Regulation of metabolism and food intake by enteropancreatic hormones

Thesis submitted for the degree of Doctor of Philosophy

at

Section of Investigative Medicine, Imperial College London

2014

Tricia Mei-Mei Tan

BSc (Sci), MBChB, FRCP

Section of Investigative Medicine

Division of Diabetes, Endocrinology & Metabolism

Faculty of Medicine

Imperial College London

To Bernard, Katie and James

Abstract

Enteropancreatic hormones such as pancreatic polypeptide (PP), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) are secreted post-prandially by the gut and the pancreas. They act to regulate metabolism and appetite. An understanding of the physiology of these hormones and how they can be delivered in a practical manner is required to allow their translation into clinical treatments for obesity and diabetes.

Work in this thesis investigated the effect of subcutaneously injected PP in healthy human volunteers, and demonstrated a significant reduction in food intake. A novel peptidase resistant analogue of PP, PP 1420, was administered in combination with metformin to rodents. This combination reduced food intake and body weight additively, suggesting that the combination of PP 1420 and metformin may well be beneficial in patients with obesity and diabetes. A subsequent study of PP 1420 in human volunteers, in a first-in-man Phase 1 trial, confirmed that PP 1420 was safe, well tolerated and possessed an extended terminal elimination half-life compared to native PP.

In this thesis, I also explored the physiological effects of gut hormone combinations. The administration of single gut hormones such as PP, PYY or GLP-1 can reduce food intake, but may cause side effects such as nausea. The combination of gut hormones offers the possibility of increased efficacy with fewer side effects, for example, PYY+GLP-1 in combination have previously been shown to possess additive effects on food intake. The effects of a PYY+GLP-1 combination on carbohydrate metabolism have not yet been investigated. Work in this thesis

examined the effects of a PYY+GLP-1 combination intravenous infusion on insulin secretion and sensitivity in healthy volunteers. Administration of PYY alone did not significantly affect insulin secretion. PYY+GLP-1 in combination stimulated insulin secretion to a similar extent to GLP-1 alone. There were no significant acute effects of PYY, GLP-1 or PYY+GLP-1 on insulin sensitivity.

These findings suggest that gut hormone analogues may represent safe, effective and practical treatments for obesity. Combination PYY+GLP-1 treatment may provide the metabolic benefits of bariatric surgery without the surgery itself.

Table of Contents

List of Tables

List of Figures

Declaration of Originality

I declare that the text is of my own writing. Where appropriate, I have cited other researchers' work. Where excerpts of copyrighted works are shown in this thesis, these have been included for the purposes of criticism and review and falls under the exemption of fair dealing for these purposes.

The majority of the work carried out in this thesis was performed by myself. All collaborators and assistance are described below.

Chapter 2: The study was carried out in collaboration with Dr Benjamin Field, with the assistance of Dr Kevin Baynes. I carried out the clinical study, analysis of samples and data analysis.

Chapter 3: The pre-clinical studies were carried out by myself in collaboration with Dr James Minnion, Ms Joy Shillito-Cuenco, Dr Benjamin Field, and Dr Jordan Baxter.

Chapter 4: I designed the clinical study, wrote the ethics application and the clinical protocol for the study. The clinical team that performed the study was myself, Dr Benjamin Field, Dr Kevin Baynes, Dr Edward Chambers, Dr Sagen Zac-Varghese with assistance from the nursing staff in the Sir John McMichael Clinical Research Centre. The pharmacokinetic analysis was done in collaboration with Dr Charlie Brindley (KinetAssist), and statistical analysis was done with the assistance of Prof

Deborah Ashby, Dr Francesca Fiorentino, and Dr Shahrul Mt-Isa (Imperial Clinical Trials Unit). The development of the LC/MS-MS assay for PP 1420 and the bioanalysis of the pharmacokinetic study was led by Dr Ian Ward (Quotient Bioresearch).

Chapter 5: I designed the clinical study, wrote the ethics application and the clinical protocol for the study. The study was conducted in collaboration with Dr Benjamin Field, and Dr Victoria Salem, with assistance from Dr Rachel Troke, Dr Ali Alsafi, Dr Shivani Misra, Dr Kevin Baynes, Dr Akila De Silva. Dr Ian Godsland performed the minimal modeling analysis. Assistance with sample analysis was provided by Dr Mandy Donaldson.

All in-house radioimmunoassays used in this research were established and are maintained by Prof Mohammad Ghatei.

Copyright Declaration

Acknowledgements

I would like to thank Prof Bloom for giving me this opportunity to carry out this research and for providing continuing guidance and support. I am also very grateful to Dr Niamh Martin for her supervision, and also to Prof Mohammad Ghatei for his guidance and support. Special thanks are due to my collaborators as listed above, and particularly to Dr Benjamin Field and Dr James Minnion.

Dedication

This thesis is dedicated to Bernard, Katie and James, without whose support and love

I would not have been able to complete this work.

Abbreviations

α-MSH	α-Melanocyte Stimulating Hormone
AcN	Acetonitrile
ADP	Adenosine Diphosphate
AE(s)	Adverse Effect(s)
AgