1 and exendin-4 the C-terminal part of the helix between residues 18-27 is amphiphilic, allowing hydrophilic and hydrophobic residues to interact with the receptor through opposite faces of the helix. The hydrophobic residues are generally conserved between GLP-1 and exendin-4 whereas the hydrophilic face consists of divergent residues (Runge et al. 2008; Underwood et al. 2010).

Whereas N-terminal truncation of 8 amino acids of GLP-1 reduces receptor affinity by 500 fold, similar truncation of exendin-4 produces the potent GLP-1 receptor antagonist exendin\(^{9-39}\) which maintains equivalent affinity to GLP-1 at both the human and rat receptors (Thoresen et al. 1993; Montrose-Rafizadeh et al. 1997). It is postulated that GLP-1 receptor affinity is maintained due to an interaction between the receptor and the 9 amino acid C-terminal extension of exendin-4 because affinity is diminished with exendin-4\(^{1-30}\) and exendin\(^{9-30}\) (Montrose-Rafizadeh et al. 1997; Mann et al. 2010). Similarly, adding the 9 amino acid tail of exendin-4 to glucagon improves GLP-1 receptor activity 4 fold, although activity remains 15 fold lower than native GLP-1 (Day et al. 2011). NMR analysis of exendin-4 in the solution state indicates that the 9 C-terminal residues form a tertiary
structure termed the ‘TRP cage’ (Neidigh et al. 2001) which was proposed to be responsible for the affinity of exendin\textsubscript{9-39} (Al-Sabah & Donnelly 2003; Lopez de Maturana et al. 2003). However this ‘TRP cage’ structure is absent in the NMR analysis of exendin-4 in a micelle model (Neidigh et al. 2001), which simulates a receptor bound environment, and is also absent in the crystal structure of exendin\textsubscript{9-39} bound to the N-terminal domain (NTD) of the GLP-1 receptor (Runge et al. 2008). Instead it is postulated that the affinity of exendin\textsubscript{9-39} is the result of superior hydrophobic interactions with the receptor, coupled with enhanced C-terminal helical conformation (Runge et al. 2007; Runge et al. 2008). Exendin-4’s greater helical propensity has been suggested to be a result of stabilising interactions between the hydrophilic residues and neighbouring amino acids that are not present in GLP-1, and also a more favourable alignment of oppositely charged residues (Underwood et al. 2010). Compared to GLP-1, Exendin-4 has superior affinity for the N-terminal domain (NTD) of the rat GLP-1 receptor which was initially attributed to an interaction between the putative ‘TRP’ cage and the receptor (Al-Sabah & Donnelly 2003; Lopez de Maturana et al. 2003). However, the affinity of exendin-4 and GLP-1 is similar at the human NTD and a specific interaction has been identified between Ser\textsubscript{32} of exendin-4 and Asp\textsubscript{68} in the NTD of the rat GLP-1 receptor. Asp\textsubscript{68} is not present in the human receptor NTD and Ser\textsubscript{32} is not present in GLP-1 (Mann et al. 2010).

3.1.3.2. Important residues

Alanine scanning indicates the N-terminal residues His\textsuperscript{1}, Gly\textsuperscript{4}, Phe\textsuperscript{6}, Thr\textsuperscript{7}, and Asp\textsuperscript{9} of GLP-1 are essential for receptor binding and activation (Adelhorst et al. 1994; Gallwitz et al. 1994; Parker et al. 1998). These residues are conserved in GLP-1, glucagon and exendin-4 and to some extent in GIP and secretin (Figure 3.1). This suggests that these residues may be essential for a common binding or activating mechanism of family B GPCRs. Eight out of the first 9 residues of GLP-1(7-36) are conserved in exendin-4 and the finding that exendin(9-39) is a potent GLP-1 receptor antagonist that retains a high binding affinity (Goke et al. 1993; Montrose-Rafizadeh et al. 1997) indicates that these N-terminal residues are required for GLP-1 receptor activation. Substituting the 4 non-conserved N-terminal residues Ser\textsuperscript{2}, Glu\textsuperscript{3}, Tyr\textsuperscript{10} and Lys\textsuperscript{12} from glucagon in to GLP-1 has a very minor effect on GLP-1 receptor affinity and potency (Hjorth et al. 1994; Runge et al. 2003b), which suggests the N-terminal residues of GLP-1 are not responsible for selective recognition or binding by the GLP-1 receptor. On the other hand, a chimeric peptide consisting of the N-terminal of glucagon and the C-terminal of GLP-1 has substantially greater affinity and activity at the GLP-1 receptor than glucagon (Hjorth et al. 1994; Runge et al. 2003a). Even the addition of the final 4 residues of GLP-1 to
glucagon increase GLP-1 receptor binding 169 fold (Hjorth et al. 1994) indicating that the C-terminal is essential for GLP-1 receptor selectivity. Similarly, swapping the divergent C-terminal residues of GLP-1 with the corresponding glucagon amino acids causes a 475 fold reduction in GLP-1 receptor affinity (Hjorth et al. 1994). The C-terminal of GLP-1 appears to be important for selective receptor recognition, whereas the glucagon receptor seems to selectively recognise the divergent N-terminal residues. It has been suggested that the C-terminal binding of GLP-1 enables the orientation of the N-terminal to enable maximum affinity and efficacy at the GLP-1 receptor (Murage et al. 2008). Residues 22 and 23 have also been identified as critical for GLP-1 receptor activity (Adelhorst et al. 1994; Gallwitz et al. 1994). Phe^{22} is a conserved residue in GLP-1, glucagon and exendin-4 whereas valine replaces Ile^{23} in glucagon. Rather than direct interaction with the receptor, it has been postulated that these two residues may enable a specific conformation of the C-terminal which contributes to the selective recognition of GLP-1 by the receptor (Adelhorst et al. 1994).

3.1.4. Dual agonists at the GLP-1 and glucagon receptors

Oxyntomodulin is a naturally occurring dual agonist of the glucagon and GLP-1 receptors, albeit with reduced potency compared to the native ligands (Jørgensen et al. 2007; Kerr et al. 2010; Santoprete et al. 2011; Kosinski et al. 2012). Structurally oxyntomodulin is the 29 amino acids of glucagon with a C-terminal octapeptide tail. As glucagon exhibits very low affinity and activity at the GLP-1 receptor it can be assumed that the octapeptide tail of oxyntomodulin is responsible for its increased efficacy at the GLP-1 receptor compared to glucagon. Oxyntomodulin has a very short half-life in vivo due to rapid renal clearance and susceptibility to proteolytic enzymes, in particular DPPIV (Druce et al. 2009). Numerous structure-function experiments have been conducted with oxyntomodulin in an effort to produce a long lasting analogue that could be used as a treatment for obesity (Day et al. 2009; Druce et al. 2009; Pocai et al. 2009; Liu et al. 2010; Santoprete et al. 2011; Lynch et al. 2014). The N-terminal dipeptide with Pro, Ala or Ser as the penultimate residue are the specific substrate sequences recognised by DPPIV; hence glucagon, oxyntomodulin and, to a greater extent, GLP-1 are susceptible to degradation by this enzyme (Mentlein 1999; Druce et al. 2009). A number of studies have demonstrated that replacing Ser^{2} of oxyntomodulin provides resistance to DPPIV degradation (Druce et al. 2009; Lynch et al. 2014). However position 2 is an important residue for receptor activation, particularly for the glucagon receptor (Runge et al. 2003b), therefore resistance to degradation is often at the expense of activity and binding at one or both receptors (Druce et al. 2009; Pocai et al. 2009; Kerr et al. 2010; Santoprete et al. 2011; Lynch et al. 2014).
Another common strategy to increase the circulating half-life of a peptide is to attach a fatty acid (Druce et al. 2009; Kerr et al. 2010; Lynch et al. 2014), cholesterol moiety (Pocai et al. 2009; Santoprete et al. 2011) or a polyethylene glycol (PEG) sidechain (Day et al. 2009; Bianchi et al. 2013); all of which promote peptide binding to plasma proteins and slow glomerular filtration due increased hydrodynamic radius (Harris & Chess 2003). However, despite increasing peptide half-life these modifications often have unfavourable effects on receptor binding and activity. C-terminal derivitization of oxyntomodulin reduced GLP-1 receptor activity 1.5 - 38 fold (Pocai et al. 2009; Santoprete et al. 2011). Bianchi et al (2013) found that PEGylation of their D-Ser² oxyntomodulin analogue reduced activity by 42 fold and more than 134 fold at the GLP-1 and glucagon receptors respectively. Alternatively, attaching a C-terminal cholesterol moiety via a mini PEG linker to the same D-Ser² oxyntomodulin analogue actually improved receptor activity to such an extent that it cancelled out the detrimental receptor effects of the D-Ser² substitution. The authors suggested that the addition of the cholesterol moiety may have increased the affinity of the peptide for the lipid raft compartment of the membrane which is where GPCRs are thought to be enriched (Santoprete et al. 2011). This particular analogue was an effective anti-obesity treatment in mice when administered at 1.9µmol/kg every other day (Pocai et al. 2009). A PEGylated chimera of glucagon and GLP-1 with an AIB² substitution and a lactam bridge between Glu¹⁶ and Lys²⁰ had 10 fold lower efficacy at the glucagon receptor and 2 fold lower efficacy at the GLP-1 receptor compared to the native peptides (Day et al. 2009). When administered at a dose of 70nmol/kg once weekly to obese mice this particular analogue reduced fat mass and normalised blood glucose after a month of treatment. The in vivo effects of this dual analogue appeared to last up to a week in mice and therefore could last longer in humans. These findings indicate that producing a chimeric glucagon/GLP-1 peptide is a viable option for generating a potent dual agonist, as opposed to trying to extend the half-life of native oxyntomodulin.

There is a paucity of information in the literature comparing the level of glucagon receptor and GLP-1 receptor activity of native oxyntomodulin; however it appears that oxyntomodulin has a greater activity at the glucagon receptor relative to the GLP-1 receptor (Jorgensen et al. 2007; Kerr et al. 2010; Kosinski et al. 2012). Previous experiments have found that oxyntomodulin administration in humans (Wynne et al. 2005; Wynne et al. 2006a) and rodents (Du et al. 2012; Kosinski et al. 2012) does not negatively impact blood glucose homeostasis, indicating that the concomitant glucagon receptor and GLP-1 receptor activity of oxyntomodulin may balance each other out. It has been suggested that in a DIO mouse model the ratio of glucagon receptor activity to GLP-1 receptor activity of a dual agonist needs to be approximately equal to maintain balanced
glycaemia (Day et al. 2012). However, the peptides used in that particular study contained the unnatural amino acid aminoisobutyric acid (AIB) and were PEGylated and it is unclear whether this ratio can be generalised to other analogues and species.

Using current strategies it is unlikely that a dual glucagon/GLP-1 receptor agonist will be developed with sufficient half-life and potency to be administered once a month in man for the treatment of obesity. The addition of large sidechains like PEG or cholesterol is also likely to prevent oxyntomodulin accessing the brain, which has been suggested as a potential site of action (Dakin et al. 2004; Polor Biotech Inc 2012). As an alternative, previous research in this group has developed a slow release formulation that facilitates peptide-zinc chelation and subsequently depot formation, allowing gradual peptide release over an extended period of time. This will be discussed in greater detail in Chapter 4. The aim of this project was to develop a dual analogue of the glucagon receptor and GLP-1 receptor that was compatible with the slow release formulation, with a view to producing a pharmacological obesity therapy with the potential for once a month administration in man. Previous studies have shown that the N-terminal of glucagon is crucial for glucagon receptor binding and activation. The majority of residues in this region are conserved between glucagon, oxyntomodulin, GLP-1 and exendin-4, and substituting the non-conserved residues from GLP-1 into glucagon doesn’t improve GLP-1 receptor efficacy, but has a large negative impact on glucagon receptor efficacy. Oxyntomodulin has lower efficacy and affinity at the glucagon receptor and the GLP-1 receptor compared to the native hormones (Kerr et al. 2010; Kosinski et al. 2012; Santoprete et al. 2011). Therefore it was decided that the basis of the dual analogue would be the amino acid structure of glucagon with conservative substitutions between residues 16-29 to increase GLP-1 receptor activity.

### 3.1.5. Aims

1. To make conservative amino acid substitutions between residues 16-29 of the glucagon sequence in order to increase GLP-1 receptor activity.
2. To produce a dual agonist with increased efficacy at the glucagon receptor and the GLP-1 receptor compared to oxyntomodulin, but with a similar ratio of glucagon receptor to GLP-1 receptor activity.
3. To enhance analogue compatibility with the slow release formulation without compromising glucagon or GLP-1 receptor efficacy.

**For details of the materials and methods used please see Chapter 2. Please see Appendix A for the structures of the analogues described in this chapter.**
3.2. Results

3.2.1. Effect of OXL analogues on human GLP-1 receptor mediated cAMP accumulation

The efficacy of the OXL peptides at the human GLP-1 receptor was investigated in vitro by assessing their ability to stimulate cAMP production in a cell line overexpressing the human GLP-1 receptor. OXL analogue efficacy was compared to native GLP-1 and oxyntomodulin (Figure 3.3. A-E & Table 3.2). At the human GLP-1 receptor, glucagon and oxyntomodulin had 573 and 35 fold lower efficacy than GLP-1 respectively, whereas exendin-4 was as potent as GLP-1 (Figure 3.2 & Table 3.2). OXL1 had similar efficacy to oxyntomodulin (Figure 3.3A) whereas all other OXL analogues had 2 to 13 fold greater efficacy (Figure 3.3). OXL10 and OXL11 were the most potent analogues at the human GLP-1 receptor with only a 3 fold lower efficacy than GLP-1 and exendin-4, and 12 and 13 fold greater efficacy than oxyntomodulin respectively (Figure 3.3E & Table 3.2).

![Graph showing cAMP production](image)

**Figure 3.2:** Effect of native peptides on cAMP accumulation in CHO-K1 cells over-expressing the human GLP-1 receptor.
cAMP was measured after incubation with the endogenous ligands GLP-1, exendin-4, glucagon or oxyntomodulin. Each peptide concentration was tested in duplicate or triplicate in each experiment and mean EC50 values were calculated from a minimum of three separate experiments. Data shown are the mean of at least three separate experiments ± SEM.
Figure 3.3: Effect of OXL analogues on cAMP accumulation in CHO-K1 cells over-expressing the human GLP-1 receptor.

cAMP was measured after incubation with GLP-1, oxyntomodulin or oxyntomodulin-like analogues OXL1 to OXL11. A: OXL1, OXL2, B: OXL3, OXL4, C: OXL5, OXL6, D: OXL7, OXL8, E: OXL9, OXL10, OXL11. Peptide concentration was tested in duplicate or triplicate in each experiment and mean EC50 values were calculated from a minimum of three separate experiments. Data shown are the mean ± SEM. Results are summarised in Table 3.2.2.
3.2.2. Effect of select OXL analogues on rat GLP-1 receptor mediated cAMP accumulation

The OXL analogues OXL10 and OXL11 were the most potent at the human GLP-1 receptor so their \textit{in vitro} efficacy was also investigated at the rat GLP-1 receptor (Figure 3.4 and Table 3.3). At the rat GLP-1 receptor glucagon and oxyntomodulin had 313 and 158 fold lower efficacy respectively compared to GLP-1. OXL10 and OXL11 were respectively 20 and 11 times less potent than GLP-1 at the rat GLP-1 receptor, however they remained more potent than oxyntomodulin by 8 and 14 fold respectively.

Figure 3.4: Effect of native peptides and analogues OXL10 and OXL11 on cAMP accumulation in CHL cells over-expressing the rat GLP-1 receptor.  
cAMP was measured after incubation with GLP-1, oxyntomodulin or oxyntomodulin-like analogues OXL10 or OXL11. Peptide concentrations were tested in duplicate or triplicate in each experiment and mean EC_{50} values were calculated from a minimum of three separate experiments. Data shown are the mean ± SEM.
3.2.3. Affinity of select OXL analogues at the human, rat and mouse GLP-1 receptors

The affinity of the most potent OXL analogues OXL10 and OXL11 was investigated at the human (Figure 3.5), rat and mouse GLP-1 receptors and compared to the affinity of the natural ligands (Table 3.1). Receptor binding affinity differed between the human, rat and mouse receptors; however glucagon consistently had the poorest binding affinity. The OXL analogues tested had greater binding affinity than oxyntomodulin at all three receptors; ranging from 4-7 fold greater for OXL10 and 10-14 fold greater for OXL11.

![Graph showing receptor binding affinity](image)

Figure 3.5: Competitive receptor binding affinity of the native peptides versus $^{125}$I-GLP-1 at the human GLP-1 receptor.

Purified cell membranes of CHO cells overexpressing the human GLP-1 receptor were incubated with $^{125}$I-GLP-1 in the presence of increasing concentrations of unlabelled GLP-1, oxyntomodulin, OXL11 or OXL10. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment and mean IC$_{50}$ values were calculated from a minimum of 3 separate experiments. Data shown are the mean ± SEM.
**Table 3.1:** Summary of mean IC₅₀ values for the native peptides and OXL analogues at the human, rat and mouse GLP-1 receptors.

Purified cell membranes of CHO cells overexpressing the human or rat GLP-1 receptors, or purified mouse lung tissue were incubated with ¹²⁵I-GLP-1 in the presence of increasing concentrations of unlabelled GLP-1, oxyntomodulin, exendin-4, glucagon, OXL11 or OXL10. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment. IC₅₀ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue IC₅₀/GLP-1 IC₅₀.

<table>
<thead>
<tr>
<th></th>
<th>human GLP-1r</th>
<th>Ratio to native peptide</th>
<th>rat GLP-1r</th>
<th>Ratio to native peptide</th>
<th>mouse GLP-1r</th>
<th>Ratio to native peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>0.42 ± 0.02</td>
<td>1.0</td>
<td>0.18 ± 0.04</td>
<td>1.0</td>
<td>0.18 ± 0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>Exendin</td>
<td>0.53 ± 0.10</td>
<td>1.3</td>
<td>0.12 ± 0.07</td>
<td>0.7</td>
<td>0.31 ± 0.05</td>
<td>1.7</td>
</tr>
<tr>
<td>Glucagon</td>
<td>733 ± 54</td>
<td>1761</td>
<td>827 ± 27</td>
<td>4694</td>
<td>659 ± 23</td>
<td>3709</td>
</tr>
<tr>
<td>OXM</td>
<td>142 ± 18</td>
<td>340</td>
<td>355 ± 46</td>
<td>2016</td>
<td>279 ± 56</td>
<td>1571</td>
</tr>
<tr>
<td>OXL10</td>
<td>32 ± 10</td>
<td>78</td>
<td>68 ± 16</td>
<td>384</td>
<td>40 ± 6</td>
<td>225</td>
</tr>
<tr>
<td>OXL11</td>
<td>9.9 ± 2.5</td>
<td>24</td>
<td>33 ± 6</td>
<td>188</td>
<td>20 ± 4</td>
<td>110</td>
</tr>
</tbody>
</table>

3.2.4. Effect of OXL analogues on human glucagon receptor mediated cAMP accumulation

The efficacy of the OXL peptides at the human glucagon receptor was investigated in vitro by assessing their ability to stimulate cAMP production in a cell line overexpressing the human glucagon receptor. OXL analogue EC₅₀’s were compared to EC₅₀’s for native glucagon and oxyntomodulin to assess their relative bioactivity (Figure 3.7. A-E & Table 3.2). At the human glucagon receptor, the efficacy of oxyntomodulin was 11 fold lower than glucagon (Figure 3.6 & Table 3.2). Exendin-4 did not stimulate cAMP production in this cell line at a maximum concentration of 3000nM (Figure 3.6). All OXL analogues were more potent at the human glucagon receptor than oxyntomodulin; ranging from 5-22 fold more efficacious at stimulating cAMP production. OXL6, OXL10 and OXL11 were of similar potency as glucagon and OXL7 was 2 fold more potent (Figure 3.7C, E & D). At the human receptors oxyntomodulin, OXL10 and OXL11 had 3 times more activity at the glucagon receptor than the GLP-1 receptor (Table 3.2).
Figure 3.6: Effect of native peptides on cAMP accumulation in CHO-K1 cells over-expressing the human glucagon receptor.

cAMP was measured after incubation with glucagon, oxyntomodulin, GLP-1 or exendin-4. Each peptide concentration was tested in duplicate or triplicate in each experiment and mean EC₅₀ values were calculated from a minimum of three separate experiments. Data shown are the mean of at least three separate experiments ± SEM.
Figure 3.7: Effect of OXL analogues on cAMP accumulation in CHO-K1 cells over-expressing the human glucagon receptor.
cAMP was measured after incubation with glucagon, oxyntomodulin or oxyntomodulin-like analogues OXL1 to OXL11. A: OXL1, OXL2, B: OXL3, OXL4, C: OXL5, OXL6, D: OXL7, OXL8, E: OXL9, OXL10, OXL11. Each peptide concentration was tested in duplicate or triplicate in each experiment and mean EC<sub>50</sub> values were calculated from a minimum of three separate experiments. Data shown are the mean ± SEM.
Table 3.2: Summary of the mean EC$_{50}$ values for the native peptides and OXL analogues at the human glucagon receptor and GLP-1 receptor.

CHO-K1 cells over-expressing either the human glucagon receptor or human GLP-1 receptor were incubated for 30 minutes with a range of peptide concentrations. Cells were lysed and intracellular cAMP levels quantified by ELISA. EC$_{50}$ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC$_{50}$/native peptide EC$_{50}$. ND – not determined.

### 3.2.5. Effect of select OXL analogues on rat glucagon receptor mediated cAMP accumulation

The *in vitro* efficacy of OXL10 and OXL11 was also investigated at the rat glucagon receptor and compared to the efficacy of the native ligands (Figure 3.8 and Table 3.3). At the rat glucagon receptor oxyntomodulin was 41 fold less potent than glucagon. The potency of OXL11 was similar to that of glucagon at the rat glucagon receptor, whereas OXL10 was twice as efficacious at stimulating cAMP production. At the rat receptor, oxyntomodulin had approximately 4 times greater activity at the glucagon receptor than the GLP-1 receptor, whereas OXL10 had 40 times greater activity at the glucagon receptor and OXL11 had 11 times more activity at the glucagon receptor compared to the GLP-1 receptor (Table 3.3).
Figure 3.8: Effect of native peptides and analogues OXL10 and OXL11 on cAMP accumulation in CHO-K1 cells over-expressing the rat glucagon receptor.

cAMP was measured following incubation with glucagon, oxyntomodulin or analogues OXL10 and OXL11. Peptide concentrations were tested in duplicate or triplicate in each experiment and mean EC$_{50}$ values were calculated from a minimum of three separate experiments. Data shown are the mean ± SEM.

Table 3.3: Summary of the mean EC$_{50}$ values for the native peptides and analogues OXL10 & OXL11 at the rat glucagon receptor and GLP-1 receptor.

CHO-K1 cells over-expressing the rat glucagon receptor or CHL cells overexpressing the rat GLP-1 receptor were incubated for 30 minutes with a range of peptide concentrations. Cells were lysed and intracellular cAMP levels quantified by ELISA. EC$_{50}$ values are the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC$_{50}$/native peptide EC$_{50}$.
3.2.6. Affinity of select OXL analogues at the human, rat and mouse glucagon receptors

The affinity of the most potent OXL analogues OXL10 and OXL11 was investigated at the human (Figure 3.9) rat and mouse glucagon receptors and compared to the affinity of the natural ligands (Table 3.4). Receptor binding affinity was similar between the human, rat and mouse receptors. IC\textsubscript{50}'s for exendin-4 and GLP-1 could not be calculated as sufficient displacement of \textsuperscript{125}I-glucagon could not be achieved at the highest peptide concentration of 5000nM. Across the 3 receptors OXL10 and OXL11 had an average of 5-6 fold greater affinity for the glucagon receptor than oxyntomodulin.

![Graph showing receptor binding affinity for OXL10 and OXL11](image_url)

Figure 3.9: Competitive receptor binding affinity for the native peptides and analogues OXL10 & OXL11 versus \textsuperscript{125}I-glucagon at the human glucagon receptor.

Purified cell membranes of CHO cells overexpressing the human glucagon receptor were incubated with \textsuperscript{125}I-glucagon in the presence of increasing concentrations of unlabelled glucagon, oxyntomodulin, OXL10 or OXL11. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment and mean IC\textsubscript{50} values were calculated from a minimum of 3 separate experiments. Data shown are the mean ± SEM.
Table 3.4: Summary of mean IC50 values for native peptides and analogues OXL10 & OXL11 at the human, rat and mouse glucagon receptors.

Purified cell membranes of CHO cells overexpressing the human or rat glucagon receptors, or purified mouse liver tissue were incubated with 125I-glucagon in the presence of increasing concentrations of unlabelled glucagon, GLP-1, oxyntomodulin, exendin-4, OXL10 or OXL11. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment. IC50 values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue IC50/glucagon IC50.

### 3.2.7. Effect of OXL analogues on acute food intake in fasted mice

Single SC administration of glucagon and oxyntomodulin at all doses tested significantly reduced food intake in fasted C57BL/6 mice in the first 30 minutes post injection compared to vehicle treated animals. Only the 1000nmolkg⁻¹ doses of glucagon (P<0.01) and oxyntomodulin (P<0.001) continued to significantly inhibit food intake during the 1-2 hour time period and food intake was not significantly reduced beyond this time period for any of the groups (Figure 3.10).
Except for 0.3nmolkg\(^{-1}\) exendin-4 all doses of GLP-1 and exendin-4 tested significantly reduced food intake during the first hour following injection. All 4 doses of exendin-4 significantly inhibited food intake during the 1-2 hour time period as did the 300nmolkg\(^{-1}\) and 1000nmolkg\(^{-1}\) doses of GLP-1. The 1000nmolkg\(^{-1}\) dose of GLP-1 (\(P < 0.01\)) and the 3nmolkg\(^{-1}\) and 10nmolkg\(^{-1}\) doses of exendin-4 (\(P < 0.001\)) continued to significantly reduce food intake in the 2-4 and 4-8 hour time periods. Only the 10nmolkg\(^{-1}\) dose of exendin-4 continued to inhibit food intake between 8-12 hours post injection (\(P < 0.05\); Figure 3.11).

Figure 3.11: Effect of GLP-1 and exendin-4 on acute food intake in mice.
Acute food intake in overnight fasted C57BL/6 mice following a single SC injection of various doses of GLP-1 or exendin-4. Data shown as the mean percentage food intake compared to vehicle ± SEM. \(n=7-8\) mice per group. Statistical analysis carried out using one way ANOVA and post hoc tests with Dunnett correction, *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) compared to vehicle controls.

OXL analogues OXL1 and OXL2 significantly decreased food intake compared to vehicle controls during the first hour post injection only (\(P < 0.001\); Figure 3.12). All other OXL analogues also significantly inhibited food intake during the 0-1 hour time period (Figure 3.13 & Figure 3.14). OXL3, OXL4, OXL5, OXL6, OXL7 and OXL8 reduced food intake up to 4 hours post injection. OXL7 significantly inhibited food intake up to 8 hours (\(P < 0.001\)) and OXL8 continued to reduce food intake up to 24 hours post injection (\(P < 0.001\); Figure 3.13). Although OXL9, OXL10 and OXL11 did not significantly reduce food intake during the 2-4 hour time period, food intake was significantly reduced during the 4-8 hour period compared to vehicle controls (\(P < 0.05\); Figure 3.14).
Figure 3.12: Effect of OXL1 and OXL2 on acute food intake in mice. Acute food intake in overnight fasted C57BL/6 mice following a single SC injection of 200nmolkg\(^{-1}\) OXL1 or OXL2 in slow release formulation. Data shown as the mean percentage food intake compared to vehicle ± SEM. n = 7-8 mice per group. Statistical analysis carried out using one way ANOVA and post hoc tests with Dunnett correction, ***P < 0.001 compared to vehicle controls.

Figure 3.13: Effect of OXL analogues on acute food intake in mice. Acute food intake in overnight fasted C57BL/6 mice following a single SC injection of 200nmolkg\(^{-1}\) of analogues OXL3 TO OXL8 in slow release formulation. Data shown as the mean percentage food intake compared to vehicle ± SEM. n = 9-10 mice per group. Statistical analysis carried out using one way ANOVA and post hoc tests with Dunnett correction, *P < 0.05, **P < 0.01, ***P < 0.001 compared to vehicle controls.
3.2.8. The effect of amino acid substitutions on pharmacokinetic parameters of OXL analogues

Measuring plasma peptide levels after administration of a single 1mg SC injection to rats enabled the comparison of several pharmacokinetic parameters (Table 3.5). A subset of peptides including OXL1, OXL2 (Figure 3.15 A), OXL3 and OXL7 had an early spike in plasma peptide levels with a $T_{\text{max}}$ of 0.5-3 hours and peptide was undetectable beyond 2-3 days post injection.

The remaining peptides tested reached maximum plasma concentrations, $T_{\text{max}}$ at a later timepoint, and levels remained elevated for an extended period. In particular, OXL6 plasma levels did not fall below half of the peak concentration, ($T_{1/2\text{max}}$) by the final plasma sampling on day 7 (Figure 3.15 B) and 39% of the peak concentration of OXL5 remained in circulation at day 7.

Plasma levels of OXL4, OXL8, OXL9, OXL10 and OXL11 remained detectable for up to 10 days, with a small percentage of the peak levels remaining. OXL10 levels remained at 99% of the peak concentration at 4 days but were not detectable at 7 days post injection (Figure 3.15 C). Plasma levels of OXL11 remained detectable at 10 days post injection with 19% of the peak concentration present (Figure 3.15 D).
Table 3.5: Summary of pharmacokinetic parameters of OXL analogues.

<table>
<thead>
<tr>
<th></th>
<th>Time post injection (hours)</th>
<th>% $C_{\text{max}}$ remaining at $T = 168$</th>
<th>% $C_{\text{max}}$ remaining at $T = 240$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXL1</td>
<td>0.5 14 0 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL2</td>
<td>3 30 0 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL3</td>
<td>3 15 0 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL4</td>
<td>3 48 11 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL5</td>
<td>24 77 39 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL6</td>
<td>3 &gt;168 61 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL7</td>
<td>0.5 2 0 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL8</td>
<td>3 31 4 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL9</td>
<td>3 43 9 -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL10</td>
<td>24 129 0 0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL11</td>
<td>3 63 17 19*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Male Wistar rats were given a single SC injection of 1mg peptide, injection volume 20µl, formulated in slow release diluent. Peptides were measured in rat plasma using RIA. $T_{\text{max}}$ = sampling time with the maximum peptide concentration; $T_{\frac{1}{2}\text{max}}$ = time of $\frac{1}{2}$ maximum concentration; %$C_{\text{max}}$ = percentage of the maximum plasma peptide concentration remaining at $T = 168$ or $T = 240$. n = 4 animals/group, data was calculated using a line graph and mean group plasma peptide levels at each time point. *Studies with the final samples taken on day 10 (240 hours), the rest had final samples taken on day 7 (168 hours).

Figure 3.15: Line graphs illustrating the pharmacokinetic profile of four OXL analogues.

Plasma peptide levels after a single SC injection of 1mg peptide, injection volume 20µl, formulated in slow release diluent. Peptides were measured in rat plasma using RIA. A) OXL2, B) OXL6, C) OXL10, D) OXL11. n = 4 animals/group, data shown is the mean plasma peptide level ± SEM.
3.2.9. Effect of chronic administration of select OXL analogues on energy homeostasis in rats

Daily SC administration of 50nmolkg\(^{-1}\) OXL10 and OXL11 in slow release formulation to rats significantly decreased bodyweight from day 2 compared to both vehicle controls and respective pair-fed groups \((P<0.001; \text{Figure 3.16 A})\). By final study day 7 there was also a significant reduction in bodyweight in the pair-fed groups compared to vehicle controls (OXL11 pair-fed: \(P<0.01\), OXL10 pair-fed: \(P<0.001\); Figure 3.16 A).

Daily administration of OXL10 and OXL11 resulted in a reduction of food intake from day 2 of administration which reached significance, compared to vehicle controls, from day 5 onwards \((P<0.001)\). Food intake did not differ between the peptide treatment groups and their respective pair-fed controls (Figure 3.16 B).
Figure 3.16: Effect of chronic OXL10 and OXL11 administration on food intake and bodyweight in Wistar rats.

Bodyweight change A) and food intake B) after daily SC injections of 50nmolkg\(^{-1}\) OXL10 or OXL11 in slow release formulation. Pair-fed groups were given a weighed amount of chow equivalent to the mean amount eaten in the previous 24 hours by the animals in the corresponding treatment group. Daily injections, food and bodyweight measurements and feeding of the paired groups were carried out at 4.30pm. \(n = 10\)/group, mean initial bodyweight = 666g. Data shown as the mean ± SEM. Statistical analysis carried out using two-way repeated measures ANOVA and post hoc tests with Bonferroni correction, \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.001\) versus vehicle control; \(\#P < 0.01\), \(\###P < 0.001\) versus corresponding pair-fed group.
3.3. Discussion

The aim of the work detailed in this chapter was to develop a dual glucagon and GLP-1 receptor agonist with increased potency at both receptors compared to oxyntomodulin. The analogue was also required to be compatible with a novel slow release formulation in order to have a prolonged duration of action, with the ultimate aim of producing a once monthly pharmacological treatment for obesity. The structure of the naturally occurring dual agonist of the glucagon and GLP-1 receptors, oxyntomodulin, is the 29 amino acids of glucagon with an octapeptide ‘tail’ (Bataille et al. 1982). However, the structural basis for the development of the dual agonists described in this chapter was the 29 amino acids of glucagon rather than oxyntomodulin, due to the reduced efficacy of oxyntomodulin at the glucagon receptor compared to native glucagon (Kerr et al. 2010; Santoprete et al. 2011; Kosinski et al. 2012). The N-terminal of glucagon is critical for glucagon receptor binding and activation (Hjorth et al. 1994), therefore amino acid substitutions were only made from residue 16 onwards. In vivo and in vitro methods were used to assess the receptor efficacy of the oxyntomodulin-like dual analogues and their effect on food intake and bodyweight, in order to identify a potent peptide with a longer half-life.

Several experimental parameters were measured to assess whether introducing key residues from GLP-1 and exendin-4 into the C-terminal of glucagon were effective. It is important to consider several properties in order to correctly interpret the data for each analogue. For example, acute food intake inhibition was monitored after a single analogue dose in rodents; however, because peptides were administered in the long acting formulation, the in vivo food intake data provides information about the combined effects of GLP-1 receptor activity and peptide pharmacokinetics. For example, a peptide with poor activity at the GLP-1 receptor but a long T½ could produce a similar in vivo effect on food intake as a more potent peptide with a shorter T½. Therefore the effects of amino acid substitutions on the pharmacokinetic parameters of analogues were also assessed. GLP-1 and exendin-4 are potent satiety inducing agents, whereas glucagon requires larger doses to generate a small transient reduction in food intake. All three peptides have short in vivo half-lives, although that of exendin-4 is slightly longer than GLP-1; approximately the same as the glomerular filtration rate (Parkes et al. 2001b). The ideal peptide would slowly reach a sustained maximum blood level, preventing nausea that is often associated with rapid increases in concentrations of satiety factors in man (Fineman et al. 2004).

A further example of where multiple data sets are required to interpret efficacy is when predicting whether results seen in rodents are likely to be translated in to man; therefore a cell based bioactivity assay quantifying cAMP production was employed to measure analogue activity at the
human glucagon and GLP-1 receptors. There was not expected to be a difference in activity between the human and rat glucagon and GLP-1 receptors, due to the high degree of homology, however the cAMP assays were also conducted using cells expressing the rat receptors to confirm this, aiding interpretation of in vivo results. Receptor binding assays enabled investigation of analogue affinity at the mouse glucagon and GLP-1 receptors as well as rat and human, to provide in vitro comparison to in vivo food intake inhibition data. Unlike the cAMP assays, receptor binding assays are unable to provide information on peptide efficacy or distinguish between agonists and antagonists, as both can bind to the receptor equally well. However, due to the absence of cell lines expressing the mouse glucagon and GLP-1 receptors that could be used in an in vitro bioactivity assay, it was necessary to perform receptor binding studies using membranes prepared from mouse liver and lung tissue. While this is not a perfect comparison, as binding does not always correlate with activity, it does provide a bridge to compare across species. Additionally, receptor binding and activity assays often don’t correlate with in vivo activity, in this case effects on food intake and body weight. In vivo, peptides are exposed to enzymes and renal filtration which can rapidly reduce the amount of peptide available for binding to and activating the receptor.

There was a good correlation between activity and binding ratios for both the human and rat glucagon receptors; although oxyntomodulin activity was 4 fold lower at the rat glucagon receptor compared to the human. There are currently no published studies that measure the activity of oxyntomodulin at the glucagon receptor in more than one species; therefore it is unclear whether this reduced activity at the rat glucagon receptor is consistent with previous observations. Surprisingly, the binding ratio for oxyntomodulin at the rat glucagon receptor was approximately 2 fold greater than at the human receptor, which is better than expected considering its poorer activity ratio at the rat glucagon receptor. However it is well known that binding and activity do not necessarily correlate, for example several potent glucagon antagonists have been developed that retain high binding affinity but have little or no activity at the glucagon receptor (Unson et al. 1987; Azizeh et al. 1996; Ahn et al. 2001b). There was a good correlation between receptor activation and binding for the OXL10 and OXL11 at both the human and rat glucagon receptors. Additionally the binding ratios for these analogues and oxyntomodulin were similar across the human, mouse and rat glucagon receptors.

On the other hand, the ratios for GLP-1 receptor activity and binding were much more inconsistent across species. Activity ratios at the human GLP-1 receptor were on average 5 fold higher for oxyntomodulin, OXL10 and OXL11 compared to at the rat receptor. Binding ratios at the GLP-1 receptor were relatively consistent between the rat and mouse receptors; however on average they
were 5 fold better at the human GLP-1 receptor. The mouse and rat GLP-1 receptors are 88% homologous with the human receptor, whereas they share 97% homology with each other (Moon et al. 2012), which could be a possible reason for the differences in receptor activities and why the binding ratios were more consistent between the mouse and rat GLP-1 receptors.

As with the glucagon receptor, there are no previous reports comparing oxyntomodulin activity or binding at the rat, mouse and human GLP-1 receptors, making it difficult to compare the current findings to the wider research environment. Compared to OXL11 and OXL10, oxyntomodulin had the lowest receptor affinity across all 3 species tested and OXL11 had the highest, despite the differences in the actual ratio values between species. Similarly, the analogues had greater efficacy than oxyntomodulin at the GLP-1 receptor. The relative efficacy of the peptides differed slightly between the human and rat GLP-1 receptors. Analogue OXL11 had a 2 fold greater efficacy at the rat GLP-1 receptor compared to OXL10, whereas they had similar efficacies at the human receptor. However it is unclear whether a 2 fold or 5 fold difference in in vitro binding or activity would translate into a measurable difference in biological effect in vivo. Overall, in vitro data indicates that the substitution of residues from GLP-1 and exendin-4 into the glucagon sequence increased the GLP-1 receptor affinity and efficacy of glucagon beyond that seen with oxyntomodulin and this was consistent across species. Furthermore glucagon receptor affinity and efficacy was maintained.

As previously discussed (Pg. 53), glucagon administration can increase energy expenditure. To ensure that in vivo glucagon activity had not been diminished by residue substitutions, a pair feeding study was conducted in rats to determine the effects of OXL10 and OXL11 on energy expenditure. Bodyweights of animals given daily analogue injections were compared to those in a ‘pair-fed’ matched group who ate the same amount of food but received vehicle injections. Any extra weight lost by the analogue-treated animals was attributed to increased energy expenditure. Pair-feeding is a commonly employed technique used to control for the effect a reduction in food intake may have on an experimental outcome (Hau & Schapiro. 2010). In the experiments I conducted, it separates weight loss due to a reduction in food intake from weight loss due to an increase in energy expenditure. The results indicate that OXL analogues OXL10 and OXL11 increased energy expenditure after chronic administration in a rat model, thus suggesting that sufficient glucagon receptor activity had been retained. It was not possible to use native glucagon as a control in this experiment due to its very short half-life. The pair-feeding paradigm has limitations, for example it does not control for any weight loss due to increased urine output. Therefore care is needed when drawing conclusions from these studies, and confirmation of increased energy expenditure is desirable by other experimental approaches.
There were several reasons for investigating the specific residue substitutions detailed in this chapter for the development of a dual glucagon GLP-1 receptor agonist. The initial substitution of leucine for methionine at position 27 was related to the practicalities of developing and distributing the analogue as a potential pharmacological agent. The sulphur containing R-group of methionine is readily oxidised to methionine sulphoxide (Stadtman et al. 2003) which would require any drug produced containing methionine to be stored in the complete absence of oxygen. This is not practical; therefore the structurally similar amino acid leucine was substituted at this position. The hydrophobic nature of this residue was also maintained by the use of leucine, which is important as it falls within the conserved hydrophobic region consisting of residues 22-27 of glucagon and GLP-1 and 22-26 of exendin-4 (see Appendix A, Figure 7.3). The results demonstrate that Leu$^{27}$ substitution substantially increased human GLP-1 receptor activity by 21 fold compared to glucagon. Position 27 is the terminal residue of the helix in GLP-1 and the valine observed at this position in the native peptide has been suggested to interact with several receptor amino acid residues via a backbone carbonyl hydrogen bond and hydrophobic sidechain contacts (Underwood et al. 2010). Leucine has a similar sidechain to valine which could facilitate these interactions and enhance the GLP-1 receptor binding of the glucagon based analogue and may explain the observed increase in receptor activity.

Both GLP-1 and exendin-4 are amidated at the C-terminal, along with a large number of other biological peptides (Kim & Seong 2001). In line with previous findings (Day et al. 2009) the addition of a C-terminal amide group to the glucagon based analogue (OXL2) improved human GLP-1 receptor activity 5 fold but it also generated a 2 fold reduction in glucagon receptor activity. Although these two substitutions resulted in increased activity at the human GLP-1 receptor compared to glucagon, there was no observed improvement in acute food intake reduction in mice with either OXL1 or OXL2. However this could be due to several reasons. Firstly, despite respective 20 and 120 fold increases in GLP-1 receptor activity compared to glucagon, their activity was still similar to that of oxyntomodulin. Also, the analogues compatibility with the long acting formulation had not been enhanced by the addition of histidine and glutamate residues; therefore they would not necessarily be able to form a good depot, as evidenced by their poor pharmacokinetic profiles (Table 3.5 and Figure 3.15 A). Consequently clearance from the circulation, and hence their effect on food intake, would be of a very short duration. Additionally, analogue activity was measured at the human rather than the mouse receptor and, as previously mentioned; receptor binding affinities differ between the mouse and human GLP-1 receptors.

Histidine residues were added to the analogue C-terminal to increase compatibility with the long acting formulation and enhance depot formation. Glutamate residues from GLP-1 and exendin-4
were specifically chosen to be substituted into the glucagon sequence to increase GLP-1 receptor activity because glutamate also interacts well with the long acting formulation and benefits depot formation. Additionally, there are a number of hydrophobic residues in the C-terminal of glucagon, GLP-1 and exendin-4 which form conserved patches; glutamate is hydrophilic, therefore substituting glutamate into the glucagon sequence can increase the amphiphilic nature of the analogue. The greater amphiphilic nature of exendin-4 has been suggested to stabilise its helical structure and enhance binding to the GLP-1 receptor N-terminal (Runge et al. 2008; Underwood et al. 2010). Glutamate can also form intramolecular interactions with amino acids arginine and lysine which can further stabilise the peptide α-helix and improve receptor binding (Sturm et al. 1998; Ahn et al. 2001c; Runge et al. 2008; Underwood et al. 2010). Isoleucine at position 23 of GLP-1 has previously been shown to be a critical residue for receptor binding and activity (Adelhorst et al. 1994; Gallwitz et al. 1994; Underwood et al. 2010). It is part of the conserved hydrophobic receptor binding face and therefore is also present in exendin-4. Similarly glycine is present at position 29 in both GLP-1 and exendin-4. Although not part of the peptide helix which directly interacts with the receptor, the flexible nature of glycine may allow optimal orientation of the terminal NH₂ of GLP-1 or the formation of the disputed TRP cage of exendin-4 (Neidigh et al. 2001). Threonine at residue 29 of glucagon was suggested to be important for optimal glucagon receptor binding but had little effect on activity (Frandsen et al. 1981; England et al. 1982), therefore Gly²⁹ was substituted into the analogue in order to increase C-terminal flexibility and aid the interaction of the histidine ‘tail’ with the long acting formulation and enhance depot formation. Glucagon has a high tendency to form fibrils in solution but reducing the β-sheet potential and substituting hydrophobic residues, in particular Val²³ and Met²⁷, can dramatically decelerate the rate of fibrillation and improve solubility (Pedersen et al. 2006).

The substitution or addition of histidine at residues 20, 30 and 31 (OXL3, OXL4 and OXL6 respectively) had very little effect on human GLP-1 or glucagon receptor activity, with the exception of His³¹ which increased glucagon receptor activity by approximately 2 fold. It is unclear from the current literature why the addition of His³¹ would increase glucagon receptor activity, although oxyntomodulin also has positively charged residues at positions 30 and 31. As predicted, the addition of histidines to the analogue sequence improved the pharmacokinetic profile measured in rat and also significantly extended the length of time that food intake was reduced in mice. Despite the improved pharmacokinetic profile of analogue OXL6, with 60% of the 3 hour peak concentration remaining in circulation at 7 days, acute food intake reduction remained similar to OXL4. Depot formation is dependent upon peptide concentration and injection volume (Mrsny & Daugherty. 2009) and both of these variables differed in the PK and food intake studies; therefore it should not
be expected that the results of the two studies will correlate. Also, although in vitro receptor binding results suggested that rat and mouse responses to analogues were similar, there may be differences in vivo; for example peptides may be filtered by the kidneys at different rates in rats and mice, or there may be a difference in exposure to peptidases (Gillette 1971; Litterst et al. 1975).

The substitution of specific residues from GLP-1 and exendin-4 to the corresponding positions in the glucagon sequence generally increased human GLP-1 receptor activity in vitro without negatively affecting human glucagon receptor activity. Interestingly, several such substitutions also improved in vitro human glucagon receptor activity. In particular, substituting glutamate at position 21 in OXL7 increased glucagon receptor activity by approximately 2 fold compared to native glucagon (Table 3.2). Glutamate has the same charge but a greater α-helical propensity than aspartate (Chou & Fasman 1975), which is the amino acid at position 21 in native glucagon. This could explain the increase in both GLP-1 and glucagon receptor activity with Glu21, as it has previously been shown that increasing the helical propensity of the C-terminal helix increases receptor binding and activity of both native peptides at their respective receptors (Krstenansky et al. 1986; Murage et al. 2008), and Glu21 in particular increases glucagon receptor binding and activity (Ahn et al. 2001c). Additionally, it is possible that intramolecular interactions between Glu21 and Arg17 or Arg18 in analogue OXL7 contributed to C-terminal helix stability and the increased efficacy of this particular analogue at both the glucagon and GLP-1 receptors (Krstenansky et al. 1986). In this instance increased in vitro receptor activity corresponded with a greater percentage reduction in food intake in vivo with analogue OXL7 administration. The reduction in food intake was also extended into the 4-8 hour time period (Figure 3.13). Interestingly, the pharmacokinetic profile of OXL7 was one of the poorest of all the analogues (Table 3.5); therefore the extension of food intake reduction was likely due to a large immediate release of peptide due to poor depot formation, combined with the increased potency of the analogue. It is possible that Glu21 substitution altered the helical structure in such a way that one or more of the glutamate or histidine residues required for interaction with the long acting formulation were less accessible, therefore reducing depot formation and producing the poor PK profile for OXL7 compared to the other analogues.

Glutamate substitutions at residues 16 (OXL8) and 24 (OXL5) both had a negative impact on in vitro GLP-1 receptor activity. Glutamate is present at these positions in exendin-4 whereas GLP-1 has glycine and alanine respectively. The Ala24 residue is part of the hydrophobic face of GLP-1 consisting of residues 22-27 which interacts with the receptor, although Ala24 has not been shown to specifically interact with receptor residues (Underwood et al. 2010). Glucagon also has a hydrophobic patch between residues 22-27. Glutamate is hydrophilic so the substitutions at residues
16 and 24 increase the amphiphilic nature of the C-terminal helix of the analogues. The more pronounced amphiphilic character of exendin-4 has been suggested to strengthen and stabilise the C-terminal α-helix and contribute to its greater binding propensity at the N-terminal of the GLP-1 receptor (Runge et al. 2008; Underwood et al. 2010). Therefore it seems somewhat anomalous that substituting glutamate at analogue positions 16 and 24 generated a reduction in in vitro human GLP-1 receptor activity. However Glu\textsuperscript{16} and Glu\textsuperscript{24} are also postulated to stabilise the helix of exendin-4 via intramolecular interactions with Arg\textsuperscript{20} and Lys\textsuperscript{27} (Underwood et al. 2010), neither of which were present in the analogues. Additionally it has been suggested that GLP-1 and exendin-4 may bind to the receptor in a slightly different manner and that the less rigid Gly\textsuperscript{16} is beneficial for GLP-1 binding, perhaps by allowing the specific change in conformation necessary for the postulated second step of the binding process (Underwood et al. 2010). Thus it is likely that helix stability was increased by the residue 16 and 24 substitutions, but in a conformation less conducive for GLP-1 receptor binding or activation.

The Glu\textsuperscript{16} substitution in analogue OXL8 also resulted in a 4 fold reduction in human glucagon receptor activity which is in contrast to previous findings (Day et al. 2009). However, this substitution was previously made in glucagon amide and the current analogue had several other additional substitutions. It has previously been shown that flexibility is required between residues 10-15 to enable agonist activity at the glucagon receptor (Ahn et al. 2001c; Sapse et al. 2002); thus one can hypothesise that intramolecular interactions between the side chains of Lys\textsuperscript{12} and the substituted Glu\textsuperscript{16} stabilised a helical conformation in this region resulting in reduced activity at the glucagon receptor. However further analysis using NMR or circular dichroism would be required to investigate whether helicity was increased in this region in the analogue OXL8. Although they had reduced in vitro efficacy, both OXL5 and OXL8 had good pharmacokinetic parameters (Table 3.5) and produced a sustained reduction in food intake in vivo (Figure 3.13).

When designing analogues, increasing helical structure in the C-terminal region was a primary aim to improve GLP-1 receptor activity. Previous studies have shown that substituting Lys\textsuperscript{17} and Lys\textsuperscript{18} into glucagon in conjunction with Glu\textsuperscript{21} results in the formation of superagonists due to a substantial increase in α-helical content (Krstenansky et al. 1988; Sturm et al. 1998; Ahn et al. 2001c). Thus lysine was substituted into position 17 in analogue OXL9 resulting in a 2.6 fold increase in activity at the human GLP-1 receptor compared to OXL8. Interestingly, there was no improvement in glucagon receptor activity with this analogue.
Isoleucine at position 23 is part of the conserved hydrophobic region in both GLP-1 and exendin-4 and is a critical residue for GLP-1 receptor binding (Adelhorst et al. 1994; Gallwitz et al. 1994), although its importance lies in secondary structure formation rather than direct receptor interaction (Adelhorst et al. 1994). Substituting Ile\textsuperscript{23} into OXL10 increased activity at the human GLP-1 receptor 2.3 fold and also resulted in a 2 fold increase in glucagon receptor activity compared to OXL9. Previously this particular substitution had been reported to have little effect on activity at either receptor (Day et al. 2009). However, the current analogue had several additional substitutions compared to the analogue in the previous study therefore their secondary structures are likely different, hence the dissimilar response to Ile\textsuperscript{23} substitution. Much of the work investigating glucagon structure-function has focused on the N-terminal region due to the interest in producing glucagon receptor antagonists for the potential treatment of T2DM; subsequently the importance of specific C-terminal residues for glucagon receptor binding and activity is unclear.

3.3.1. Conclusion

Although there is an apparent lack of correlation between the various in vivo and in vitro studies, by comparing the analogues with each other under the different experimental conditions, it is possible to assess the overall contribution of amino acid substitutions to analogue efficacy. The results detailed in this chapter demonstrate that the dual analogues OXL10 and OXL11 have increased efficacy at the human and rat GLP-1 receptors compared to oxyntomodulin and they also prolong acute food intake reduction in mice. Glucagon receptor efficacy is maintained both in vitro at the human and rat receptors and in vivo in the rat. Both peptides have an extended pharmacokinetic profile compared to OXL1 as measured in a rat model. However the pharmacokinetic profiles of OXL10 and OXL11 differ slightly; OXL10 takes longer to reach peak concentration but plasma levels of OXL11 are still measurable at 10 days post injection. Due to these differences it was decided that both OXL10 and OXL11 would be used as the initial peptides for the second generation analogue development which aimed to further extend the length of time the peptide was present in the circulation.
Chapter 4

C-terminal amino acid extensions to OXL analogues: Assessment of an optimised dual agonist as a potential obesity therapy
4.1. Introduction

4.1.1. Slow release formulation

Rapid changes in blood levels of anorectic gut hormones, for example GLP-1 and PYY, are associated with nausea and vomiting (Fineman et al. 2004; Sloth et al. 2007; Astrup et al. 2009). Therefore the ideal dual agonist of the glucagon and GLP-1 receptors would slowly reach a sustained maximum blood level, allowing once monthly administration for the treatment of obesity in humans. One method of achieving this is the formation a peptide depot. Previous research in our group has developed a slow release peptide formulation using zinc chloride (ZnCl₂). The peptide-zinc combination forms a depot when administered subcutaneously and slowly releases peptide as it is broken down by the body over a period of time.

Zinc plays a crucial role in vivo in the formation of endogenous insulin hexamers that enable crystalline insulin storage in the pancreas (Dunn 2005). Three insulin dimers are co-ordinated by two Zn²⁺ to form a hexamer, which decreases insulin solubility and protects it from proteolytic degradation, enabling it to be stored in the pancreas until required (Li 2014). Exogenous insulin has long been co-administered with zinc in order to prolong its period of action for the treatment of diabetes. The understanding of endogenous insulin hexamer formation has aided the development of specific acute acting and long acting insulin therapeutics that can be administered to mimic prandial and basal insulin levels, and hence reduce the incidence of hypoglycaemia (Havelund et al. 2015). Zinc has also been used to stabilise sustained release formulations of other pharmacological proteins including recombinant human growth hormone and α-interferon (Bartus et al. 1998).

Histidine and cysteine are classical zinc chelating ligands commonly found in several classes of metalloproteins, including zinc-finger proteins (Laity et al. 2001) and metallothioneins (Blindauer 2008). Insulin hexamers interact with zinc via imidazole nitrogen atoms on the histidine residues of insulin beta chains (His B10 binding sites) situated in the central core of the hexamer (Dunn 2005). Recently ‘zinc-stapled’ insulin hexamers have been investigated as a potential new treatment for the maintenance of basal insulin levels in diabetic patients (Phillips et al. 2010). This approach involves histidine substitutions at the α-helical surface of insulin hexamers, enabling interaction with Zn²⁺ and the formation of multi-hexamer crystals at physiological pH; reducing insulin solubility and improving depot formation. Theoretical modelling has also predicted that glutamate and aspartate residues have relatively high affinities for zinc ions (Trzaskowski et al. 2008). Consequently, in the studies detailed in Chapter 3, histidine and glutamate residues found in GLP-1 and exendin-4 were preferentially substituted in to the glucagon sequence to increase compatibility of the dual analogue
with Zn$^{2+}$. Each zinc ion can form covalent bonds with two amino acid residues, subsequently forming a depot of peptide molecules interconnected via zinc ions. Increasing the number of histidine and glutamate residues in the analogues increases the likelihood of peptide-zinc binding and hence depot formation.

Histidine is hydrophilic and the incorporation of histidine residues can increase peptide solubility, as is often observed with proteins that have C or N-terminal His-tags to facilitate protein purification (Terpe 2003). The pH of the peptide-zinc solution outside of the body is approximately pH 4-5, at which point histidine is positively charged (Campbell & Farrell 2011); therefore the addition of histidines increases the overall positive charge of the peptide below pH7, and hence increases its solubility. Peptide molecules with an overall positive or negative charge will repel each other and as a result will be less likely to self-aggregate prior to injection. As glucagon is well known to form fibrils in solution (Pedersen et al. 2006) this is of particular importance for oxyntomodulin analogues which share a high degree of homology with glucagon. Similarly, as zinc ions carry a Z$^{2+}$ charge they will be repelled by positively charged peptides, reducing the likelihood of chelation occurring and ensuring the peptide remains in solution prior to injection. At physiological pH histidine residues have no net charge (Campbell & Farrell 2011); therefore post injection peptide solubility will rapidly decrease due to the loss of positive charge, which will contribute to the peptide crashing out of solution. Glutamate residues remain negatively charged at physiological pH (Campbell & Farrell 2011), which is important as a neutral or small negative overall peptide charge is desirable to facilitate zinc chelation. (See Appendix A Figure 7.3 for a summary of native peptide and analogue residue charges).

4.1.2. Rationale for second generation analogue design

Chapter 3 detailed the investigation of conservative amino acid substitutions and additions to the C-terminal region of glucagon to increase GLP-1 receptor activity and produce a dual agonist at the glucagon and GLP-1 receptors. The two OXL analogues, OXL10 and OXL11 had a similar ratio of activity at the GLP-1 and glucagon receptors as oxyntomodulin, but were more potent at both receptors. When administered with ZnCl$_2$ as part of a slow release formulation both peptides took longer to reach peak plasma concentrations in rats and subsequent levels plateaued for several days. However by 7 days post injection, plasma levels of OXL11 were a small percentage of the peak concentration and plasma levels of OXL10 were below the assay detection limit. This chapter details further amino acid additions to the C-terminus of OXL10 and OXL11 to produce a histidine ‘tail’ in order to enhance peptide-zinc chelation and subsequently improve depot formation and extend in vivo peptide availability.
4.1.3. Aims

The aim of this section of work was to investigate the effect of C-terminal amino acid extensions and substitutions to OXL analogues on PK parameters and food intake and bodyweight in rodents, in order to extend the duration of peptide action following a single SC injection. Analogue activity at GLP-1 and GCG receptors was also assessed.

For details of the materials and methods used please see Chapter 2. Please see Appendix A for the structures of the analogues described in this chapter.

4.2. Results

4.2.1. Effect of C-terminal amino acid extensions on the pharmacokinetic profiles of OXL-Tail analogues

All of the peptides tested had a T\text{max} of 3 hours or greater and all except OXL10 had measurable plasma levels at 7 days post injection (Table 4.1). Analogues OXLT4 (Figure 4.1B) and OXLT8 reached their peak concentrations by 3 hours and had low percentage peak levels remaining at 7 days (12% and 5% respectively). Analogues OXLT3 (Figure 4.1C), OXLT6 and OXLT9 (Figure 4.1D) reached their maximum plasma concentration at the 24 hour timepoint and subsequent levels remained above 50% of the peak concentration between 95-207 hours post injection. OXLT3 (Figure 4.1C) and OXLT6 had relatively high percentages of peak levels present at 7 days (30% and 26% respectively). OXLT9 plasma levels remained elevated at the final sampling point, 10 days post injection, with 41% of the peak plasma levels still present (Figure 4.1D).
Table 4.1: Summary of pharmacokinetic parameters of OXL-Tail analogues.

Male Wistar rats were given a single SC injection of 1mg peptide in 20µl formulated in ZnCl₂ slow release diluent (1:1, zinc to peptide). Peptides were measured in rat plasma using RIA. \( T_{\text{max}} \) = sampling time with the maximum peptide concentration; \( T_{\frac{1}{2}\text{max}} \) = a theoretical value calculated by determining the time at which plasma concentrations reached 50% of \( C_{\text{max}} \), assuming a linear clearance rate; % of \( C_{\text{max}} \) = percentage of the maximum plasma peptide concentration remaining at \( T=168 \) or \( T=240 \). \( n = 4 \) animals/group, data was calculated using a line graph and mean group plasma peptide levels at each time point. *Studies with the final samples taken on day 10 (240 hours), the rest had final samples taken on day 7 (168 hours).

```
<table>
<thead>
<tr>
<th></th>
<th>( T_{\text{max}} )</th>
<th>( T_{\frac{1}{2}\text{max}} )</th>
<th>% ( C_{\text{max}} ) remaining at ( T=168 )</th>
<th>% ( C_{\text{max}} ) remaining at ( T=240 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXL10</td>
<td>24</td>
<td>129</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>OXL11</td>
<td>3</td>
<td>63</td>
<td>17</td>
<td>19*</td>
</tr>
<tr>
<td>OXL12</td>
<td>24</td>
<td>56</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>OXL13</td>
<td>24</td>
<td>110</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>OXL14</td>
<td>3</td>
<td>66</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>OXL15</td>
<td>24</td>
<td>95</td>
<td>26</td>
<td>16*</td>
</tr>
<tr>
<td>OXL16</td>
<td>3</td>
<td>32</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>OXL17</td>
<td>3</td>
<td>73</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>OXL18</td>
<td>3</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OXL19</td>
<td>24</td>
<td>207</td>
<td>61</td>
<td>41*</td>
</tr>
</tbody>
</table>
```

Figure 4.1: Line graphs illustrating the pharmacokinetic profile of four OXL-Tail analogues. Analogue levels in rat plasma were measured using RIA at various time points following a single 20µl SC injection (50 mg/mL), formulated in ZnCl₂ slow release diluent (1:1, zinc to peptide). A) OXL11, B) OXL4, C) OXL3, D) OXL9. \( n = 4 \) animals/group, data shown is the mean plasma peptide level ± SEM.
4.2.2. Effect of OXL-Tail analogues on food intake and bodyweight in fasted DIO mice

A single SC 50nmolkg⁻¹ dose of OXLT1 or OXLT2 administered in ZnCl₂ slow release formulation (1:1, zinc to peptide) in fasted DIO mice resulted in a significant reduction in food intake up to 4 hours post injection (P <0.001, Figure 4.2A). 50nmolkg⁻¹ of all other analogues tested significantly reduced food intake up to at least 8 hours post injection (P <0.001, Figure 4.2A). Analogues OXLT3 and OXLT9 continued to inhibit food intake up to 24 hours post injection (P <0.001; Figure 4.2A). There was a significant increase in food intake during the 8-24 hour time period in the OXLT6 group compared to vehicle controls (P <0.05; Figure 4.2A).

Administration of OXLT6, OXLT3 and OXLT9 resulted in a significant reduction in bodyweight compared to vehicle controls during the 48 hours post injection (P <0.05 & P <0.001 and P <0.001 respectively; Figure 4.2B). Several groups, including OXLT9 continued to lose weight compared to controls during the 48-144 hour time period, however this was not significant (OXLT1 P =0.08, OXLT9 P =0.42). Overall OXLT3 and OXLT9 administration resulted in a respective 8% and 5% bodyweight loss over 6 days compared to vehicle controls (P <0.001; Figure 4.2B).
Figure 4.2: Effect of acute administration of OXL-Tail analogues on food intake and bodyweight in mice.
Cumulative food intake (A) and bodyweight change (B) in overnight fasted male DIO C57BL/6 mice after a single SC injection of vehicle or 50nmolkg\(^{-1}\) analogues (OXLT1 to OXLT9) formulated in ZnCl\(_2\) slow release diluent (1:1, zinc to peptide). Data shown as the mean percentage food intake or mean percentage change in bodyweight compared to vehicle ± SEM. n=6 mice per group; initial mean bodyweight 50.3g. Statistical analysis carried out using one way ANOVA and post hoc tests with Dunnett correction, *P <0.05, **P <0.01, ***P <0.001 compared to vehicle controls.
4.2.3. Effect of OXL-Tail analogues on food intake and bodyweight in fasted rats

Analogues were formulated in ZnCl$_2$ slow release diluent (1:1, zinc to peptide) and administered SC to fasted rats. Out of the 8 analogues tested, a single dose of 50nmolkg$^{-1}$ of OXLT5 OXLT3 and OXLT8 resulted in a significant reduction in food intake during the 0-4 hour time period ($P <0.001$, $P <0.01$ & $P <0.01$ respectively; Figure 4.3A). OXLT5 continued to inhibit food intake up to 24 hours post injection ($P <0.001$, Figure 4.3A) and food intake was significantly increased compared to vehicle controls during the 24-48 hour time period ($P <0.05$). Food intake in the groups administered analogues OXLT8 and OXLT9 was non-significantly decreased compared to vehicle controls during the 4-8 hour time period (OXLT8 $P =0.23$, OXLT9 $P =0.20$) and significantly decreased during 8-24 hours post injection ($P <0.001$; Figure 4.3A).

Administration of OXLT6 ($P <0.001$), OXLT3 ($P <0.01$), OXLT8 ($P <0.01$) and OXLT9 ($P <0.001$) significantly decreased bodyweight during the 0-48 hour time period compared to vehicle controls (Figure 4.3B). Bodyweight was significantly increased in the OXLT3 ($P <0.01$) and OXLT9 ($P <0.001$) groups during the 72-144 hour time period compared to vehicle controls (Figure 4.3B). Overall, except for OXL1 and OXL3, all analogue treated groups had significantly lower bodyweights by 6 days post injection compared to vehicle controls (Figure 4.3B).
Figure 4.3: Effect of acute administration of OXL-Tail analogues on food intake and bodyweight in rats.
Cumulative food intake (A) and bodyweight change (B) in 24 hour fasted male Wistar rats after a single SC injection of vehicle or 50nmolkg\(^{-1}\) OXL-Tail peptides (Analogues OXLT1 to OXLT9) formulated in ZnCl\(_2\) slow release diluent (1:1, zinc to peptide). Data shown as the mean percentage food intake or mean percentage change in bodyweight compared to vehicle ± SEM. n=6 rats per group; initial mean bodyweight 569g. Statistical analysis carried out using one way ANOVA and post hoc tests with Dunnett correction, *P <0.05, **P <0.01, ***P <0.001 compared to vehicle controls.
4.2.4. Effect of OXL-Tail analogues on human glucagon receptor and GLP-1 receptor mediated cAMP accumulation

The activity of the native peptides and analogues OXL11 and OXL10 at the human glucagon and GLP-1 receptors was similar to the results reported in Chapter 3 (Table 3.2).

All analogues tested had greater activity at the human glucagon receptor compared to oxyntomodulin, ranging from 5-50 fold increased efficacy (Table 4.2). OXLT5, OXLT6, OXLT8 and OXLT9 had similar efficacy to glucagon, whereas OXLT1, OXLT2 and OXLT3 had approximately 2 fold greater activity and OXLT7 had 5 fold greater activity than glucagon.

All analogues tested also had greater efficacy at the human GLP-1 receptor compared to oxyntomodulin, ranging from 7-31 fold greater efficacy. The majority of peptides had approximately 2 fold lower efficacy than GLP-1, except for OXLT7 which had similar receptor activity to GLP-1 and OXLT3 which was 5 fold less potent than GLP-1 (Table 4.2).

Comparison of the native peptide ratios at the human receptors indicated that oxyntomodulin had 3 times greater activity at the glucagon receptor compared to the GLP-1 receptor (Table 4.2). Three analogues, including OXLT9, had 2 fold greater activity at the glucagon compared to the GLP-1 receptor. OXLT7 and OXLT1 had approximately 5 times greater activity at the glucagon receptor compared to the GLP-1 receptor, whereas OXLT3 had 9 times greater activity at the glucagon receptor compared to the GLP-1 receptor. OXLT2 and OXLT6 had 3 fold greater activity at the glucagon receptor compared to the GLP-1 receptor, which was equivalent to the ratio of activity achieved for the first generation analogues OXL10 and OXL11 (Table 4.2 OXL10 and OXL11 highlighted in green).
<table>
<thead>
<tr>
<th></th>
<th>human GCGr</th>
<th></th>
<th>human GLP-1r</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP EC$_{50}$ (nM) ± SEM</td>
<td>Ratio to native peptide</td>
<td>cAMP EC$_{50}$ (nM) ± SEM</td>
<td>Ratio to native peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>&gt;1000</td>
<td></td>
<td>0.33 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.015 ± 0.001</td>
<td>10</td>
<td>185 ± 79</td>
<td>569</td>
</tr>
<tr>
<td>OXM</td>
<td>0.16 ± 0.02</td>
<td>0.9</td>
<td>11 ± 2</td>
<td>34</td>
</tr>
<tr>
<td>OXL10</td>
<td>0.014 ± 0.001</td>
<td>0.4</td>
<td>0.92 ± 0.10</td>
<td>2.8</td>
</tr>
<tr>
<td>OXLT1</td>
<td>0.007 ± 0.001</td>
<td>0.6</td>
<td>0.69 ± 0.18</td>
<td>2.1</td>
</tr>
<tr>
<td>OXLT2</td>
<td>0.009 ± 0.002</td>
<td>0.5</td>
<td>0.79 ± 0.03</td>
<td>2.4</td>
</tr>
<tr>
<td>OXLT3</td>
<td>0.008 ± 0.002</td>
<td>0.7</td>
<td>1.6 ± 0.2</td>
<td>4.8</td>
</tr>
<tr>
<td>OXL11</td>
<td>0.011 ± 0.002</td>
<td>1.8</td>
<td>0.89 ± 0.03</td>
<td>2.7</td>
</tr>
<tr>
<td>OXLT4</td>
<td>0.027 ± 0.003</td>
<td>0.2</td>
<td>0.70 ± 0.07</td>
<td>2.1</td>
</tr>
<tr>
<td>OXLT5</td>
<td>0.016 ± 0.002</td>
<td>1.1</td>
<td>0.58 ± 0.06</td>
<td>1.8</td>
</tr>
<tr>
<td>OXLT6</td>
<td>0.016 ± 0.001</td>
<td>0.1</td>
<td>0.77 ± 0.02</td>
<td>2.4</td>
</tr>
<tr>
<td>OXLT7</td>
<td>0.004 ± 0.001</td>
<td>1.0</td>
<td>0.37 ± 0.03</td>
<td>1.1</td>
</tr>
<tr>
<td>OXLT8</td>
<td>0.016 ± 0.002</td>
<td>1.0</td>
<td>0.69 ± 0.10</td>
<td>2.1</td>
</tr>
<tr>
<td>OXLT9</td>
<td>0.015 ± 0.002</td>
<td>1.0</td>
<td>0.70 ± 0.13</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 4.2: A summary of receptor efficacy for the native peptides and OXL-Tail analogues at the human glucagon receptor and GLP-1 receptor.
CHO-K1 cells over-expressing either the human glucagon receptor (GCGr) or human GLP-1 receptor (GLP-1r) were incubated for 30 minutes with a range of peptide concentrations. Cells were lysed and intracellular cAMP levels quantified by ELISA. EC$_{50}$ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC$_{50}$/native peptide EC$_{50}$. The results highlighted in green show the two peptides from Chapter 3 which were the starting structures of the analogues detailed in the current chapter.

### 4.2.5. Effects of OXLT9 on rat glucagon receptor and GLP-1 receptor mediated cAMP accumulation

Oxyntomodulin had 45 fold lower activity at the rat glucagon receptor compared to glucagon. OXLT9 had similar activity to glucagon, OXL10 and OXL11 at the rat glucagon receptor (Table 4.3 OXL10 and OXL11 highlighted in green). At the rat GLP-1 receptor oxyntomodulin activity was 153 fold lower than GLP-1. OXLT9 activity at the rat GLP-1 receptor was 8.8 fold lower than GLP-1 and 20 fold greater than oxyntomodulin. OXLT9 activity at the rat GLP-1 receptor was similar to that of OXL11 and approximately 2.5 fold greater than OXL10 (Table 4.3 highlighted in green). Subsequent comparison of the native peptide ratios for OXLT9 indicated that this peptide had 11 fold greater activity at the rat glucagon receptor than the rat GLP-1 receptor, which was more similar to the ratio of 15 calculated for OXL11 than the ratio of 44 for OXL10 (Table 4.3 highlighted in green).
Table 4.3: A summary of receptor efficacy for the native peptides and OXL-Tail analogues at the rat glucagon receptor and GLP-1 receptor.

CHO-K1 cells over-expressing the rat glucagon receptor (GCGr) or CHL cells overexpressing the rat GLP-1 receptor (GLP-1r) were incubated for 30 minutes with a range of peptide concentrations. Cells were lysed and intracellular cAMP levels quantified by ELISA. EC50 values are the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC50/native peptide EC50.

<table>
<thead>
<tr>
<th></th>
<th>rat GCGr</th>
<th></th>
<th>rat GLP-1r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP EC50 (nM) ±</td>
<td>Ratio to native</td>
<td>cAMP EC50 (nM) ±</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>peptide</td>
<td>SEM</td>
</tr>
<tr>
<td>GLP-1</td>
<td>&gt;1000</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.27 ± 0.02</td>
<td>21 ± 6</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>OXM</td>
<td>12 ± 2</td>
<td>45</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>OXL10</td>
<td>0.14 ± 0.04</td>
<td>0.5</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>OXL11</td>
<td>0.23 ± 0.04</td>
<td>0.8</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>OXLT9</td>
<td>0.21 ± 0.04</td>
<td>0.8</td>
<td>0.56 ± 0.12</td>
</tr>
</tbody>
</table>

4.2.6. Affinity of OXLT9 for the human, rat and mouse glucagon receptor and GLP-1 receptor

The affinity of the most potent OXL-Tail analogue, OXLT9 was investigated at the human, rat and mouse glucagon receptors (Table 4.4) and GLP-1 receptors (Table 4.5) and compared with the affinity of the natural ligands and analogues OXL10 and OXL11. The affinity of the native peptides and analogues OXL11 and OXL10 at the human, rat and mouse glucagon and GLP-1 receptors were similar to the results reported in Chapter 3 (Table 3.1 & Table 3.4).

Receptor affinity was consistent between the human, rat and mouse glucagon receptors. OXLT9 had similar affinity to glucagon at the glucagon receptor for all three species. Comparing the receptor affinity to that of oxyntomodulin at the human, rat and mouse glucagon receptors, OXLT9 had 9, 3 and 6 fold greater affinity respectively. Compared to OXL11, OXLT9 had similar affinity at the human rat and mouse glucagon receptors and 2-2.5 fold greater affinity than OXL10.

Receptor binding affinity differed between the human, rat and mouse GLP-1 receptors (Table 4.5). However OXLT9 consistently had greater affinity than oxyntomodulin, ranging from 15 and 22 fold greater at the rat and mouse receptors respectively, to 100 fold greater affinity at the human GLP-1 receptor. The affinity of OXLT9 at the human GLP-1 receptor was 22 fold and 6 fold greater than OXL10 and OXL11 respectively. At the rat and mouse GLP-1 receptors OXLT9 affinity was 3 fold and 1.5 fold greater than OXL10 and OXL11 respectively (Table 4.5).
### Table 4.4: A summary of receptor affinity for the native peptides and select OXL analogues at the human, rat and mouse glucagon receptors.

Purified cell membranes of CHO cells overexpressing the human or rat glucagon receptors, or purified mouse liver tissue were incubated with $^{125}$I-glucagon in the presence of increasing concentrations of unlabelled glucagon, GLP-1, oxyntomodulin, exendin-4, OXL11, OXL10 or OXLT9. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment. IC$_{50}$ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue IC$_{50}$/glucagon IC$_{50}$. The results highlighted in green show the two peptides from Chapter 3 which were the starting structure of the analogues detailed in the current chapter.

<table>
<thead>
<tr>
<th></th>
<th>human GCGr</th>
<th>Rat GCGr</th>
<th>mouse GCGr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM) ± SEM</td>
<td>Ratio to native peptide</td>
<td>IC$_{50}$ (nM) ± SEM</td>
</tr>
<tr>
<td>GLP-1</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.94 ± 0.02</td>
<td>1.2 ± 0.1</td>
<td>0.86 ± 0.01</td>
</tr>
<tr>
<td>OXM</td>
<td>6.9 ± 0.7</td>
<td>7.4</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>OXL10</td>
<td>1.7 ± 0.2</td>
<td>2.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>OXL11</td>
<td>0.73 ± 0.02</td>
<td>0.9</td>
<td>0.93 ± 0.05</td>
</tr>
<tr>
<td>OXLT9</td>
<td>0.71 ± 0.02</td>
<td>0.8</td>
<td>0.94 ± 0.21</td>
</tr>
</tbody>
</table>

### Table 4.5: A summary of receptor affinity for the native peptides and select OXL analogues at the human, rat and mouse GLP-1 receptors.

Purified cell membranes of CHO cells overexpressing the human or rat GLP-1 receptors, or purified mouse lung tissue were incubated with $^{125}$I-GLP-1 in the presence of increasing concentrations of unlabelled GLP-1, oxyntomodulin, exendin-4, glucagon, OXL11, OXL10 or OXLT9. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment. IC$_{50}$ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue IC$_{50}$/GLP-1 IC$_{50}$. The results highlighted in green show the two peptides from Chapter 3 which were the starting structure of the analogues detailed in the current chapter.

<table>
<thead>
<tr>
<th></th>
<th>human GLP-1r</th>
<th>rat GLP-1r</th>
<th>mouse GLP-1r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM) ± SEM</td>
<td>Ratio to native peptide</td>
<td>IC$_{50}$ (nM) ± SEM</td>
</tr>
<tr>
<td>GLP-1</td>
<td>0.48 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Glucagon</td>
<td>735 ± 43</td>
<td>1536</td>
<td>663 ± 25</td>
</tr>
<tr>
<td>OXM</td>
<td>144 ± 12</td>
<td>301</td>
<td>274 ± 33</td>
</tr>
<tr>
<td>OXL10</td>
<td>32 ± 11</td>
<td>66</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>OXL11</td>
<td>9.2 ± 1.9</td>
<td>19</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>OXLT9</td>
<td>1.6 ± 0.1</td>
<td>3.4</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>
4.2.7. Effect of chronic OXLT9 administration on food intake, bodyweight, blood glucose and body composition in DIO mice

In DIO mice, thrice weekly administration of 15nmolkg\(^{-1}\) and 30nmolkg\(^{-1}\) of OXLT9 formulated in ZnCl\(_2\) slow release diluent (1:1, zinc to peptide) resulted in a significant reduction in food intake from day 10 and day 5 respectively compared to vehicle controls (\(P<0.05, P<0.01\) respectively; Figure 4.4A). By day 24 cumulative food intake was reduced by 14\% and 21\% by 15nmolkg\(^{-1}\) and 30nmolkg\(^{-1}\) administration respectively compared to vehicle controls (\(P<0.001\); Figure 4.4A).

A significant reduction in bodyweight was observed by day 5 and day 3 after administration of 15nmolkg\(^{-1}\) and 30nmolkg\(^{-1}\) OXLT9 respectively (\(P<0.001\)). OXLT9 7.5nmolkg\(^{-1}\) also significantly reduced bodyweight from day 10 (\(P<0.01\); Figure 4.4B). By day 24, thrice weekly administration of 7.5, 15 and 30nmolkg\(^{-1}\) OXLT9 significantly reduced bodyweight by 3\% (\(P<0.01\)), 14\% and 28\% (\(P<0.001\)) respectively compared to vehicle controls (Figure 4.4B).

An IP glucose tolerance test (GTT) was carried out on day 24 to investigate the effect of chronic thrice weekly OXLT9 administration on blood glucose homeostasis. Basal blood glucose was significantly decreased after administration of OXLT9 at 15- or 30nmolkg\(^{-1}\) compared to vehicle controls (Figure 4.5A). Following an IP glucose bolus, peak blood glucose concentration was lower than controls for all three groups administered OXLT9 and levels returned to baseline concentrations within 90 minutes (Figure 4.5B). The area under the curve (AUC) for the groups administered 15nmolkg\(^{-1}\) and 30nmolkg\(^{-1}\) doses of OXLT9 was significantly reduced compared to the vehicle control (\(P<0.01\); Figure 4.5C) indicating that the change in blood glucose profiles shown in Figure 4.5B were significantly different from controls. A trend towards a reduction in the AUC for glucose levels over a 90 minute test period was observed for the lowest dose of OXLT9 tested, 7.5nmolkg\(^{-1}\) (\(P=0.08\) compared to controls).

Thrice weekly administration of all three doses significantly reduced percentage fat (7.5nmolkg\(^{-1}\) \(P<0.05\), 15 & 30nmolkg\(^{-1}\) \(P<0.001\)) and significantly increased percentage lean mass compared to vehicle controls (7.5nmolkg\(^{-1}\) \(P<0.05\), 15 & 30nmolkg\(^{-1}\) \(P<0.001\); Figure 4.6). A reduction in percentage protein was also seen after 30nmolkg\(^{-1}\) OXLT9 administration (\(P<0.05\); Figure 4.6).
Figure 4.4: Effect of chronic OXLT9 administration on food intake and bodyweight in mice.
Cumulative food intake (A) and bodyweight change (B) in male C57Bl/6 DIO mice after thrice weekly SC injections of vehicle or various doses of OXLT9 formulated in ZnCl₂ slow release diluent (1:1, zinc to peptide). Injections and food and bodyweight measurements were carried out at 4.30pm on Monday, Wednesday and Friday. n = 10/group, mean initial bodyweight = 40.6g. Data shown as the mean ± SEM. Statistical analysis carried out using two-way repeated measures ANOVA and post hoc tests with Bonferroni correction, *P <0.05, **P <0.01, ***P <0.001 versus vehicle controls, NS - non-significant at all timepoints versus vehicle controls.
Figure 4.5: Effect of chronic OXLT9 administration on blood glucose homeostasis in mice.
An IP GTT was carried out on study day 24. Animals were fasted for 6 hours and given 2mg/kg IP glucose at t=0. (A) Basal blood glucose concentration (t= -30); (B) Change in blood glucose concentration, both expressed as mean ± SEM. (C) The area under the curve for the change in blood glucose between 0-90 minutes, expressed as a percentage of vehicle AUC ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Dunnett correction. *P <0.05; **P <0.01 versus vehicle controls.
4.2.8. Effect of chronic OXLT9 administration on food intake and bodyweight in rats

Daily SC administration of 43.3nmolkg$^{-1}$ OXLT9 formulated in ZnCl$_2$ slow release diluent (1:1, zinc to peptide) to rats significantly decreased bodyweight from day 1 compared to vehicle controls ($P < 0.05$) and the respective pair-fed group ($P < 0.001$; Figure 4.7A). After 7 days of administration the 43.3nmolkg$^{-1}$ group had lost 10% bodyweight compared to vehicle controls and 9% compared to the pair-fed group ($P < 0.001$; Figure 4.7A). There was also a reduction in bodyweight with 14.4nmolkg$^{-1}$ OXLT9 administration which reached significance on day 6 versus vehicle controls ($P < 0.001$) and day 7 versus pair-fed controls ($P < 0.05$; Figure 4.7A). The bodyweight of the 14.4nmolkg$^{-1}$ OXLT9 group was 4% and 2% lower than vehicle and pair-fed controls respectively by day 7.

Daily administration of 43.3nmolkg$^{-1}$ OXLT9 resulted in a significant reduction in food intake from day 6 reaching a 14% reduction in food intake compared to vehicle controls by day 7 ($P < 0.001$; Figure 4.7B). Food intake did not differ between the 14.4nmolkg$^{-1}$ OXLT9 and vehicle groups or between the treatment groups and their respective pair-fed controls (Figure 4.7B).
Figure 4.7: Effect of chronic OXLT9 administration on food intake and bodyweight in rats.
Cumulative bodyweight change (A) and food intake (B) in rats after daily SC administration of various doses of OXLT9 formulated in ZnCl₂ slow release diluent (1:1, zinc to peptide). Pair-fed groups were given a weighed amount of food equivalent to the mean amount eaten in the previous 24 hours by the animals in the corresponding treatment group. Daily injections, food and bodyweight measurements and feeding of paired groups were carried out at 4.30pm. n= 11/group, mean initial bodyweight 582g. Data shown as mean ± SEM. Statistical analysis carried out using two-way repeated measures ANOVA and post hoc tests with Bonferroni correction. *P <0.05, **P <0.01, ***P <0.001 versus vehicle controls. *P <0.05, ***P <0.001 versus corresponding pair-fed group.
4.2.9. Effect of acute OXLT9 administration on energy expenditure in rats

It has previously been shown that substituting glutamate (Glu/E) for glutamine (Gln/Q) at residue 3 of oxyntomodulin and other dual glucagon and GLP-1 receptor analogues greatly diminishes glucagon receptor activity without affecting GLP-1 receptor activity. OXLT9E3, the Glu-3 equivalent of OXLT9, was synthesised and used to investigate the contribution of glucagon receptor activity to OXLT9 effects on energy expenditure. The rat glucagon receptor activity of OXLT9E3 was 140 fold lower than that of OXLT9 whereas activity at the rat GLP-1 receptor was similar (Table 4.6).

<table>
<thead>
<tr>
<th>GLP-1</th>
<th>Glucagon</th>
<th>OXLT9</th>
<th>OXLT9E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP EC_{50} (nM) ± SEM</td>
<td>0.27 ± 0.02</td>
<td>0.21 ± 0.04</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Ratio to native peptide</td>
<td>0.06 ± 0.00</td>
<td>0.56 ± 0.12</td>
<td>0.72 ± 0.05</td>
</tr>
</tbody>
</table>

Table 4.6: A summary of receptor efficacy for OXLT9 and Glu-3 equivalent OXLT9E3 at the rat glucagon receptor and GLP-1 receptor.

CHO-K1 cells over-expressing the rat glucagon receptor or CHL cells overexpressing the rat GLP-1 receptor were incubated for 30 minutes with a range of peptide concentrations. Cells were lysed and intracellular cAMP levels quantified by ELISA. EC_{50} values are the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC_{50}/native peptide EC_{50}.

In male Wistar rats, a single SC administration of 8nmolkg^{-1} OXLT9 in 1:3 molar ratio with ZnCl_2 slow release formulation resulted in a significant increase in the volume of oxygen consumed in both the first and second light phase compared to vehicle controls (both \( P < 0.05 \) versus Vehicle controls) (Figure 4.8). There was no significant difference in oxygen consumption following OXLT9E3 administration compared to Vehicle or OXLT9 treatment. Oxygen consumption did not differ in any of the groups during either the first or second dark phase (one dark phase = 1900hrs-0700hrs). There was no significant difference in RER or total ambulatory movement for any of the groups (Figure 4.9 & Figure 4.10 A). Bodyweight did not differ significantly over the 48 hour period for any of the groups (Figure 4.10 B). OXLT9E3 administration resulted in a small non-significant decrease in food intake in both the 0-24 and 24-48 hour time period versus vehicle controls (Mean 0-24hr food intake, Vehicle: 25.9 ± 1.5g; OXLT9E3: 22.38 ± 1.6g, \( P = 0.37 \). Mean 24-48hr food intake, Vehicle: 26.1 ± 2.5g; OXLT9E3: 22.6 ± 0.8g, \( P = 0.25 \), Figure 4.11).
Figure 4.8: The acute effect OXLT9 and OXLT9E3 on oxygen consumption in rats.

A single SC injection of vehicle or 8nmolkg⁻¹ OXLT9 or Glu-3 equivalent OXLT9E3, formulated in ZnCl₂ slow release diluent (3:1, zinc to peptide) was administered at 1500 hours on study day 1. (A) The mean volume of oxygen consumed for 48 hours post injection. (B) The AUC for the volume of oxygen consumption shown in A, separated by light and dark phases. Data expressed as mean ± SEM. Shaded areas indicates the dark phases. n = 5-6/group. Statistical analysis was carried out on AUC data using one-way ANOVA and post hoc tests with Dunnett correction *P <0.05, **P <0.01, ***P <0.001 versus Vehicle controls.
Figure 4.9: The acute effect of OXLT9 and OXLT9E3 on respiratory exchange ratio (RER) in rats.

A single SC injection of vehicle or 8nmolkg\(^{-1}\) OXLT9 or Glu-3 equivalent OXLT9E3, formulated in ZnCl\(_2\) slow release diluent (3:1, zinc to peptide) was administered at 1500 hours on study day 1. (A) The mean RER for 48 hours post injection. (B) The area under the curve for the RER shown in A, separated by light and dark phases. Data expressed as mean ± SEM. Shaded areas indicate the dark phases. n = 5-6/group.
Figure 4.10: The acute effect of OXLT9 and OXLT9E3 on movement and bodyweight in rats.
A single SC injection of vehicle or 8nmolkg⁻¹ OXLT9 or Glu-3 equivalent OXLT9E3, formulated in ZnCl₂ slow release diluent (3:1, zinc to peptide) was administered at 1500 hours on study day 1. (A) The mean ambulatory movement for 48 hours post injection. (B) The mean cumulative weight change over 48 hours post injection. Data expressed as mean ± SEM. Shaded area indicates the dark phase. n = 5-6/group.
Figure 4.11: The acute effect of OXLT9 and OXLT9E3 on food intake in rats.

A single SC injection of vehicle or 8nmolkg⁻¹ OXLT9 or Glu-3 equivalent OXLT9E3, formulated in ZnCl₂ slow release diluent (3:1, zinc to peptide) was administered at 1500 hours on study day 1. (A) Mean cumulative food intake for up to 48 hours post injection. (B) Bar graph of the cumulative food intake shown in A. Data expressed as mean ± SEM. Shaded areas indicate the dark phases. n = 5-6/group.
4.3. Discussion

The aim of the work detailed in this chapter was to develop an optimised dual glucagon receptor and
GLP-1 receptor agonist by further enhancing peptide interaction with zinc, in order to improve
subcutaneous depot formation and extend the duration of peptide action. While improving the PK
properties of the dual analogues was the main aim, it was important to ensure efficacy at the
glucagon and GLP-1 receptors was maintained, therefore the effect of each amino acid change on
receptor activity and affinity was also assessed. OXL10 and OXL11 were selected from the work
completed in Chapter 3 as the basic structures to be modified. The peptides were selected based on
their strong efficacy at the glucagon and GLP-1 receptors, their similar ratio of receptor activity as
oxyntomodulin and their already improved PK properties.

Histidine residues were preferentially added to the C-terminal of OXL10 and OXL11 due to the ability
of histidine to chelate with zinc ions at physiological pH (Laity et al. 2001; Trzaskowski et al. 2008).
The addition of 3 histidines to form a C-terminal 5-histidine ‘tail’ (OXLT3 and OXLT6) had beneficial
effects in vivo, improving PK parameters and reducing food intake and bodyweight in both mouse
and rat models. The results suggest that depot formation was enhanced, probably due to an
increased likelihood of chelation between histidine and zinc ions. Comparing the PK profile of
peptides administered in ZnCl₂ versus saline to see if the enhanced PK parameters are dependent on
the presence of zinc would help to confirm the mechanism of action. Interestingly, a 4-histidine tail
(OXLT1, OXLT4) did not have the same beneficial in vivo effects. It could be speculated that the 5ᵗʰ
histidine alone at position 34 would be sufficient to enhance depot formation, due to its location
being adequately distant from the main part of the molecule that potential interference with zinc
interaction is reduced. This could be investigated with an analogue that has the 3ʳᵈ and 4ᵗʰ histidines
at positions 32 and 33 replaced with Arg or Lys.

Extending the ‘tail’ to 5 histidines had small effects on in vitro efficacy at the human glucagon and
GLP-1 receptors. However, because OXLT3 activity at the glucagon receptor was slightly increased
and GLP-1 receptor activity was slightly decreased, the overall ratio of GLP-1 receptor to glucagon
receptor activity tripled from 3.1 for OXL10 to 9.6 for OXLT3. The ratio of GLP-1 receptor to glucagon
receptor activity has been suggested to be important to balance maximal weight loss with the
potential hyperglycaemic effects of glucagon (Day et al. 2012). The ratio of GLP-1 receptor to
glucagon receptor activity for OXLT6 was 2.4, which was more similar to the value of 3.4 achieved for
native oxyntomodulin; for this reason further analogue development was carried out with OXLT6.
A single glycine substitution was introduced at amino acid position 31 to investigate whether increasing the flexibility of the C-terminal histidine ‘tail’ could enhance peptide-zinc interaction and hence depot formation. This was assessed by measuring PK parameters in rats and 6 day food intake and bodyweight reduction in rodents following a single SC injection. As the smallest amino acid, with a single hydrogen atom ‘side-chain’, glycine has greater conformational flexibility than other amino acids (Campbell & Farrell 2011). Glycine is often found in peptide loops where flexibility is essential and has been suggested to play an important role in the active sites of enzymes, providing the flexibility to enable conformational changes required for activation (Yan & Sun 1997). Substituting glycine for the second histidine residue in the 4-histidine ‘tail’ (OXLT2 and OXLT5) resulted in an improved $T_{1/2}\text{max}$ and increased the percentage of peptide remaining in the circulation at 7 days, suggesting enhanced depot formation. However this was not reflected in the biological actions of the analogues, as the reduction in food intake and bodyweight in rodents following single SC injections was not extended compared to other analogues with poorer PK profiles. Substituting glycine for the first histidine residue of the 5-histidine tail (analogue OXLT7) to form a flexible Gly-Gly linker between the histidine ‘tail’ and the rest of the peptide, had a detrimental effect on PK parameters (Table 4.1). It can be speculated that the interaction with zinc, and hence depot formation, was hindered by the high degree of flexibility in this region resulting from the Gly substitution. Alternatively, the spacing between the zinc binding histidine atoms in the ‘tail’ and the rest of the molecule may be important; therefore, as glycine is much smaller than histidine, optimal zinc chelation may have been hindered by the histidine ‘tail’ being closer to the rest of the molecule. It can also be postulated that the first tail histidine atom itself is important for zinc interaction.

The addition of glutamine at position 35 between the 5th histidine and the terminal amide (OXLT9) substantially improved PK parameters, with over half of the maximal blood peptide levels remaining at 10 days post injection. This corresponded with a sustained significant reduction in food intake up to 24 hours and a significant reduction in bodyweight over 6 days in both rat and mouse models. Substituting alanine at position 35 abolished the beneficial effects on PK parameters and food intake and bodyweight reduction in mice, indicating that the favourable effects were specific to glutamine at this residue.

The efficacy of OXLT9 at the human glucagon receptor and GLP-1 receptor was 10 fold and 16 fold greater than oxyntomodulin respectively and similar to that of OXL11, the first generation OXL analogue. In line with the results from Chapter 3, OXLT9 efficacy at the rat glucagon receptor was similar to that at the human receptor whereas efficacy at the rat GLP-1 receptor was lower compared to the human receptor. There were also differences in OXLT9 receptor affinity at the
human, rat and mouse GLP-1 receptors as discussed in Chapter 3. In vitro OXLT9 demonstrated 4 fold greater efficacy at the human GLP-1 receptor compared to the rat receptor, which could suggest that OXLT9 would have a greater effect on food intake reduction in humans compared to rats. However, it is difficult to predict how the difference between in vitro human and rat efficacy might translate to a biological effect in vivo, as species differences in peptide degradation, renal filtration and distribution space will also affect peptide efficacy in vivo (Mahmood 2007).

Chronic administration of OXLT9 in both mice and rats demonstrated a dose dependent reduction in food intake and bodyweight. Body composition analysis in mice after chronic 30nmolkg\(^{-1}\) OXLT9 administration demonstrated a 5.9% reduction in fat mass. It is not possible to determine whether fat mass was decreased as a result of an increase in energy expenditure or due to a reduction in food intake. Groups of animals pair-fed to the treatment groups would control for the effect of reduced food intake, but this was not possible as the pair-fed mice failed to consume all of their daily rations. However, food intake following administration of 7.5nmolkg\(^{-1}\) OXLT9 did not differ significantly from food intake in the Vehicle group, yet weight gain in the OXLT9 group was significantly reduced. This suggests that part of the weight loss following OXLT9 administration was due to an increase in energy expenditure. There was also a significant reduction in protein mass following chronic administration of the highest dose of 30nmolkg\(^{-1}\) OXLT9. This suggests that the increased energy expenditure following OXLT9 administration may have resulted in the loss of lean mass. This could be a possible safety concern that would need to be closely monitored if the compound undergoes formal assessment for human studies. It is of note that lean mass increased following OXLT9 administration despite protein mass being decreased. It is currently unclear why this is the case. Given the contribution of bone to lean mass, these data may reflect an increase in bone density. However, further investigation is necessary to confirm whether this is the case. Importantly, glucose tolerance was improved during an IP GTT in mice chronically administered OXLT9 and basal blood glucose was also significantly lower after 15 and 30nmolkg\(^{-1}\) OXLT9 administration. These results indicate that at doses up 30nmolkg\(^{-1}\) glucagon receptor activity of OXLT9 did not negatively impact glucose tolerance or cause hyperglycaemia in mice. Although no formal behavioural analysis was performed, animals were observed post injection and did not display any abnormal behaviour or signs of aversion that might indicate hyperglycaemia. Day et al (2012) reported that in rodents, a 1:1 ratio of glucagon receptor activity to GLP-1 receptor activity for their PEGylated dual analogues was optimal for minimal food intake reduction, good weight loss and improvements in blood glucose. However, their results also showed that up to 6 fold greater activity at the glucagon receptor compared to the GLP-1 receptor (i.e. a ratio of 1:6) was not detrimental to glucose homeostasis. The optimal ratio of glucagon receptor : GLP-1 receptor activity that maximises weight loss and
minimises hyperglycaemia has not been calculated for humans and is likely to vary between species (Day et al. 2012). Acute and chronic administration of native oxyntomodulin in humans increases energy expenditure and reduces food intake without negatively affecting glycaemia (Wynne et al. 2005; Wynne et al. 2006a), therefore the receptor activity ratio of oxyntomodulin is the best indicator available for what the ratio may be in humans. For native oxyntomodulin the ratio of glucagon receptor activity to GLP-1 receptor activity at the human receptors was 1:3 and the ratio for OXLT9 was 1:2. Further attempts to make the ratio for OXLT9 more similar to that of oxyntomodulin substantially reduced analogue efficacy at one or both receptors. Detailed investigation of the acute effects of OXLT9 on glucose homeostasis is required and effects on blood glucose will need to be closely monitored if OXLT9 proceeds to human studies.

Daily OXLT9 treated rats lost significantly more weight than pair-fed control animals indicating that analogue treatment increased energy expenditure and this was indeed confirmed with the use of metabolic cages in a separate study. Results indicated that a single SC injection of 8nmolkg⁻¹ OXLT9 significantly increased resting energy expenditure (measured as oxygen consumption during the light phase) over 48 hours compared to weight matched vehicle controls. Interestingly, resting energy expenditure following administration of GLP-1 receptor selective analogue OXLT9E3 was also slightly increased. Previous findings regarding the effect of exogenous GLP-1 or its analogues on energy expenditure are inconsistent. Several studies in rodents (Osaka et al. 2005; Lockie et al. 2012; Yang et al. 2014) and humans (Horowitz et al. 2012) suggested that administration of GLP-1, exendin-4 or liraglutide increased energy expenditure, whereas others reported a reduction (Flint et al. 2000; Flint et al. 2001; Baggio et al. 2004a; Hayes et al. 2008). Additionally several clinical studies with GLP-1 analogues reported no change in energy expenditure in obese subjects (Harder et al. 2004; Bradley et al. 2012). It appears that a number of factors may affect the relationship between GLP-1 and energy expenditure including the route and duration of administration, the index of metabolic rate that is measured and in animals the type of chow they are fed. Lockie (2012) reported that oxyntomodulin requires the GLP-1 receptor to generate an increase in energy expenditure in mice, however they administered oxyntomodulin via ICV injection and they measured BAT thermogenesis rather than VO₂. The current results suggest that both the glucagon receptor and GLP-1 receptor activity of peripherally administered oxyntomodulin analogues is necessary to generate a significant increase in energy expenditure in rats.

The number of available metabolic cages was limited; therefore rather than including a group of animals pair-fed to the OXLT9 group, a low dose of OXLT9 was used so that food intake was similar to that of vehicle controls. Subsequently, the observed increase in energy expenditure following
OXLT9 administration was not due to differences in food intake. It is interesting to note that OXLT9E3 caused a small reduction in food intake compared to the equivalent dose of OXLT9. It is possible that OXLT9 treated animals ate more than OXLT9E3 animals in response to the increase in energy expenditure resulting from glucagon receptor activity of OXLT9. Increased food intake following administration of low doses was consistently observed during OXL analogue screening and is discussed further in Chapter 5.

4.3.1. Conclusion

In conclusion, a range of experimental methods were utilised to develop an optimised dual glucagon and GLP-1 receptor agonist which demonstrates improved efficacy at the human glucagon and GLP-1 receptors compared to the native dual agonist oxyntomodulin. The extension of the C-terminal histidine ‘tail’ and the addition of glutamine at position 35 extend the PK profile of OXLT9, presumably due to improved depot formation resulting from enhanced peptide interaction with zinc ions present in the slow release diluent. OXLT9 generates a sustained decrease in bodyweight in rodent models by reducing food intake, increasing resting energy expenditure and reducing fat mass. Glucose homeostasis is improved following chronic administration of OXLT9 in DIO mice, indicating that the enhanced glucagon receptor activity of OXL-Tail analogues does not appear to cause hyperglycaemia and that OXLT9 may be suitable for the treatment of obese patients with T2DM, although extensive further testing will be necessary to determine this.
Chapter 5

Increased food intake with OXL analogues
5.1. Introduction

Numerous studies have shown that oxyntomodulin and long acting oxyntomodulin analogues reduce both food intake and bodyweight in rodents (Dakin et al. 2001; Baggio et al. 2004a; Dakin et al. 2004; Maida et al. 2008; Liu et al. 2010; Kosinski et al. 2012; Bianchi et al. 2013) and reductions in food intake and bodyweight have also been achieved after oxyntomodulin administration in humans (Cohen et al. 2003; Wynne et al. 2005; Wynne et al. 2006a). Based on findings in mice it has been suggested that oxyntomodulin reduces food intake via activity at the GLP-1 receptor, whilst its activity at the glucagon receptor is postulated to cause weight loss by increasing energy expenditure (Kosinski et al. 2012). During the course of developing the oxyntomodulin-like, dual glucagon and GLP-1 receptor agonists detailed in Chapters 3 and 4, it was consistently observed that a number of analogues generated a significant reduction in bodyweight and food intake when administered peripherally to rats at 50nmolkg⁻¹; but at 25nmolkg⁻¹ the same analogues unexpectedly caused an increase of food intake, without increasing bodyweight.

A similar increase in food intake with concomitant weight loss is classically associated with patients suffering from thyrotoxicosis (Franklyn & Boelaert 2012). It was widely assumed that the increased consumption was a compensatory response to the hypermetabolic state caused by elevated plasma levels of thyroid hormones. However, the appetites of approximately 5-10% of individuals with thyrotoxicosis are sufficiently increased as to cause weight gain (Gurney et al. 1970), indicating that thyroid hormones directly stimulate feeding. More recently peripheral and central administration of biologically active thyroid hormone tri-iodothyronine (T₃) and the biogenic amine derivative tri-iodothyronamine (T₁AM) have been shown to increase food intake in rodents via direct effects in the hypothalamus and independently of changes in energy expenditure (Kong et al. 2004; Dhillo et al. 2009). These findings were not replicated in a similar study using human subjects, however T₃ was administered orally rather than subcutaneously and participants were fasted which would have made it difficult to observe small increases in food intake (Martin et al. 2007).

GLP-1 and oxyntomodulin are known to exert their effects on satiety via central actions within the hypothalamus and brainstem in key areas involved in the regulation of feeding (Simpson et al. 2009; Suzuki et al. 2010; Yu & Kim 2012; Sam et al. 2012), and it is likely that they cross the blood brain barrier in order to do so (Kastin et al. 2002; Dakin et al. 2004; Larsen & Holst 2005; Wynne et al. 2006a). GLP-1 receptor mRNA is present throughout the hypothalamus and brainstem (Navarro et al. 1996; Merchenthaler et al. 1999) and GLP-1 immunoreactive neurons project from the NTS in the brainstem to the PVN and DMN of the hypothalamus (Larsen et al. 1997a). One can hypothesise that the oxyntomodulin analogues investigated in the current study directly increased food intake via
central effects, independently of any weight loss. The analogues detailed in this chapter are not derivatised and are therefore of a similar size and length to oxyntomodulin and GLP-1, subsequently their ability to cross the blood brain barrier and interact with hypothalamic and brainstem receptors should be similar to that of the native peptides.

Alternatively, the unexpected increase in food intake could be a compensatory response to the analogues causing an increase in energy expenditure. Numerous mammalian species have evolved a variety of biological signals and systems that allow them to adapt their energy intake and expenditure to maintain bodyweight (Keesey & Powley 1986; Morton et al. 2006). Glucagon increases energy expenditure in rodents via BAT activation leading to a subsequent increase in non-shivering thermogenesis (Billington et al. 1987; Billington et al. 1991b; Lockie et al. 2013) and it is thought a similar mechanism may be responsible for the increase in energy expenditure following glucagon administration in humans (Tan et al. 2013). The mechanism by which oxyntomodulin mediates its effects on body weight and energy expenditure is currently unclear. Evidence suggests a glucagon receptor mediated mechanism is at least partly responsible (Kosinski et al. 2012) and it has recently been suggested that GLP-1 receptors are also involved in BAT mediated thermogenesis in mice (Lockie et al. 2013). Energy expenditure is also increased in humans following oxyntomodulin administration (Wynne et al. 2006a), possibly via similar mechanisms as in rodents, raising the possibility that the oxyntomodulin analogues could also increase food intake in humans.

The observed increase in food consumption may be a consequence of unbalanced GLP-1 receptor and glucagon receptor activity, with insufficient GLP-1 receptor activity to prevent an increase in food intake in response to a glucagon receptor mediated increase in energy expenditure. Increased feelings of hunger and increased food intake as a result could be an undesirable side-effect in a drug targeted at treating obesity. Therefore a representative sample of oxyntomodulin analogues that increased food intake was further investigated to assess the impact of over-eating on the design and potential therapeutic use of oxyntomodulin-like analogues.
5.1.1. Aims

4. To investigate whether the increase in food intake is related to the analogues ratio of GLP-1 to glucagon receptor activity.

5. To investigate the mechanism behind the increase in food intake.

6. To assess the impact of increased food intake on the design and use of oxyntomodulin-like analogues as a potential obesity therapy.

For the details of materials and methods used please see Chapter 2. Please see Appendix A for the sequences of peptides investigated in this chapter.
5.2. Results

5.2.1. Chronic OXL analogue administration increases food intake in rats

Daily 25nmolkg\(^{-1}\) injections of oxyntomodulin-like analogue OXL15 significantly increased 6 day cumulative food intake by a mean of 17% in male Wistar rats compared to vehicle treated animals (Mean 6 day food intake; Vehicle: 201.9 ± 4.8g, OXL15: 253.6 ± 9.0g, \(P = 0.042\)) (Figure 5.1A & Table 5.1). Animals administered OXL15 weighed a mean of 3.4% less than vehicle treated animals after 6 days, although this was not significant (Mean 6 day weight change; Vehicle: 13.1 ± 2.1g, OXL15: -5.9 ± 5.3g, \(P = 0.051\)) (Figure 5.1B & Table 5.1). Analogue OXL24 also significantly increased 6 day food intake by a mean of 20% compared to vehicle treated animals (Mean 6 day food intake; Vehicle: 197.6 ± 6.9g, OXL24: 237.1 ± 13.6g, \(P = 0.019\)) (Figure 5.2A and Table 5.2). Mean bodyweight was 1.6% lower in the OXL24 treated group compared to vehicle which was not significant (Mean 6 day weight change; Vehicle: 16.0 ± 2.3g, OXL24: 7.4 ± 3.2g, \(P = 0.401\)) (Figure 5.2B and Table 5.2).

Daily 25nmolkg\(^{-1}\) administration of OXL21, OXL23, OXL31 and OXL33 for 6 days significantly decreased bodyweight compared to vehicle controls by 4-6.2% (Mean 6 day weight change; Vehicle: 13.1 ± 2.1g, OXL21: -20.5 ± 7.9g, \(P < 0.0001\), OXL23: -19.5 ± 7.1g, \(P < 0.0001\), OXL31: -8.7 ± 3.3g, \(P < 0.016\), OXL33: -14.5 ± 7.5g, \(P = 0.001\)) (Figure 5.1B & Table 5.1) Administration of OXL21 and OXL31 resulted in a non-significant increase in food intake of 8.2% and 14.5% respectively (Mean 6 day food intake; Vehicle: 201.9 ± 4.8g, OXL21: 218.5 ± 7.8g, \(P = 0.666\), OXL31: 231.1 ± 9.7g, \(P = 0.108\)) (Figure 5.1A & Table 5.1). OXL20 and OXL14 daily administration for 6 days significantly reduced bodyweight gain compared to vehicle treated animals by 2.7 and 2.6% respectively (Mean 6 day weight change; Vehicle: 16.0 ± 2.3g, OXL20: 2.0 ± 3.2g, \(P = 0.034\), OXL14: 2.6 ± 7.4g, \(P = 0.049\)) (Figure 5.2B & Table 5.2). OXL20 and OXL14 administration caused a non-significant 13% and 14% increase in food intake respectively (Mean 6 day food intake; Vehicle: 197.6 ± 6.9g, OXL20: 223.1 ± 9.7g, \(P = 0.275\), OXL14: 225.2 ± 5.6g, \(P = 0.198\)) (Figure 5.2A & Table 5.2).

As expected the specific GLP-1 receptor agonist exendin-4 significantly reduced food intake and bodyweight over 6 days of administration at 5nmolkg\(^{-1}\) compared to vehicle treated animals. Food intake was reduced by 31% (Mean 6 day food intake; Vehicle: 201.9 ± 4.8g, exendin-4: 144.2 ± 5.7g, \(P < 0.0001\)) (Figure 5.1A & Table 5.1) and 23% (Mean 6 day food intake; Vehicle: 197.6 ± 6.9g, exendin-4: 151.3 ± 9.0g, \(P = 0.003\)) (Figure 5.2A & Table 5.2) compared to vehicle in the first and second study respectively. Exendin-4 administration significantly reduced bodyweight by 4.2% (Mean 6 day weight change; Vehicle: 13.1 ± 2.1g, exendin-4: -9.3 ± 2.1g, \(P = 0.009\)) (Figure 5.1B &
Table 5.2) and 2.8% (Mean 6 day weight change; Vehicle: 16.0 ± 2.3g, exendin-4: 1.33 ± 3.5g, $P=0.018$) (Figure 5.2 B and Table 5.2) over 6 days compared to vehicle treated animals.

All OXL analogue treated animals in both studies consumed significantly more food than exendin-4 treated animals (Figure 5.1A and Table 5.1; Figure 5.2A and Table 5.2; $P<0.01$ or $P<0.001$). However, apart from OXL13 group in study 1 (Figure 5.1B and Table 5.1) and OXL16 and OXL28 groups in study 2 (Figure 5.2B and Table 5.2) the bodyweights of all OXL analogue treated groups did not differ significantly from that of exendin-4 treated groups.
Figure 5.1: Effect of OXL analogue administration on food intake and bodyweight in rats.
6 day cumulative food intake (A) and bodyweight change (B) in male Wistar rats in response to daily 25nmolkg⁻¹ injections of OXL analogues (Odds, OXL13-OXL33), 5nmolkg⁻¹ exendin-4 or vehicle. Mean starting weight 528g; n= 7-8/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Bonferroni correction. *P <0.05, **P <0.01, ***P <0.001 versus vehicle controls; ## P < 0.01 versus exendin-4 group.
Figure 5.2: Effect of OXL analogue administration on food intake and bodyweight in rats.
6 day cumulative food intake (A) and bodyweight change (B) in male Wistar rats in response to daily 25nmolkg⁻¹ injections of OXL analogues OXL12-14 and Evens OXL16-OXL28), 5nmolkg⁻¹ exendin-4 or vehicle. Mean starting weight 506g; n= 8-9/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Bonferroni correction. *P <0.05, ***P <0.001 versus vehicle controls; #P <0.05, ##P < 0.01, ###P <0.001 versus exendin-4 group.
<table>
<thead>
<tr>
<th></th>
<th>0-7day FI (g)</th>
<th>Difference from Vehicle (g)</th>
<th>0-7day BW change (g)</th>
<th>Difference from Vehicle (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>201.9</td>
<td>13.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exendin</td>
<td>140.1</td>
<td>-61.8</td>
<td>-9.4</td>
<td>-22.5</td>
</tr>
<tr>
<td>OXL13</td>
<td>214.8</td>
<td>12.9</td>
<td>18.1</td>
<td>5.0</td>
</tr>
<tr>
<td>OXL15</td>
<td>235.6</td>
<td>33.7</td>
<td>-5.9</td>
<td>-19.0</td>
</tr>
<tr>
<td>OXL17</td>
<td>215.6</td>
<td>13.7</td>
<td>2.9</td>
<td>-10.2</td>
</tr>
<tr>
<td>OXL19</td>
<td>219.9</td>
<td>18.0</td>
<td>2.5</td>
<td>-10.6</td>
</tr>
<tr>
<td>OXL21</td>
<td>218.5</td>
<td>16.6</td>
<td>-20.3</td>
<td>-33.4</td>
</tr>
<tr>
<td>OXL23</td>
<td>194.8</td>
<td>-7.1</td>
<td>-19.5</td>
<td>-32.6</td>
</tr>
<tr>
<td>OXL25</td>
<td>199.0</td>
<td>-2.9</td>
<td>11.9</td>
<td>-1.2</td>
</tr>
<tr>
<td>OXL27</td>
<td>224.5</td>
<td>22.6</td>
<td>9.7</td>
<td>-3.4</td>
</tr>
<tr>
<td>OXL29</td>
<td>210.7</td>
<td>8.8</td>
<td>0.7</td>
<td>-12.4</td>
</tr>
<tr>
<td>OXL31</td>
<td>231.1</td>
<td>29.2</td>
<td>-8.7</td>
<td>-21.8</td>
</tr>
<tr>
<td>OXL33</td>
<td>206.8</td>
<td>4.9</td>
<td>-14.6</td>
<td>-27.7</td>
</tr>
</tbody>
</table>

Table 5.1: Summary of day 6 cumulative food intake and bodyweight change data compared to vehicle. Numerical summary of the data presented in Figure 5.1. Bolded numbers in the difference from vehicle columns indicate analogues which increase food intake and decrease bodyweight compared to vehicle.

<table>
<thead>
<tr>
<th></th>
<th>0-7day FI (g)</th>
<th>Difference from Vehicle (g)</th>
<th>0-7day BW change (g)</th>
<th>Difference from Vehicle (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>197.6</td>
<td>16.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exendin</td>
<td>151.3</td>
<td>-46.3</td>
<td>1.3</td>
<td>-14.7</td>
</tr>
<tr>
<td>OXL12</td>
<td>219.1</td>
<td>21.5</td>
<td>11.5</td>
<td>-4.5</td>
</tr>
<tr>
<td>OXL13</td>
<td>205.6</td>
<td>8.0</td>
<td>15.0</td>
<td>-1.0</td>
</tr>
<tr>
<td>OXL14</td>
<td>225.2</td>
<td>27.7</td>
<td>2.6</td>
<td>-13.4</td>
</tr>
<tr>
<td>OXL16</td>
<td>201.5</td>
<td>3.9</td>
<td>16.5</td>
<td>0.5</td>
</tr>
<tr>
<td>OXL18</td>
<td>210.1</td>
<td>12.6</td>
<td>7.6</td>
<td>-8.4</td>
</tr>
<tr>
<td>OXL20</td>
<td>223.1</td>
<td>25.5</td>
<td>2.0</td>
<td>-14.0</td>
</tr>
<tr>
<td>OXL22</td>
<td>208.8</td>
<td>11.3</td>
<td>12.0</td>
<td>-4.0</td>
</tr>
<tr>
<td>OXL24</td>
<td>237.1</td>
<td>39.5</td>
<td>7.4</td>
<td>-8.6</td>
</tr>
<tr>
<td>OXL26</td>
<td>232.2</td>
<td>34.6</td>
<td>6.1</td>
<td>-9.9</td>
</tr>
<tr>
<td>OXL28</td>
<td>225.6</td>
<td>28.1</td>
<td>18.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 5.2: Summary of day 6 cumulative food intake and bodyweight change data compared to vehicle. Numerical summary of the data presented in Figure 5.2 Bolded numbers in the difference from vehicle columns indicate analogues which increase food intake and decrease bodyweight compared to vehicle.
5.2.2. Effects of OXL analogues on rat and human GLP-1 receptor and glucagon receptor mediated cAMP accumulation

The efficacy of select OXL analogues at the rat and human receptors was investigated in vitro by assessing their ability to stimulate cAMP production in cell lines over expressing either the rat or human glucagon or GLP-1 receptors. At both the rat and human receptors oxyntomodulin was 3-4 times more efficacious at the glucagon receptor compared to the GLP-1 receptor. However, oxyntomodulin had greater efficacy at both human receptors compared to the rat receptors (Table 5.3).

At both rat and human glucagon receptors all OXL analogues tested, except for OXL13, had similar activity to glucagon indicated by a ratio to the native peptide of approximately 1. These ratios were consistent between rat and human glucagon receptors. Analogue OXL13 was an exception, having 4 fold and 2 fold lower efficacy at the rat and human glucagon receptors respectively compared to glucagon.

All OXL analogues tested had lower efficacy than GLP-1 at both the rat and human GLP-1 receptors, however the ratios were not consistent between the rat and human GLP-1 receptors. Apart from OXL13, the tested OXL analogues had 1.5-6.3 fold lower efficacy at the rat GLP-1 receptor compared to the human GLP-1 receptor.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC$_{50}$ ratio to native peptide (arbitrary units)</th>
<th>Ratio GLP-1r: GCGr efficacy (arbitrary units)</th>
<th>EC$_{50}$ ratio to native peptide (arbitrary units)</th>
<th>Ratio GLP-1r: GCGr efficacy (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>Exendin</td>
<td>ND</td>
<td>0.9</td>
<td>ND</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1.0</td>
<td>313</td>
<td>1 : 313</td>
<td>1 : 313</td>
</tr>
<tr>
<td>OXL13</td>
<td>41</td>
<td>158</td>
<td>1 : 3.8</td>
<td>1 : 3.8</td>
</tr>
<tr>
<td>OXL14</td>
<td>2.1</td>
<td>54</td>
<td>1 : 26</td>
<td>1 : 15</td>
</tr>
<tr>
<td>OXL15</td>
<td>0.8</td>
<td>23</td>
<td>1 : 28</td>
<td>1 : 7</td>
</tr>
<tr>
<td>OXL21</td>
<td>1.4</td>
<td>68</td>
<td>1 : 50</td>
<td>1 : 8</td>
</tr>
<tr>
<td>OXL23</td>
<td>0.7</td>
<td>34</td>
<td>1 : 45</td>
<td>1 : 12</td>
</tr>
<tr>
<td>OXL31</td>
<td>1.7</td>
<td>67</td>
<td>1 : 40</td>
<td>1 : 15</td>
</tr>
</tbody>
</table>

Table 5.3: A summary of native peptide and OXL analogue receptor efficacy ratios.

EC$_{50}$ values for cAMP accumulation were measured using CHO cells over expressing the rat glucagon receptor (GCGr), human GCGr or human GLP-1 receptor (GLP-1r) or CHL cells over expressing the rat GLP-1r. Receptor efficacy at the glucagon receptors is expressed as a ratio of peptide EC$_{50}$ to glucagon EC$_{50}$ and to GLP1 EC$_{50}$ at the GLP-1 receptors. A ratio value below 1 indicates enhanced efficacy and above 1 decreased efficacy compared to the native peptide. A value above 1 for the GLP-1:GCGr efficacy ratio indicates that particular peptide has stronger activity at the glucagon receptor than GLP-1 receptor. Ratios for each peptide are calculated from the mean EC$_{50}$’s from a minimum of 3 independent experiments. ND – not determined, OXM – oxyntomodulin.
5.2.3. Affinity of OXL analogues at the rat, human and mouse glucagon receptors and GLP-1 receptors

The affinity of two representative OXL analogues was investigated at the rat, mouse and human receptors and compared to the native ligands. The binding ratios for oxyntomodulin compared to glucagon were similar at the mouse and human receptors, with oxyntomodulin having 7 fold and 8.5 fold lower affinity respectively. At the rat glucagon receptor oxyntomodulin had 3.5 fold lower affinity compared to glucagon (Table 5.3). Binding affinity ratios for the OXL analogues were equivalent to glucagon at both the rat and mouse glucagon receptors; however the affinity of OXL14 was approximately 3 fold greater than glucagon at the human glucagon receptor and the affinity of OXL15 was 1.5 fold lower than glucagon (Table 5.3).

At the human, rat and mouse GLP-1 receptor, oxyntomodulin and OXL analogues had lower affinity than GLP-1. There was a species difference in affinity with glucagon, oxyntomodulin and OXL analogues having lower affinity at the rat GLP-1 receptor compared to the human and mouse GLP-1 receptors (Table 5.4). OXL14 had 12 fold lower affinity at the rat GLP-1 receptor than the human receptor and OXL15 had 25 fold lower affinity (Table 5.4).

<table>
<thead>
<tr>
<th>GLP-1</th>
<th>Rat glucagon receptor</th>
<th>Human glucagon receptor</th>
<th>Mouse glucagon receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM) ± SEM</td>
<td>Ratio to native peptide</td>
<td>IC$_{50}$ (nM) ± SEM</td>
</tr>
<tr>
<td>Exendin</td>
<td>&gt;1000</td>
<td>ND</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1.2 ± 0.0</td>
<td>1.0</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>OXL14</td>
<td>4.3 ± 2.0</td>
<td>3.6</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>OXL15</td>
<td>1.0 ± 0.1</td>
<td>0.8</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.79 ± 0.07</td>
<td>0.7</td>
<td>1.3 ± 0.0</td>
</tr>
</tbody>
</table>

Table 5.4: A summary of mean IC$_{50}$ values for native peptides and analogues OXL14 & OXL15 at the rat, human and mouse glucagon receptors.

Purified cell membranes of CHO cells overexpressing the human or rat glucagon receptors, or purified mouse liver tissue were incubated with $^{125}$I-glucagon in the presence of increasing concentrations of unlabelled glucagon, GLP-1, oxyntomodulin, OXL14 or OXL15. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment. IC$_{50}$ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the peptide IC$_{50}$/glucagon IC$_{50}$. ND- not determined.
Table 5.5: A summary of mean IC₅₀ values for native peptides and analogues OXL14 & OXL15 at the rat, human and mouse GLP-1 receptors.

<table>
<thead>
<tr>
<th></th>
<th>rat GLP-1 receptor</th>
<th>human GLP-1 receptor</th>
<th>mouse GLP-1 receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (nM) ± SEM</td>
<td>ratio to native peptide</td>
<td>IC₅₀ (nM) ± SEM</td>
</tr>
<tr>
<td>GLP-1</td>
<td>0.18 ± 0.04</td>
<td>1.0</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Exendin</td>
<td>0.12 ± 0.07</td>
<td>0.7</td>
<td>0.53 ± 0.10</td>
</tr>
<tr>
<td>Glucagon</td>
<td>827 ± 27</td>
<td>4694</td>
<td>733 ± 54</td>
</tr>
<tr>
<td>OXM</td>
<td>355 ± 46</td>
<td>2016</td>
<td>142 ± 18</td>
</tr>
<tr>
<td>OXL14</td>
<td>35 ± 9</td>
<td>199</td>
<td>9.8 ± 2.2</td>
</tr>
<tr>
<td>OXL15</td>
<td>44 ± 11</td>
<td>248</td>
<td>6.1 ± 0.9</td>
</tr>
</tbody>
</table>

Purified cell membranes of CHO cells overexpressing the human or rat GLP-1 receptors, or purified mouse lung tissue were incubated with ¹²⁵I-GLP-1 in the presence of increasing concentrations of unlabelled GLP-1, oxyntomodulin, OXL14 or OXL15. Unlabelled peptide concentrations were tested in duplicate or triplicate in each experiment. IC₅₀ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the peptide IC₅₀/GLP-1 IC₅₀.

5.2.4. Chronic dose response effects of OXL analogues on food intake and bodyweight in rats

The effect of daily administration of various doses of OXL14 and OXL15 on food intake and bodyweight was investigated in male Wistar rats. Daily 25nmolkg⁻¹ and 50nmolkg⁻¹ doses of OXL14 increased 6 day cumulative food intake by 11.5% and 12.2% respectively compared to vehicle controls, although this was not significant (Mean 6 day food intake; Vehicle: 197.6 ± 7.9g, OXL14 25nmolkg⁻¹: 220.3 ± 4.8g, P =0.506, 50nmolkg⁻¹: 21.7 ± 11.2g, P =0.449) (Figure 5.3A). Animals administered 100nmolkg⁻¹ of OXL14 ate 23.3% less food over 6 days than vehicle treated animals (Mean 6 day food intake; Vehicle: 197.6 ± 7.9g, OXL14 100nmolkg⁻¹: 151.5 ± 21.7g, P =0.031) (Figure 5.3A). Daily doses of 50nmolkg⁻¹ and 100nmolkg⁻¹ OXL14 significantly reduced bodyweight by 5.3% and 13.6% respectively compared to vehicle controls (Mean 6 day weight change; Vehicle: 11.9 ± 2.9g, OXL14 50nmolkg⁻¹: -19.3 ± 7.6g, P =0.041, 100nmolkg⁻¹: -68.0 ± 18.6g, P <0.0001) (Figure 5.3B). OXL15 at a daily dose of 25nmolkg⁻¹ increased food intake by 8% compared to vehicle controls, although this was not significant (Mean 6 day food intake; Vehicle: 215.4 ± 6.8g, OXL15 25nmolkg⁻¹: 232.6 ± 6.7g, P =0.385) (Figure 5.4A). OXL15 50nmolkg⁻¹ non-significantly reduced 6 day cumulative food intake by 11.6% compared to vehicle (Mean 6 day food intake; Vehicle: 215.4 ± 6.8g, OXL15 50nmolkg⁻¹: 190.2 ± 6.0g, P =0.125) (Figure 5.4A). OXL15 35nmolkg⁻¹ and 50nmolkg⁻¹ significantly reduced bodyweight by 4.5% and 8.2% respectively compared to vehicle treated animals (Mean 6 day weight change; Vehicle: 14.4 ± 1.0g, OXL15 35nmolkg⁻¹: -10.7 ± 6.0g, P<0.0001, 50nmolkg⁻¹: -31.1 ± 4.8g, P<0.0001) (Figure 5.4B).
Figure 5.3: Effect of chronic OXL14 dose response on food intake and bodyweight in rats.
6 day cumulative food intake (A) and bodyweight change (B) in male Wistar rats in response to daily injections of various doses of OXL14 or vehicle. Mean starting weight 577g; n= 8-9/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Dunnett correction. *P <0.05, **P <0.001 versus vehicle controls.
Figure 5.4: Effect of chronic OXL15 dose response on food intake and bodyweight in rats.  
6 day cumulative food intake (A) and bodyweight change (B) in male Wistar rats in response to daily injections of various doses of OXL15 or vehicle. Mean starting weight 542g; n= 8-10/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Dunnett correction. ***P <0.001 versus vehicle controls.
5.2.5. Chronic OXL analogue administration does not increase food intake in mice

Daily administration of various doses of OXL14 did not significantly increase food intake compared to vehicle controls in mice (Figure 5.5A). Daily 100nmolkg$^{-1}$ OXL14 significantly reduced 6 day bodyweight by 4.8% compared to vehicle treated controls (Figure 5.5B, $P=0.031$).

![Figure 5.5: Effect of chronic OXL14 dose response on food intake and bodyweight in mice.](image)

6 day cumulative food intake (A) and bodyweight change (B) in male C57BL/6 mice in response to daily injections of various doses of OXL14 or vehicle. Mean starting weight 37.9; n=7-8/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Dunnett correction. *$P<0.05$ versus vehicle controls.
5.2.6. OXL analogues do not increase acute food intake in rats

A single injection of various doses of OXL15 did not cause a consistent, significant increase in acute food intake in *ad libitum* fed rats during the dark phase (Figure 5.6) or in fasted rats during the light phase (data not shown).

Figure 5.6: Effect of OXL15 dose response on acute food intake in fed rats.
Acute food intake in male Wistar rats in response to a single SC injection of various doses of OXL15 or vehicle. Peptide was administered immediately prior to the onset of the dark phase in fed animals. Food intake measurements were carried out throughout the dark phase and interval food intake data calculated for A) 0-1 hour, B) 1-2 hours, C) 2-4 hours and D) 4-8 hours post injection. Mean starting weight 597g; n= 7-8/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Dunnett correction. **P <0.01, ***P <0.001 versus vehicle controls.
5.2.7. Effect of chronic administration of Glu-3 OXL analogues on food intake and bodyweight in rats

Substituting glutamate (Glu/E) for glutamine (Gln/Q) at residue 3 of oxyntomodulin and other dual glucagon and GLP-1 receptor analogues has previously been shown to greatly diminish glucagon receptor activity without affecting GLP-1 receptor activity (Pocai et al. 2009; Kosinski et al. 2012). The Glu-3 equivalent analogues of OXL14 and OXL15, referred to as OXL14E3 and OXL15E3 respectively, were synthesised and used to investigate the contribution of glucagon receptor activity to the increase in food intake observed in rats. The rat GLP-1 receptor efficacy of OXL14E3 was 174 fold lower than OXL14 and the efficacy of OXL15E3 was 26 fold lower than OXL15 (Table 5.6).

<table>
<thead>
<tr>
<th></th>
<th>Glu-3</th>
<th>GLP-1</th>
<th>Glu-3</th>
<th>GLP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>&gt;1000</td>
<td>0.07</td>
<td>± 0.00</td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.28</td>
<td>20</td>
<td>± 6</td>
<td></td>
</tr>
<tr>
<td>OXL14</td>
<td>0.23</td>
<td>1.5</td>
<td>± 0.2</td>
<td></td>
</tr>
<tr>
<td>OXL14E3</td>
<td>40</td>
<td>2.2</td>
<td>± 0.1</td>
<td></td>
</tr>
<tr>
<td>OXL15</td>
<td>0.38</td>
<td>4.4</td>
<td>± 1.2</td>
<td></td>
</tr>
<tr>
<td>OXL15E3</td>
<td>10</td>
<td>7.0</td>
<td>± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.6: A summary of mean EC50’s for the native peptides, OXL analogues and Glu-3 analogues at the rat glucagon receptor and GLP-1 receptor.

As shown previously, daily administration of 25nmolkg⁻¹ OXL14 and OXL15 significantly increased 6 day food intake by 18.6% and 16.3% respectively (Mean 6 day food intake; Vehicle: 213.9 ± 7.8g, OXL14: 253.9 ± 16.0g, \( P = 0.021 \), OXL15: 248.7 ± 6.9g, \( P = 0.038 \)) (Figure 5.7A), whereas the Glu-3 equivalent peptides OXL14E3 and OXL15E3 did not significantly alter food intake compared to vehicle or their respective Gln-3 peptides (Mean 6 day food intake; OXL14E3: 232.3 ± 7.8g, \( P = 0.561 \), OXL15E3: 227.6 ± 3.7g, \( P = 0.829 \)) (Figure 5.7A). Animals administered 25nmolkg⁻¹ OXL14E3 gained significantly more weight than vehicle controls and OXL14 treated animals (Mean 6 day weight change; Vehicle: 12.9 ± 2.7g, OXL14: 9.2 ± 3.8g, OXL14E3: 27.5 ± 2.2g, \( P = 0.006 \) versus, \( P = 0.0019 \) versus OXL14) (Figure 5.7B). OXL15E3 treated animals gained significantly more weight than OXL15 treated animals (Mean 6 day weight change; OXL15: 4.5 ± 2.7g, OXL15E3: 19.0 ± 3.3g, \( P = 0.012 \)) (Figure 5.7B).
Figure 5.7: Effect of chronic OXL analogue or Glu-3 analogue administration on food intake and bodyweight in rats.

6 day cumulative food intake (A) and bodyweight change (B) in male Wistar rats in response to daily injections of vehicle or 25nmolkg⁻¹ of OXL14 or OXL15 or their respective Glu-3 equivalents OXL14E3 and OXL15E3. Mean starting weight 468g; n= 7-8/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Bonferroni correction. *P <0.05, **P <0.01 versus vehicle controls; *P <0.05 versus OXL15, ##P <0.01 versus OXL14.
5.3. Discussion

The aim of the work detailed in this chapter was to investigate the mechanism behind the increase in food intake generated by lower doses of oxyntomodulin-like analogues and to assess the impact of this upon the design and use of these analogues to treat obesity.

There are several possible reasons why numerous studies may not have previously observed or reported the increase in food intake that has been demonstrated in this chapter. Indeed a wide range of studies using native oxyntomodulin and analogues with extended half-lives have consistently reported a reduction in food intake after repeated peripheral administration in rodents (Dakin et al. 2004; Pocai et al. 2009; Kerr et al. 2010; Liu et al. 2010; Day et al. 2012) and humans (Wynne et al. 2005; Wynne et al. 2006a).

Studying the effects of chronic administration of oxyntomodulin, GLP-1 and glucagon is inherently challenging. The peptides have short circulatory half-lives under 10 minutes (Kervran et al. 1990; Vilsboll et al. 2003a) making repeated daily dosing necessary to achieve 24 hour coverage; this involves significant handling and consequential animal stress which could mask a small increase in food intake. All three native peptides are relatively unstable in solution and glucagon in particular is known to form fibrils (Pedersen et al. 2006; Pedersen 2010), subsequently limiting the use of continuous administration systems such as Alzet pumps. These limitations of the native peptides are possibly one reason why numerous feeding studies have not reported an increase in food intake.

As observed with the detailed dose response studies completed with several oxyntomodulin analogues in this chapter, there is a narrow dose range where food intake is increased. Indeed, higher doses used to achieve significant weight loss are likely to exceed the dose range where food intake is increased. While dose comparisons are difficult to make between studies, particularly for those using analogues, higher doses were administered in numerous prior studies investigating the effect of oxyntomodulin and its analogues on energy homeostasis (Dakin et al. 2004; Kerr et al. 2010; Kosinski et al. 2012). This could be another reason why previous studies have not observed or reported an increase in food intake; as the studies detailed in this chapter show that higher doses of the analogues caused a reduction in food intake. Liu et al (2010) did administer their oxyntomodulin analogue to rats at lower doses, however their particular analogue had a much greater efficacy at the GLP-1 receptor, compared to the oxyntomodulin analogues in the current study. Subsequently any potential increase in food intake could have been masked by enhanced GLP-1 receptor activity causing a dominant reduction in food intake.
The increase of food intake was only observed after several days of repeat administration, and only observed in rats. As the majority of previous work investigating the effects of oxyntomodulin and analogues on energy homeostasis has been conducted in mice (Pocai et al. 2009; Heppner et al. 2010; Day et al. 2012; Lynch et al. 2014) this may be yet another reason why increased food intake has not previously been reported. In the current work, acute feeding studies in both ad libitum fed rats at the onset of the dark phase and fasted rats in the early light phase did not demonstrate over eating. Finally, an increase in food intake was not demonstrated in mice in any of the various study paradigms used in the current work, indicating that the increase in rats may be a species specific effect.

In the initial feeding studies, all groups administered OXL analogues demonstrated a reduction in bodyweight similar to that of the exendin-4 group, despite eating significantly more food (Figure 5.1, Figure 5.2, Table 5.1 & Table 5.2). Exendin-4 is a naturally occurring, GLP-1 receptor selective agonist with a longer half-life than GLP-1 and the weight loss caused by administration of this peptide has been shown to be mainly due to reduction in food intake (Szayna et al. 2000; Yang et al. 2014). In the current study, several oxyntomodulin analogues generated weight loss equivalent to that of exendin-4 treatment, despite exendin-4 treated animals eating significantly less food. As previously reported in the literature oxyntomodulin causes an increase in energy expenditure (Dakin et al. 2001; Dakin et al. 2002; Wynne et al. 2006), which could account for the weight loss without reduced food intake seen with several analogues in the current study. However, in this chapter I also report a number of analogues which cause an increase in food intake, in particular OXL15 and OXL14. The finding that these analogues increased food intake despite no significant reduction in bodyweight (Figure 5.1, Table 5.1, Figure 5.2 & Table 5.2) opens up the possibility that the increase could be a result of a direct stimulatory effect of the analogue on food intake, rather than a compensatory response to an increase in energy expenditure. An alternative possibility is that greater weight loss may have been prevented by the increase in food intake.

The oxyntomodulin analogues investigated in the current work bind to and activate the glucagon and GLP-1 receptors with greater affinity and efficacy than native oxyntomodulin (Table 5.3, Table 5.4 & Table 5.5). There is a paucity of information in the literature comparing native oxyntomodulin activity at the rat glucagon and GLP-1 receptors. Similar to previous findings at the human (Kerr et al. 2010) and mouse (Kosinski et al. 2012) receptors, the current work demonstrated that oxyntomodulin and OXL analogue activity at the rat receptors favoured glucagon receptor activity (Table 5.3).
Peptide affinity and activity were also studied at the mouse and human receptors to investigate whether analogue effects were similar across species. Interestingly, oxyntomodulin and the majority of the OXL analogues tested in the *in vitro* cAMP assay had lower efficacy at the rat GLP-1 receptor compared to the human GLP-1 receptor, whereas glucagon receptor efficacy was relatively consistent across species (Table 5.3). Similarly oxyntomodulin, OXL14 and OXL15 had much lower binding affinity at the rat GLP-1 receptor than either the human or the mouse GLP-1 receptors; although affinity was better at the human receptor than the mouse (Table 5.5). It seems that in this instance activity and binding data are consistent and indicate reduced affinity and efficacy at the rat GLP-1 receptors compared to the human and mouse receptors. It is unlikely that poor affinity and efficacy at the rat GLP-1 receptors is an artefact of the CHL over expressing cell line, as similar results were observed using membranes isolated from rat lung tissue in the binding affinity experiments (data not shown). No increase in food intake was seen in any of the mouse feeding studies, which could be a result of the differential affinity at the mouse GLP-1 receptor compared to the rat receptor. Further investigation of oxyntomodulin analogue activity using cell lines over-expressing the mouse glucagon receptor or GLP-1 receptor would be necessary to further investigate this. As the receptor binding at the human GLP-1 receptor was more similar to that of the mouse receptor this may suggest that no increase in food intake would be observed after oxyntomodulin analogue administration in humans. However it is not clear from the current results whether *in vitro* activity correlates with *in vivo* food intake.

Detailed dose response studies were conducted with two representative analogues to investigate whether the increase in food intake was dose dependent. There was a trend towards an increase in food intake with lower doses of both OXL14 and OXL15 up to a ‘threshold dose’, beyond which food intake was then decreased (Figure 5.3A & Figure 5.4A). A possible hypothesis is that low dose glucagon receptor activity increases food intake and the threshold point may reflect the dose at which there is sufficient GLP-1 receptor activity to overcome this and cause a reduction in food intake. Both OXL14 and OXL15 caused a dose dependent reduction in bodyweight (Figure 5.3B & Figure 5.4B). At lower doses, food intake increased as bodyweight decreased (Figure 5.4 & Figure 5.5), indicating that below the ‘threshold point’ bodyweight loss was likely to be due to the proposed increase in energy expenditure, mediated via glucagon receptor activity. There appears to be a difference in threshold dose between OXL14 and OXL15, with a higher dose of OXL14 required to cause a reduction in food intake. It is possible this can be attributed to differences in their ratio of rat GLP-1 receptor to rat glucagon receptor activity (Table 5.3). However, The ratio of rat GLP-1:glucagon receptor efficacy was approximately 2 fold lower for OXL14 compared to OXL15.
indicating OXL14 had greater GLP-1 receptor activity (Table 5.3) which would suggest a lower dose would be required to reduce food intake.

Indeed for several OXL analogues investigated in this chapter in vitro EC₅₀ ratios appeared to be poor predictors of in vivo effects on food intake and bodyweight. OXL15 had similar activity at both rat receptors as OXL23 (Table 5.3) as well as a similar pharmacokinetic profile (data not shown); however OXL15 significantly increased food intake despite generating less weight loss than OXL23 (Figure 5.1). Analogue OXL31 had 4 fold greater efficacy at the rat GLP-1 receptor than OXL15 (Table 5.3), therefore it might be expected that there would be greater reduction in food intake with OXL31 administration. However there was a similar increase in food intake and decrease in bodyweight with both peptides (Figure 5.1A). These results suggest that the underlying receptor interactions involved with oxyntomodulin-like dual analogues, and probably oxyntomodulin, are complex and possibly include an interaction which is not represented by their relative in vitro receptor efficacies. Co-administration of glucagon and GLP-1 in rodents (Parker et al. 2013) and man (Cegla et al. 2014) appears to produce synergistic effects on food intake, indicating probable interaction of the two peptides. A number of intracellular accessory proteins can interact with GPCRs and alter their activity (Magalhaes et al. 2012). Recently there has been interest in the effects of the arrestin family of adaptor proteins which are recruited to, and deactivate, GPCRs. Differential recruitment of β-arrestins to the activated GLP-1 receptor may result in signalling bias which alters the effects of the ligand (Jorgensen et al. 2007; Appleton & Luttrell 2013). A recent study suggested that ligand binding and activation at the glucagon receptor is modified by receptor-activity modifying proteins (RAMPs) (Weston et al. 2015). Neither of these protein families is specifically expressed in the cell lines used, which could be one explanation for the differences between in vitro and in vivo findings.

Further investigation of the mechanism behind the increase in food intake was difficult for several reasons. Firstly, not being able to reproduce the increase in food intake in mice prevented the use of transgenic glucagon receptor or GLP-1 receptor knockout mice. Secondly, it was not possible to identify an acute time period in any of the rat feeding studies where food intake was consistently increased. This prevented the use of glucagon receptor and GLP-1 receptor antagonists, as a 6 day chronic study would have required a large amount of antagonists and multiple daily injections due to their short half-lives and instability in solution. Using antagonists of the glucagon receptor or GLP-1 receptor would also block the activity of the endogenous hormones which would limit the conclusions that could be drawn from these studies.

Previous studies had reported the use of oxyntomodulin analogues with a glutamate substitution at amino acid position 3 as a tool to investigate the contribution of glucagon receptor activity to the
effects on food intake and bodyweight (Pocai et al. 2009). Changing the position 3 glutamine (Gln/Q) of oxyntomodulin to glutamate (Glu/E) (as found in GLP-1 and its natural analogue exendin-4) severely diminished glucagon receptor activity without affecting the activity at the GLP-1 receptor, essentially producing a GLP-1 receptor selective analogue (Du et al. 2012; Kosinski et al. 2012). Comparing the effects of OXL analogues with their Glu-3 counterparts in a chronic study therefore enabled investigation of the contribution of glucagon receptor activity to oxyntomodulin analogue function. In the current study Glu-3 analogues OXL14E3 and OXL15E3, with reduced glucagon receptor activity, did not increase food intake suggesting that a glucagon receptor mediated mechanism is involved. One previous study has reported a hyperphagic response to IP glucagon administration in rats and the authors attributed this to the low dose of 50µg kg\(^{-1}\) (14nmol kg\(^{-1}\)) that was used (Hell & Timo-Iaria 1985). However, a number of other studies have found that similar low doses of glucagon reduce food intake in humans (Geary et al. 1992) and rodents (Geary & Smith 1982b; Geary et al. 1993; Parker et al. 2013), although several of these administered glucagon into the rodent portal circulation rather than SC or IP. Intra-portal administration would have caused higher glucagon concentrations local to the liver, which may have resulted in different effects on food intake. No previous studies have investigated the effects of chronic exposure to glucagon on rodent food intake, probably due to the difficulties imposed by glucagon’s short half-life and instability in solution.

Interestingly, in the current study bodyweight was significantly increased by both Glu-3 peptides, despite there being no increase in food intake (Figure 5.7). None of the previous studies have reported an increase in bodyweight with Glu-3 peptides; however they were conducted in mice rather than rats (Pocai et al. 2009; Kosinski et al. 2012). This suggests that the increased bodyweight may also be a species specific effect. The effects of the Glu-3 peptides will be investigated further in Chapter 6.

A direct method to examine the relationship between increased food intake and energy expenditure would be to use metabolic CLAMS cages. Several studies to investigate the mechanism of increased food intake failed to establish a dose of oxyntomodulin analogues which reliably increased food intake when the rats were in the CLAMS cages. The variation in food intake was likely a consequence of the increased stress associated with acclimatisation to the CLAMS environment (Martí et al. 1994). A further difficulty with the experimental design of CLAMS studies is the limited number of cages and study slots which makes achieving a suitably powered study to observe a small effect challenging. For future work, an extensive cross-over dose range finding study will be carried out to try to establish a suitable dose to investigate the mechanism of increased food intake using the
CLAMS. Another future study will investigate if a thermal imaging camera is adequately sensitive to detect and quantify levels of activated BAT in rats administered oxyntomodulin analogues. Increased BAT activation is an indirect indicator of an increase in energy expenditure, therefore if increased BAT activation was observed in over-eating animals this could suggest that increased energy expenditure was driving the hyperphagia.

Measuring BAT weight and expression levels of markers of BAT activation, such as UCP1 levels, would provide other indirect indicators of increased energy expenditure, again providing a mechanism by which the hyperphagia could be linked to increased energy expenditure. Ideally this would be carried out using rodents housed at thermoneutral temperatures of approximately 30°C, in order to prevent the BAT activation that is likely to occur at the considerably lower normal housing temperature of 22°C.

Measuring neuronal activation within hypothalamic and brainstem regions known to be involved in appetite regulation is a common method used to investigate peptide effects on feeding. Investigating the levels of c-Fos in these brain areas after high and low doses of oxyntomodulin analogues that do and don’t increase food intake may provide information as to which regions, if any, are differentially activated or deactivated by analogues which increase food intake.

5.3.1. Conclusion

The results shown in this chapter demonstrate that in rats low doses of oxyntomodulin analogues generate an increase in food intake with a concomitant significant reduction in bodyweight. The increase in food intake appears to be dependent on glucagon receptor activity; however investigating the exact mechanism involved is difficult due to the lack of increased food intake in mice and no acute increase in rats. It is unlikely that these findings will negatively impact the design and use of oxyntomodulin analogues for obesity treatment in humans as results indicate that increased food intake may be a rat specific species effect. Increased food would not necessarily be a problem in humans; nausea limits the dose of GLP-1 analogues that can be used in man (Fineman et al. 2004) and this could theoretically be less with agents that increase appetite. The findings do highlight the importance of testing analogues in more than one species, particularly for dual analogues where differential efficacy may occur.
Chapter 6

Glucagon receptor antagonism with Glu-3 oxyntomodulin-like analogues
6.1. Introduction

The gut derived hormone oxyntomodulin is a naturally occurring dual agonist of the glucagon receptor and GLP-1 receptor. Administration of oxyntomodulin to rodents and humans reduces food intake and increases energy expenditure, generating significant weight loss and highlighting oxyntomodulin as a potential pharmacological treatment for obesity (Dakin et al. 2004; Wynne et al. 2006a). However, the short half-life of oxyntomodulin (Kervran et al. 1990) and its reduced receptor efficacy and affinity compared to native glucagon and GLP-1 (Dakin et al. 2001; Jorgensen et al. 2007; Druce et al. 2009; Kosinski et al. 2012), necessitate the design of long lasting efficacious analogues for clinical use.

The lack of a specific oxyntomodulin receptor coupled with the peptide’s agonist activity at both the glucagon receptor and GLP-1 receptor makes it difficult to delineate the mechanisms related to the specific effects of endogenous oxyntomodulin. Similarly, it is difficult to assess the contribution of glucagon receptor activity and GLP-1 receptor activity to the effects of exogenously administered oxyntomodulin and synthetic dual agonists, as using glucagon receptor and GLP-1 receptor specific antagonists also blocks the actions of the endogenous hormones. Glucagon administration increases energy expenditure (Davidson et al. 1960; Tan et al. 2013) and may increase satiety (Martin & Novin 1977; Geary et al. 1992; Parker et al. 2013) (see section 1.4.3.4.2), whereas GLP-1 administration potently decreases food intake (Rodriquez de Fonseca et al. 2000; Verdict et al. 2001) but effects on energy expenditure are unclear (Baggio et al. 2004a; Beiroa et al. 2014; Heppner et al. 2015b) (see section 1.4.2.4.1). Administration of GLP-1 and GLP-1 analogues is associated with high incidence of nausea and vomiting (Parkinson et al. 2009; Lean et al. 2014). Therefore it is important to be able to assess the contribution of activity at the glucagon receptor and GLP-1 receptor to the weight loss generated by administration of synthetic dual analogues, in order to maximise weight loss and minimise food intake reduction and nausea.

The substitution of glutamine at position 3 of oxyntomodulin (Gln/Q) with glutamate (Glu/E) has previously been reported to diminish glucagon receptor activity without affecting GLP-1r activity (Pocai et al. 2009; Kosinski et al. 2012). These Glu-3 analogues have been utilised in mice to investigate the contribution of glucagon receptor activity and GLP-1 receptor activity to the function of both native oxyntomodulin (Du et al. 2012; Kosinski et al. 2012) and a derivatised analogue (Pocai et al. 2009). Previous results using Glu-3 analogues have indicated that oxyntomodulin activity at the GLP-1 receptor is responsible for reduction in food intake and activity at the glucagon receptor increases energy expenditure (Kosinski et al. 2012).
Glu-3 versions of several oxyntomodulin-like analogues were synthesised to enable comparison of the weight loss achieved with and without glucagon receptor activity, in order to investigate the relative contribution of food intake reduction and energy expenditure. As reported in Chapter 5, an increase in bodyweight was observed following chronic administration of two Glu-3 oxyntomodulin-like analogues in male Wistar rats, without a change in food intake (Figure 6.1).

### 6.1.1. Aims

- To investigate whether the increase in bodyweight following Glu-3 administration is a dose dependent effect
- To investigate the potential mechanism behind the increase in bodyweight

For details of the main materials and methods used please see Chapter 2. Please refer to Appendix A for the sequences of peptides investigated in this chapter.
Figure 6.1: The effects of chronic OXL and OXLE3 analogue administration on food intake and bodyweight in rats.
6 day cumulative food intake (A) and bodyweight change (B) in response to daily injections of vehicle or 25nmolkg⁻¹ of OXL14 or OXL15 or their respective Glu-3 equivalents OXL14E3 and OXL15E3 in male Wistar rats. Mean starting weight 468g; n = 7-8/group. Data represent mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Bonferroni correction. *P <0.05, **P <0.01 versus vehicle controls; #P <0.05 versus OXL15, ##P <0.01 versus OXL14.
6.2. Methods

6.2.1. In vitro glucagon receptor antagonism

Cells were cultured and maintained as described in Chapter 2 (Section 2.4.1).

For glucagon receptor antagonism studies CHO cells overexpressing either the rat glucagon receptor or human glucagon receptor were seeded at a density of 150 000 cells/ml in their respective supplemented DMEM, 250µl/well of a 48 well plate (Nunc, VWR International Inc, Chicago, USA), then incubated for 24 hours. Cells were serum starved in DMEM + 1% antibiotic (100U/ml Penicillin and 100µg/ml Streptomycin, Invitrogen) for 1 hour prior to 30 minutes incubation at room temperature with peptide concentrations diluted in serum free DMEM with 1mM IBMX (Sigma-Aldrich) and either 0.1nM glucagon (rat glucagon receptor cells) or 0.02nM glucagon (human glucagon receptor cells). Five wells of each concentration and 12 wells of 0.1nM or 0.02nM glucagon alone were applied per experiment.

Post incubation, medium was removed and cells lysed with 120µl/well of 0.1M HCL + 0.5% Triton-X. cAMP levels were measured using an indirect ELISA according to the manufacturer’s instructions (ADI-900-066, Enzo Life Sciences, UK), optical density was read at 405nm on a Biotek ELx808 (Wolf Laboratories, York, UK).

6.3. Results

6.3.1. Chronic dose response effects of Glu-3 analogue administration on food intake and bodyweight in rats

Daily SC administration of low doses of OXL15E3 in slow release formulation dose dependently increased bodyweight (Figure 6.2 A & B) in male Wistar rats without altering food intake (Figure 6.2 C & D). Doses of 10nmolkg⁻¹ and 20nmolkg⁻¹ OXL15E3 administered daily for 6 days significantly increased bodyweight compared to vehicle controls (Day 6 weight change from initial, Vehicle: 7.9 ± 2.0g; 10nmolkg⁻¹: 15.1 ± 1.9g, P <0.01; 20nmolkg⁻¹: 17.7 ± 1.5g, P <0.001) (Figure 6.2 A & B). Bodyweight was increased from day 2 compared to vehicle control following doses of 10- or 20nmolkg⁻¹ OXL15E3 and significantly increased from day 5 (P<0.05) and day 4 (P <0.05) respectively. Doses of 30- and 50nmolkg⁻¹ did not significantly alter bodyweight (Day 6 weight change from initial, Vehicle: 7.9 ± 2.0g; 30nmolkg⁻¹: 10.4 ± 2.4g, P =0.5093; 50nmolkg⁻¹: 8.3 ± 1.5g, P =0.9992).
Figure 6.2: Dose response effects of chronic Glu-3 analogue administration.
Bodyweight and food intake following daily SC administration of various doses of OXL15E3 or vehicle in Wistar rats. (A) Cumulative bodyweight change; (B-insert) Total 6 day bodyweight change; (C) cumulative food intake; (D-insert) total 6 day food intake. Mean starting weight 542g; n=11-12/group. Data represent mean ± SEM. Statistical analysis was carried out using 2-way repeated measures ANOVA and post hoc tests with Bonferroni correction. *P <0.05, **P <0.01, ***P <0.001 versus vehicle controls.
6.3.2. Affinity and efficacy of select Glu-3 analogues at the rat glucagon receptor and rat GLP-1 receptor

The efficacy and affinity of the Glu-3 analogues OXL14E3, OXL15E3 and OXME3, the Glu-3 equivalent of native oxyntomodulin, were investigated at the rat glucagon receptor and GLP-1 receptor. The efficacy and affinity of OXL14, OXL15, OXL14E3 and OXL15E3 were similar to that reported in Chapter 5. The efficacy and affinity of glucagon, GLP-1 and oxyntomodulin were similar to that reported throughout this thesis.

At the rat glucagon receptor the efficacy of OXL14E3 was 150 fold lower compared to OXL14 and the efficacy of OXL15E3 was 32 fold lower compared OXL15. The efficacy of OXME3 was 15 fold lower than native oxyntomodulin at the rat glucagon receptor. Glu-3 substitution had minimal effect on efficacy at the rat GLP-1 receptor (Table 6.1).

At the rat glucagon receptor the affinity of OXL14E3 was 120 fold lower compared to OXL14 and the efficacy of OXL15E3 was 63 fold lower compared OXL15. The efficacy of OXME3 was 31 fold lower than native oxyntomodulin at the rat glucagon receptor. Glu-3 substitution had a small positive effect on affinity at the rat GLP-1 receptor. The affinity of OXL14E3 and OXL15E3 was approximately 4 fold greater than OXL14 and OXL15 respectively. The affinity of OXME3 at the rat GLP-1 receptor was approximately 3 fold greater than native oxyntomodulin (Table 6.1).

<table>
<thead>
<tr>
<th>Rat GCGr</th>
<th>Rat GLP-1r</th>
</tr>
</thead>
<tbody>
<tr>
<td>**EC&lt;sub&gt;50&lt;/sub&gt; ± SEM</td>
<td>**IC&lt;sub&gt;50&lt;/sub&gt; ± SEM</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>OXM</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>OXME3</td>
<td>160 ± 52</td>
</tr>
<tr>
<td>OXL14</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>OXL14E3</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>OXL15</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>OXL15E3</td>
<td>11 ± 1</td>
</tr>
</tbody>
</table>

Table 6.1: A summary of Glu-3 peptide efficacy and affinity at the rat glucagon receptor and rat GLP-1 receptor.

EC<sub>50</sub>s were calculated using a cAMP bioactivity assay and CHO cells over-expressing the rat glucagon receptor or CHL cells overexpressing the rat GLP-1 receptor. To calculate the IC<sub>50</sub>s membranes were prepared from these cells and incubated with <sup>125</sup>I-glucagon or <sup>125</sup>I-GLP-1 respectively, in the presence of increasing concentrations of the unlabelled peptides. All peptide concentrations were tested in duplicate or triplicate per experiment. Mean EC<sub>50</sub> and IC<sub>50</sub> values for the natural ligands, Glu-3 analogues and their comparative Gln-3 peptides are shown as the mean of at least 3 separate experiments ± SEM. OXM – oxyntomodulin, GCGr – glucagon receptor, GLP-1r – GLP-1 receptor.
6.3.3. Glu-3 analogue antagonism of the rat glucagon receptor *in vitro*

The ability of Glu-3 analogues OXL14E3, OXL15E3 and OXME3 (the Glu-3 analogue of native oxyntomodulin) to antagonise a glucagon stimulated increase in cAMP was investigated utilising CHO cells over-expressing the rat glucagon receptor. Concentrations of 0.03-1nM of OXL14E3 and 0.03-0.3nM OXL15E3 decreased the percentage of glucagon (0.1nM) mediated cAMP production by a mean of 18.4% and 27.5% respectively (Figure 6.3 A & B respectively). Concentrations of 3- and 30nM OXL14E3 and OXL15E3 increased cAMP levels compared to glucagon stimulation alone (OXL14E3 30nM: 155% increase, OXL15E3: 214% increase Figure 6.3 A & B respectively). Except 30nM, all concentrations of OXME3 tested decreased glucagon (0.1nM) mediated cAMP accumulation by a mean of 40.3%. Levels of cAMP were increased by 9% following co-incubation with 30nM OXME3 and 0.1nM glucagon (Figure 6.3 C).
Figure 6.3: In vitro Glu-3 analogue antagonism of the rat glucagon receptor.
The percentage change in 0.1nM glucagon stimulated cAMP accumulation in CHO cells over-expressing the rat glucagon receptor following co-incubation with various concentrations of Glu-3 analogues (A) OXL14E3, (B) OXL15E3, (C) OXME3. Data is expressed as a percentage of glucagon mediated cAMP accumulation ± SEM and results are from 3 separate cell experiments.
6.3.4. Affinity and efficacy of select Glu-3 analogues at the human glucagon receptor

The efficacy and affinity of the Glu-3 analogues OXL14E3, OXL15E3, OXLT10E3 and GCGE3, the Glu-3 equivalent of native glucagon, were investigated at the human glucagon receptor and GLP-1 receptor. The efficacy and affinity of OXL14 and OXL15 was similar to that reported in Chapter 5. The efficacy and affinity of glucagon and GLP-1 were similar to that reported throughout this thesis.

At the human glucagon receptor the efficacy of OXL14E3 was 390 fold lower compared to OXL14 and the efficacy of OXL15E3 was 32 fold lower compared OXL15. The efficacy of GCGE3 was 560 fold lower than native glucagon at the human glucagon receptor. Glu-3 substitution had minimal effect on efficacy at the human GLP-1 receptor (Table 6.1).

At the human glucagon receptor the affinity of OXL14E3 was 304 fold lower compared to OXL14 and the affinity of OXL15E3 was 63 fold lower compared OXL15. Glu-3 substitution had minimal effect on affinity at the human GLP-1 receptor (Table 6.2).

<table>
<thead>
<tr>
<th></th>
<th>Human GCGr</th>
<th>Human GLP-1r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 ± SEM (nM)</td>
<td>IC50 ± SEM (nM)</td>
</tr>
<tr>
<td>GLP-1</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.01 ± 0.00</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td>GCGE3</td>
<td>5.6 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>OXL14</td>
<td>0.01 ± 0.00</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>OXL14E3</td>
<td>3.9 ± 0.4</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>OXL15</td>
<td>0.01 ± 0.00</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>OXL15E3</td>
<td>0.60 ± 0.02</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>OXLT10</td>
<td>0.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>OXLT10E3</td>
<td>9.0 ± 3.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2: A summary of Glu-3 peptide efficacy and affinity at the human glucagon receptor.

Mean EC50s were calculated using a cAMP bioactivity assay and CHO cells over-expressing the human glucagon receptor or the human GLP-1 receptor. To calculate the mean IC50s membranes were prepared from these cells and incubated with 125I-glucagon or 125I-GLP-1 respectively, in the presence of increasing concentrations of the unlabelled peptides. All peptide concentrations were tested in duplicate or triplicate per experiment. Mean EC50 and IC50 values for the natural ligands, Glu-3 analogues and their comparative Gin-3 peptides are shown as the mean of at least 3 separate experiments ± SEM. OXM – oxyntomodulin, GCGr – glucagon receptor, GLP-1r – GLP-1 receptor.
6.3.5. Glu-3 analogue antagonism of the human glucagon receptor in vitro

The ability of Glu-3 analogues OXL14E3, GCGE3 and OXLT10E3 to antagonise glucagon stimulated increase in cAMP was investigated using CHO cells over-expressing the human glucagon receptor. Concentrations of 0.01 and 0.1nM OXL14E3 decreased glucagon (0.02nM) mediated cAMP accumulation by 33% and 31% respectively. Concentrations of 10-10000nM OXL14E3 increased cAMP levels by a mean of 56% compared to glucagon stimulation alone (Figure 6.4 A).

Concentrations of 0.0001- and 0.001nM GCGE3 decreased glucagon mediated cAMP accumulation by 33% and 20% respectively. Incubation with concentrations of 0.01-1nM GCGE3 resulted in a mean 8% reduction in cAMP accumulation. Concentrations of 10- and 100nM increased cAMP accumulation by 35% and 59% respectively compared to glucagon stimulation alone (Figure 6.4 B).

Concentrations of 0.0001-1nM OXLT10E3 decreased cAMP accumulation by a mean of 23% compared to glucagon stimulation alone. Concentrations of 10- and 100nM OXLT10E3 increased cAMP accumulation by a mean of 54% compared to glucagon stimulation alone (Figure 6.4 C).
Figure 6.4: In vitro Glu-3 analogue antagonism of the human glucagon receptor.
The percentage change in 0.02nM glucagon stimulated cAMP accumulation in CHO cells over-expressing the human glucagon receptor following co-incubation with various concentrations of Glu-3 analogues (A) OXL14E3, (B) GCGE3, (C) OXLT10E3. Data is expressed as a percentage of glucagon mediated cAMP accumulation ± SEM. OXL15E3 n= 2 experiments; GCGE3 & OXLT10E3 n= 3 experiments except concentrations 0.0001- & 0.001nM n=1.
6.4. Discussion

In order to investigate the contribution of glucagon receptor and GLP-1 receptor activity to the weight loss effects of OXL analogues, several analogues were synthesised with a glutamate (Glu/E) at amino acid position 3 instead of glutamine (Gln/Q). This single amino acid substitution has previously been shown to diminish glucagon receptor activity without affecting GLP-1 receptor activity (Pocai et al. 2009; Du et al. 2012; Kosinski et al. 2012).

The experiments detailed in this chapter were carried out in order to investigate the novel finding that bodyweight was significantly increased in male Wistar rats following chronic administration of 25nmol/kg of two Glu-3 OXL analogues, OXL14E3 and OXL15E3; without an alteration in food intake. In the current study OXL15E3 dose dependently modified bodyweight, with lower doses (10- and 20nmol/kg) significantly increasing bodyweight and higher doses causing a small increase or having no effect (30- and 50nmol/kg respectively). Two studies have previously investigated the effects of Glu-3 analogues on food intake and bodyweight and neither reported an increase in bodyweight (Pocai 2009, Kosinski 2012).

There are several possible reasons why an increase in bodyweight has not been previously been observed or reported. Firstly, previous studies investigating the effects of Glu-3 substitutions on food intake and bodyweight have only been conducted in mice; therefore it is possible that the increased bodyweight demonstrated in the current study may be a rat specific effect. Kosinski et al (2012) reported no significant difference in bodyweight following chronic administration of the Glu-3 peptide of native oxyntomodulin (OXMQ3E) in mice; whereas Pocai et al (2009) demonstrated a significant reduction in mouse bodyweight following chronic administration of the Glu-3 peptide of C-terminal derived oxyntomodulin (GLPAG).

Secondly the two mouse studies used higher daily doses of 1100nmol/kg (Kosinski) and 950nmol/kg (Pocai) compared to the 10-, 20- and 25nmol/kg/day doses which increased bodyweight in the current rat study. Therefore, the doses of Glu-3 peptides used in previous mouse studies were likely to be too large to observe an increase in bodyweight, if indeed this does occur in mice. Indeed, doses of Glu-3 OXL analogues greater than 50nmol/kg/day also cause a reduction of bodyweight in rat (data not shown), indicating an inverted U shaped dose response curve. Comparison with previous studies is further complicated by differences in the route of peptide administration and the dosing schedule. Kosinski et al (2012) infused OXMQ3E for 14 days via minipump and Pocai et al (2009) administered GLPAG by SC injection every other day over 13 days. In the current study Glu-3 OXL analogues were injected SC every day for 6 days.
The effect of Glu-3 substitution on receptor affinity has not previously been reported and the effect on receptor activity has only been reported for the mouse receptors. In the current study, the effect of Glu-3 substitution on OXL analogue efficacy and affinity at the human and rat glucagon receptors and GLP-1 receptors was investigated and the results were generally similar to those previously observed in mice with Glu-3 oxyntomodulin (Pocai 2009, Kosinski 2012). Receptor activity at the human and rat glucagon receptors was diminished by Glu-3 substitution, although Glu-3 analogues maintained some activity at the glucagon receptor. Activity at the human GLP-1 receptor was minimally affected, whereas activity at the rat GLP-1 receptor was increased 3-4 fold by Glu-3 substitution. Pocai et al (2009) reported that GLPAG maintained some efficacy at the mouse glucagon receptor and that efficacy was increased 2 fold at the mouse GLP-1 receptor. In the current study the effects of Glu-3 substitution on receptor binding are similar to the effects on receptor activity, with diminished glucagon receptor binding and a 3-4 fold increase in binding at the rat GLP-1 receptor.

A possible explanation for the increase in rat bodyweight following Glu-3 OXL analogue administration observed in the current study might be due to a reduction in energy expenditure, possibly as a result of antagonism at the glucagon receptor. Therefore, dose responses of several Glu-3 OXL analogues were tested; including Glu-3 of native oxyntomodulin (OXME3) and native glucagon (GCGE3), to examine if they could inhibit glucagon stimulated cAMP accumulation in cells expressing the rat or human glucagon receptors. Results demonstrated that at lower doses (below 1nm) Glu-3 OXL analogues did inhibit glucagon stimulated cAMP accumulation, an expected effect of an antagonist. However higher doses (above 10nm) increased cAMP accumulation suggesting the Glu-3 analogues are partial agonists. If bodyweight was increased due to Glu-3 OXL analogues blocking endogenous glucagon activity, it would suggest that endogenous glucagon has a tonic effect on energy expenditure. Interestingly, a clinical trial investigating the use of an orally administered glucagon receptor antagonist (MK-0893) for the treatment of T2DM reported a dose dependent increase in bodyweight (Christensen et al. 2011).

Future work will investigate whether Glu-3 OXL analogues can act as antagonists in vitro at the mouse glucagon receptor and whether low doses increase bodyweight in mice. The effect of Glu-3 OXL analogues on energy expenditure in rats will be investigated using either thermal imaging of BAT or measuring BAT weight and UCP1 expression levels. A wider dose response will be conducted to establish the lowest dose of Glu-3 OXL analogues which do not affect glucagon mediated cAMP accumulation at the human or rat glucagon receptors.
6.4.1. Conclusion

The results of this study indicate that Glu-3 substitution in oxyntomodulin analogues is not necessarily the benign switch it was previously suggested to be. Glu-3 OXL analogues increase bodyweight in Wistar rats possibly as a result of antagonist activity at the glucagon receptor. These analogues may block the actions of endogenous glucagon, suggesting that these analogues are not suitable tools to investigate the mechanisms of oxyntomodulin analogue activity in Wistar rats.
Chapter 7

General discussion
Widely available calorie dense foods and increased sedentary lifestyles, combined with a genetic predisposition to store excess energy have been proposed as the driving factors behind the rapidly increasing prevalence of obesity worldwide (Swinburn et al. 2011). Despite the substantial financial and medical burden that obesity and its associated co-morbidities place on healthcare systems, existing treatment options to tackle obesity are severely limited.

Currently the most successful treatment for obesity is bariatric surgery; however it is impractical to apply this treatment to the general obese population. Several currently approved pharmacological treatments for obesity are drugs that cause weight loss as a side effect of their actions on central monoamine systems, possibly by altering the hedonic or rewarding feelings associated with food and eating. However, they come with a plethora of warnings, including possible cognitive impairments, anxiety, depression and suicidal ideation, which are similar to the side effects associated with previously withdrawn obesity drugs.

Individual administration of several gut hormones, including exogenous GLP-1 (Verdich et al. 2001), oxyntomodulin (Cohen et al. 2003) and PYY (Batterham et al. 2002) have been shown to inhibit food intake in humans. Circulating levels of these hormones have been demonstrated to be significantly higher following bariatric surgery and have been suggested to contribute to the appetite reduction and weight loss observed in these patients (Holdstock et al. 2008; Laferrère et al. 2010; Falkén et al. 2011; Alexander et al. 2014). It has been postulated that mimicking post bariatric levels by peripherally administering long acting analogues of gut hormones to produce a ‘medical bypass’, may be a viable therapeutic approach for the treatment of obesity. Based on the incretin effects of GLP-1, the long acting analogue liraglutide was developed for the treatment of T2DM and has recently been approved for the treatment of obesity as it also reduces food intake and bodyweight.

Nausea and vomiting are common side effects following administration of GLP-1 analogues and the prevalence has been shown to be increased with the higher dose of liraglutide that is licenced for obesity treatment (3mg vs 1.8mg for T2DM) (Kanoski et al. 2012; Lean et al. 2014). Bodyweight reduction following GLP-1 analogue administration is minimally significant and thought to be due to a reduction in food intake. A recent clinical trial of the 3mg liraglutide dose reported a mean 4% placebo adjusted weight loss after one year of treatment and only 32.9% of subjects achieved the benchmark >5% placebo adjusted weight loss. Strong appetite inhibition activates counter regulatory mechanisms to prevent starvation, including reducing metabolic rate (Ochner et al. 2013), which could explain the failure of GLP-1 analogues to produce significant weight loss. Thus, the ideal anti-obesity drug would reduce food intake and increase energy expenditure in order to achieve clinically significant weight loss.
Previous research has demonstrated that administration of native oxyntomodulin and long acting analogues causes weight loss by reducing food intake and increasing energy expenditure (Dakin et al. 2002; Cohen et al. 2003; Dakin et al. 2004; Wynne et al. 2005; Wynne et al. 2006a) via its effects at the GLP-1 receptor and glucagon receptor respectively (Kosinski et al. 2012). Importantly, glucose homeostasis is not adversely affected, indicating that combining the activity of glucagon and GLP-1 could be a viable therapeutic option for weight loss.

As demonstrated by Wynne et al (2005) oxyntomodulin in its native form is unsuitable to be used as a drug; volunteers required three injections a day of a substantial dose (400nmol) of OXM to achieve weight loss. The study confirmed two known weaknesses; oxyntomodulin activates both receptors with reduced efficacy compared to the native hormones and as is rapidly cleared. While there is little published data which demonstrates successful modifications to oxyntomodulin that address these issues, there are several studies which highlight unsuccessful changes. Relatively simple modifications to improve the pharmacokinetics of oxyntomodulin, such as the addition of large molecules to improve its circulatory half-life, further reduce the potency of the analogues (Druce et al. 2009; Lynch et al. 2014; Kerr et al. 2010; Pocai et al. 2009; Santoprete et al. 2011) and can prevent them from crossing the blood brain barrier (Prolor Biotech Inc 2012).

This thesis aimed to design a potent dual agonist of the glucagon and GLP-1 receptors that was compatible with a ZnCl2 slow release formulation, resulting in a weight loss therapy with the potential for once monthly administration in man. Glucagon was chosen as the initial starting sequence as oxyntomodulin has reduced potency at the glucagon receptor and GLP-1 receptor compared to the native hormones, therefore to develop a potent dual analogue it was logical to start with a potent peptide. The structure-function relationship of GLP-1 has been investigated to a greater extent than that of glucagon making it easier to identify substitutions that could be introduced to improve GLP-1 receptor activity. In order to improve the pharmacokinetic (PK) profile of the drug, I further investigated analogue compatibility with a slow release formulation that facilitates peptide-zinc chelation and subsequently depot formation.

Previously-identified key amino acid residues from GLP-1 and exendin-4 were substituted in to the glucagon sequence, with the aim of increasing activity at the GLP-1 receptor. Consistent with previous findings, binding and activation of the GLP-1 receptor by glucagon was increased following C-terminal amidation and conservative substitution of GLP-1 or exendin-4 residues in to the glucagon sequence (Hjorth et al. 1994; Runge et al. 2003a; Day et al. 2009). In particular, substituting glutamate at residue 21 increased analogue activity at the human GLP-1 receptor and also at the glucagon receptor. Substitution of the exendin-4 residue glutamate at position 16 had a substantial
negative impact on activity at the human GLP-1 receptor, but this was ameliorated by subsequent substitution of lysine at residue 17. When designing the dual analogues enhancing / stabilising the helical structure of the C-terminal region was a primary aim, as this has previously been suggested to improve the binding and activity of analogues of both GLP-1 and glucagon (Ahn et al. 2001c; Runge et al. 2007; Runge et al. 2008). In solution, glucagon is well known to form insoluble inactive aggregated polymers termed fibrils. Increasing the helical potential of glucagon has been postulated to impede fibril formation (Pedersen et al. 2006), an additional reason to increase the number and / or size of helical regions when designing oxyntomodulin analogues. To further understand the potential structural changes caused by amino acid substitutions, collaborative investigation of the structure of several OXL analogues has been undertaken using X-ray crystallography, but attempts have been unsuccessful to date. Importantly, glucagon receptor affinity and efficacy was maintained following conservative substitution of GLP-1 or exendin-4 residues, resulting in dual analogues with increased potency at the glucagon receptor and GLP-1 receptor compared to native oxyntomodulin.

An analogue with enhanced potency compared to oxyntomodulin would only be useful if it did not negatively impact glycaemia. The optimal ratio of glucagon receptor activity to GLP-1 receptor activity required to maintain balanced glycaemia has not been established in man. One study has suggested that in mice the optimal ratio for a dual agonist of the glucagon receptor and GLP-1 receptor is approximately 1:1, in order to achieve maximal weight loss whilst maintaining glucose homeostasis (Day et al. 2012). Although their results also indicated that up to 6 fold greater activity at the glucagon receptor compared to the GLP-1 receptor was not detrimental to glucose homeostasis. However, the optimal ratio is likely to differ between species. Acute and chronic administration of oxyntomodulin in humans increases energy expenditure and reduces food intake without negatively impacting glucose homeostasis (Wynne et al. 2005; Wynne et al. 2006a). This could be considered the best available indication of the ratio of glucagon receptor activity to GLP-1 receptor activity to be used in humans. Therefore, during the design and development of the dual analogues I tried to match the ratio of receptor activity to that calculated for oxyntomodulin. The optimised dual analogue OXLT9 was 10 fold stronger at the human glucagon receptor and 16 fold stronger at the human GLP-1 receptor compared oxyntomodulin, with a ratio of 1:2. The ratio of oxyntomodulin was 1:3 and while further attempts were made to more closely match the analogue activity ratio to this, it resulted in substantial reductions in efficacy at one or both receptors. It is of note that at the rat receptors the ratio of receptor activity for analogue OXLT9 was 1:11 compared to 1:3.4 for oxyntomodulin, but appeared to be well tolerated. The mouse receptor activity ratio of OXLT9 and oxyntomodulin was not calculated in the current study; however a reduction in basal blood glucose and improved glucose tolerance during an IPGTT was observed following chronic
administration of OXLT9 in DIO mice. Weight loss and reductions in visceral adiposity are known to improve glucose tolerance (Pan et al. 1997); therefore the improvements observed in this experiment could be due to the significant weight loss (14% and 28%) caused by OXLT9 administration. Comparing blood glucose measurements with weight matched controls would help to assess the impact of weight loss and the effect of OXLT9. For the GTT glucose was administered IP, therefore the effects on glucose homeostasis do not take in to account the incretin effect of GLP-1 receptor activity which would further reduce hyperglycaemia following oral ingestion of glucose or chow. An oral GTT which investigates the acute effect of OXLT9 administration on glucose homeostasis in weight matched animals is necessary; although this will not control for potential differences in effects on gastric emptying. The positive effects of OXLT9 on blood glucose in DIO mice are promising, but careful monitoring of blood glucose during clinical trials will be essential, in both type-2 diabetic and non-diabetic subjects.

The native hormones glucagon, GLP-1 and oxyntomodulin have very short half-lives; therefore a second aim of this work was to develop dual analogues with substantially extended half-lives and the potential for once monthly administration in man. It is important to reduce the number of injections required in order to lessen the impact and discomfort on the patient and to reduce the overall cost of goods. The cost of treating obesity and diabetes is a huge drain on health care finances, therefore if a new drug is 5% more effective than current treatments but 10% more expensive it will struggle to compete for a share of the market, particularly in America where health insurance companies decide which medications they will cover.

Rapid changes in blood levels of anorectic gut hormones like GLP-1 are associated with nausea and vomiting (Fineman et al. 2004; Sloth et al. 2007; Astrup et al. 2009). Therefore the ideal pharmacokinetic (PK) profile of an oxyntomodulin-like dual agonist would be a slow increase in blood levels to a sustained maximum which would then be ‘topped up’ by the next injection the following month, so the blood levels never fall completely to zero. One method of achieving this is the formation of a peptide depot. Several amino acids, including histidine and glutamate, chelate with zinc ions which can reduce the solubility of co-administered peptides, resulting in the formation of a depot (Trzaskowski et al. 2008). Peptide-zinc chelation has been successfully exploited in slow release preparations of exogenously administered insulin (Havelund et al. 2015), human growth hormone and α-interferon (Bartus et al. 1998). During the development of the dual analogue, histidine and glutamate residues were preferentially substituted in to the peptide sequence in order to enhance peptide-zinc chelation and subsequently depot formation. Histidine is positively charged at low pH but uncharged at physiological pH meaning it becomes less soluble following injection,
thus adding histidine residues may also contribute to rapid peptide depot formation independent of its interaction with Zn\(^{2+}\). The effect of amino acid substitutions and additions on depot formation was investigated indirectly by measuring plasma peptide levels and evaluating the PK profiles of the analogues. Adding histidine at residue 31 (OXL6) markedly improved the PK profile of the dual analogue, increasing the estimated T\(_{\text{1/2,max}}\) from 77 hours post injection to >168 hours with 61% of the peak concentration remaining at 7 days. However the subsequent addition of glutamate at position 21 (OXL7) substantially diminished the PK profile resulting in a peak plasma level at just 30 minutes post injection, an estimated T\(_{\text{1/2,max}}\) of 2 hours and no detectable plasma levels at 7 days. The addition of a histidine ‘tail’ of varying length generally improved the PK profiles of the analogues, lengthening the time until the estimated T\(_{\text{1/2,max}}\) and increasing the level of peptide remaining at 7 or 10 days post injection, without affecting receptor efficacy.

Trying to estimate a human PK profile from the results of an experiment conducted in animals is challenging. The rate of peptide clearance will differ between species due to differences in metabolic rate, kidney filtration and exposure to endopeptidases; the volume of distribution may also differ between species. As depot formation is dependent upon peptide concentration and injection volume (Mrsny & Daugherty. 2009) it was necessary to try to match clinical formulation in rodent studies. I hypothesised that approximately 1mg of peptide administered in 20µl of ZnCl\(_2\) diluent would be the required dose to have a therapeutic effect in man and used these conditions for rat PK studies. Allometric scaling predicts that peptide duration may be up to 5 fold longer in humans compared to rats; although there is limited data available for the modelling of SC administered peptides and even less for those released from depots. It was postulated that if plasma peptide levels at 7-10 days in a rat were a high percentage of the peak concentration, the analogue may have the potential for once monthly administration in man given the slower metabolic rate in humans compared to rats. However this would also be dependent upon the efficacy of the peptide of which the PK studies tell us nothing.

Combining the findings of the PK studies with the effects of analogue administration on acute and chronic food intake and bodyweight in rodents and also the results of the in vitro receptor efficacy and affinity assays, it is possible to assess the overall contribution of amino acid substitutions to analogue efficacy. The addition of a histidine ‘tail’ improved the pharmacokinetic profile of OXL dual analogues without reducing efficacy at either the glucagon receptor or the GLP-1 receptor. This is in contrast to acylation or PEGylation which increase peptide half-life but often reduce receptor efficacy (Druce et al. 2009; Lynch et al. 2014; Kerr et al. 2010; Pocai et al. 2009; Santoprete et al. 2011). This suggests that administering oxyntomodulin analogues as a slow release formulation with
ZnCl$_2$ is a viable alternative to peptide acylation or PEGylation. The oxyntomodulin-like analogues also inhibit food intake and significantly reduce bodyweight following acute and chronic administration in rodents. Pair-feeding studies indicate that weight loss following chronic administration in rats is due to both reduced food intake and increased energy expenditure. The pair-feeding paradigm does not control for potential weight loss due to diuresis, but it was expected that any weight loss due to diuresis would correct itself after the first 24 hours and was unlikely to be a factor in weight loss at 6 or 7 days. In the chronic rat studies substantial weight loss of around 4% was observed in the first 24-48 hours following analogue injection. However, the weight loss was sustained and continued to increase over the subsequent days suggesting the initial reduction was not a result of diuresis. Metabolic cages were also used to measure the volume of oxygen consumption as an alternative method for measuring energy expenditure following analogue administration. This method does not rely on weight change to calculate energy expenditure and therefore should not be affected by diuresis. However the environment of the metabolic cages is more stressful for the animal which can affect food intake and may not give a completely accurate picture of analogue effects on energy expenditure (Martí et al. 1994). During the CLAMS experiment rat bodyweight was decreased by 2.5% in the first 24 hours following administration of OXL T9 (8nmol/kg). This appeared to be due to increased resting energy expenditure as there were no differences in food intake, locomotion or respiratory exchange ratio. Increased energy expenditure could be further investigated by measuring UCP1 expression levels in BAT; whereas measuring plasma fatty acid levels and the expression of enzymes involved in lipolysis would enable further investigation of differences in substrate utilisation.

Nausea and vomiting is a common side effect of GLP-1 analogues; in order to compete with liraglutide, the GLP-1 analogue licenced for obesity treatment, it would be desirable for OXL T9 not to cause nausea. In the current work, no experimental assessment of nausea was carried out. Mice and rats cannot vomit, but animals were monitored following analogue injection and no adverse behaviour was observed that might suggest discomfort analogous to nausea in humans, for example hunching or ingestion of bedding or faeces (pica). However, conditioned taste aversion studies or experimental assessment of pica may help to discern whether administration of the oxyntomodulin-like analogues is likely to cause nausea.

In Chapter 5 the effect of low doses of OXL analogues on food intake was investigated following repeated observations of increased food intake during analogue screening. The results indicate that doses between 25- to 50nmol/kg increase food intake without increasing bodyweight until they reach a ‘threshold point’ around 50nmol/kg, beyond which food intake and bodyweight are both
significantly decreased. These findings are in contrast to previous results that demonstrate a reduction in food intake following administration of oxyntomodulin and long lasting analogues in rodents (Dakin et al. 2004; Pocai et al. 2009; Kerr et al. 2010; Liu et al. 2010; Day et al. 2012). However previous studies used doses greater than 50nmol/kg and administration routes and dosing schedules varied making it difficult to compare between studies. The difference in analogue efficacy at the glucagon and GLP-1 receptors also makes comparison difficult. A direct comparison with a chronic dose response of native oxyntomodulin would be ideal, but unfortunately due the inherent short half-life of gut hormones this is difficult to achieve.

The increase in food intake following chronic low dose OXL analogue administration could not be demonstrated in mice, indicating this may be a rat specific phenomenon. As most previous studies investigating the effects of oxyntomodulin and analogues on energy homeostasis have been conducted in mice, this could be another reason why an increase in food intake has not previously been reported. It is not possible to predict from animal studies and receptor activity assays whether low doses of OXL analogues will increase food intake in humans. However, in vitro affinity studies indicate that OXL analogue behaviour at the human receptor is more similar to the mouse receptor, binding with higher affinity at the human and mouse receptors compared to the rat. This may indicate that the effects of low dose OXL analogues in humans would be more similar to the response in mice than rats. However, findings in vivo do not necessarily reflect in vitro results. Indeed, the EC₅₀ ratios for several OXL analogues in Chapter 5 are poor predictors of in vivo effects on food intake and bodyweight. For example the OXL analogue dose required to reduce food intake is higher for OXL14 compared to OXL15, despite OXL14 having 2 fold greater activity at the rat GLP-1 receptor. This indicates that the in vivo receptor interaction of the OXL analogues, and probably native oxyntomodulin, is complex and possibly reflects component(s) that are absent from the simplified in vitro receptor systems. For example, differences in PK would affect the in vivo actions of the analogues. Also there are a number of intracellular accessory proteins that can interact with GPCRs and subsequently alter their trafficking, localisation and activity (Magalhaes et al. 2012). β-arrestin and RAMPs have been suggested to affect ligand activity at the GLP-1 receptor and glucagon receptor respectively (Jorgensen et al. 2007; Appleton & Luttrell 2013; Weston et al. 2015); therefore co-expressing these proteins in the receptor over-expressing cell lines may provide in vitro results that more accurately reflect ligand activity in vivo.

The lack of increased food intake in mice prevented the use of transgenic GLP-1 receptor or glucagon receptor knockout models for investigating the mechanism behind the increase in food intake. Instead, analogues with glutamate (Glu/E) substituted for glutamine (Gln/Q) at residue 3 were used;
Glu-3 substitution diminishes glucagon receptor activity whilst maintaining activity at the GLP-1 receptor (Kosinski et al. 2012). In the current study, no increase in food intake was observed with the GLP-1 receptor selective Glu-3 OXL analogues, indicating that glucagon receptor activity is necessary to cause an increase in food intake. It could be postulated that the increase in food intake is a compensatory response to an increase in energy expenditure mediated by activity at the glucagon receptor. Unfortunately it was not possible to investigate the relationships between Glu-3, Gln-3, energy expenditure and food intake using the metabolic CLAMS cages. Several studies failed to identify a dose of OXL analogues that increased food intake when the animals were in the metabolic cages; possibly due to mild stress induced hypophagia resulting from the specific CLAMS environment (Martí et al. 1994). Future work will investigate the use of thermal imaging to measure levels of activated BAT as a marker of increased energy expenditure. BAT weight and measurement of UCP1 expression, an indirect marker of BAT activity (Cinti 1999; Lowell & Spiegelman 2000), will also be investigated following doses of OXL analogues that increase food intake. Using immunohistochemical staining for c-Fos to compare the brain areas demonstrating neuronal activation or deactivation following anorexigenic and orexigenic doses of OXL analogues may also provide insights into the mechanism behind the increase in food intake.

Interestingly, although chronic administration of Glu-3 OXL analogues didn’t increase food intake, it did significantly increase bodyweight. The results detailed in Chapter 6 suggest that a number of Glu-3 analogues can act as antagonists at the rat and human glucagon receptors; therefore it is possible that Glu-3 analogues could block the actions of endogenous glucagon. If bodyweight was increased as a result of Glu-3 OXL analogues antagonising endogenous glucagon, this would suggest glucagon has a tonic effect on energy expenditure. Future studies will investigate the effects of acute and chronic Glu-3 analogue administration on energy expenditure using thermal imaging of BAT and measurement of BAT UCP1 expression. In vitro results suggest antagonism occurs at the human glucagon receptor as well as the rat, therefore it will be interesting to also investigate the effects of these Glu-3 analogues at the mouse glucagon receptor in vitro and also on mouse bodyweight in vivo. Glu-3 analogues of oxyntomodulin and a long acting analogue have previously been chronically administered in mice and increased bodyweight has not been reported (Pocai et al. 2009; Kosinski et al. 2012). However, higher doses were administered (1110- and 950nmol/kg) compared to the doses which increase bodyweight in the current study (10-, 20 and 25nmol/kg).

In conclusion, the combined evaluation of the multiple in vivo and in vitro studies detailed in this thesis enabled the development of a potent dual agonist at the glucagon and GLP-1 receptors that has the potential for once monthly administration as a weight loss therapy in man. The investigation
of novel food intake and bodyweight effects of OXL and Glu-3 OXL analogues raises interesting questions about the role glucagon receptor activity plays in the energy expenditure effects of oxyntomodulin and provides a starting point for further investigation.
References


Abbott CR, Small CJ, Kennedy AR, et al. (2005a) Blockade of the neuropeptide Y Y2 receptor with the specific antagonist BIIE0246 attenuates the effect of endogenous and exogenous peptide YY(3-36) on food intake. Brain Res. 1043 (1-2), 139-144.


Arvat E, Maccagno B, Ramunni J, et al. (2000) Glucagon is an ACTH secretagogue as effective as hCRH after intramuscular administration while it is ineffective when given intravenously in normal subjects. Pluitory. 3 (3), 169-173.


Deacon CF, Nauck MA, Toft-Nielsen M, et al. (1995b) Both Subcutaneously and Intravenously Administered Glucagon-Like Peptide 1 Are Rapidly Degraded From the NH2-Terminus in Type II Diabetic Patients and in Healthy Subjects. Diabetes. 44 (9), 1126-1131.


Doi K & Kuroshima A. (1982) Modified me


196


Murphy WA, Coy DH & Lance VA. (1986) Superactive amidated COOH-terminal glucagon analogues with no methionine or tryptophan. Peptides. 7 Suppl 1, 69-74.


Parker JA, McCullough KA, Field BC, et al. (2013) Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala. *Int. J. Obes. (Lond)*. ;


Rinaman L. (2010) Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. Brain Res. 1350 (0), 18-34.


Wynne K, Park AJ, Small CJ, et al. (2006a) Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int. J. Obes. (Lond).* 30 (12), 1729-1736.


Appendix A: Peptide structures

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNT</td>
<td>GLP-1 (7-36 amide)</td>
</tr>
<tr>
<td>Oxymodulin</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNTKRNRNNIA</td>
<td>GLP-1 analogue</td>
</tr>
<tr>
<td>GLP-1 analogue</td>
<td>HEGTFTSDYSSLYGEQAAKEFWLYKRNH</td>
<td>Exendin-4</td>
</tr>
</tbody>
</table>

**Chapter 3 OXL analogues**

<table>
<thead>
<tr>
<th>Analogues</th>
<th>Amino Acid Sequence</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXL1</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNT</td>
<td>Conserved residue in glucagon &amp; GLP-1</td>
</tr>
<tr>
<td>OXL2</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNTNH2</td>
<td>Conserved residue in glucagon &amp; Exendin-4</td>
</tr>
<tr>
<td>OXL3</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNTNH2</td>
<td>Glucagon residue</td>
</tr>
<tr>
<td>OXL4</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNTNH2</td>
<td>GLP-1 residue (introduced)</td>
</tr>
<tr>
<td>OXL5</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNTNH2</td>
<td>Exendin-4 residue (introduced)</td>
</tr>
<tr>
<td>OXL6</td>
<td>HSQGTFTSDYSKLYLDSRRAQDFVQWLMLNTNH2</td>
<td>GLP-1 &amp; Exendin-4 residue (introduced)</td>
</tr>
<tr>
<td>OXL7</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Novel residue substitution</td>
</tr>
<tr>
<td>OXL8</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>2nd novel residue substitution</td>
</tr>
<tr>
<td>OXL9</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Glu3 substitution</td>
</tr>
<tr>
<td>OXL10</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td></td>
</tr>
<tr>
<td>OXL11</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td></td>
</tr>
</tbody>
</table>

**Chapter 4 OXL analogues**

<table>
<thead>
<tr>
<th>Analogues</th>
<th>Amino Acid Sequence</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXLT1</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Conserved residue in glucagon &amp; GLP-1</td>
</tr>
<tr>
<td>OXLT2</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Conserved residue in glucagon &amp; Exendin-4</td>
</tr>
<tr>
<td>OXLT3</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Glucagon residue</td>
</tr>
<tr>
<td>OXLT4</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>GLP-1 residue (introduced)</td>
</tr>
<tr>
<td>OXLT5</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Exendin-4 residue (introduced)</td>
</tr>
<tr>
<td>OXLT6</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>GLP-1 &amp; Exendin-4 residue (introduced)</td>
</tr>
<tr>
<td>OXLT7</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Novel residue substitution</td>
</tr>
<tr>
<td>OXLT8</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>2nd novel residue substitution</td>
</tr>
<tr>
<td>OXLT9</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td>Glu3 substitution</td>
</tr>
<tr>
<td>OXLT9E3</td>
<td>HSQGTFTSDYSKLYLDEKhAFVEVLNTNH2</td>
<td></td>
</tr>
</tbody>
</table>

Figure 7.1: Amino acid sequences of native peptides and analogues investigated in Chapters 3 & 4.
Figure 7.2: Amino acid sequences of analogues investigated in Chapters 5 & 6.
Figure 7.3: Summary of peptide charges.

<table>
<thead>
<tr>
<th></th>
<th>pH4</th>
<th>pH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exendin-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXL10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Summary of peptide charges**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pH4</th>
<th>pH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>GLP-1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>-1</td>
<td>-2</td>
</tr>
<tr>
<td>OXL10</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>OXL11</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>OXL10</td>
<td>6</td>
<td>-1</td>
</tr>
</tbody>
</table>

+ indicates a positive charge, - indicates a negative charge. +/- indicates the addition of a charged residue.
Appendix B: Amino acid abbreviations

**Ala** (A) Alanine

**Cys** (C) Cystine

**Asp** (D) Aspartic acid

**Glu** (E) Glutamic acid

**Phe** (F) Phenylalanine

**Gly** (G) Glycine

**His** (H) Histidine

**Ile** (I) Isoleucine

**Lys** (K) Lysine

**Leu** (L) Leucine

**Met** (M) Methionine

**Asn** (N) Asparagine

**Pro** (P) Proline

**Gln** (Q) Glutamine

**Arg** (R) Arginine

**Ser** (S) Serine

**Thr** (T) Threonine

**Val** (V) Valine

**Trp** (W) Tryptophan

**Tyr** (Y) Tyrosine
Appendix C: General principle of a radioimmunoassay

All radioimmunoassay 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 (Poole, Dorset, UK or BDH (Poole, Dorset, UK). The principle of radioimmunoassay is the competition between a radioactive and nonradioactive antigen for a fixed number of antibody binding sites. When unlabelled antigen from 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 radioimmunoassay is incubated and allowed to reach equilibrium, according to the equation:

\[ \text{Ag} + \text{Ab} + \text{Ag} \leftrightarrow x \text{AgAb} + \text{AgAb} \]

\( \text{Ag} = \) unlabelled antigen

\( \text{*Ag} = \) radiolabelled antigen

\( \text{Ab} = \) antibody

Separation of the bound from the free antigen is achieved by addition of either dextrancoated 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 \( \gamma \)-counter. The data are used to construct a standard curve from which the values of the unknowns can be obtained by interpolation.

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

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

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

\( \frac{1}{2} \times \): 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>½ X</td>
</tr>
<tr>
<td>5-6</td>
<td>2 X</td>
</tr>
<tr>
<td>7-8</td>
<td>Zero</td>
</tr>
<tr>
<td>9-10</td>
<td>Zero</td>
</tr>
<tr>
<td>11-12</td>
<td>Standard 1</td>
</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>

Figure 7.4: The general plan for a typical RIA.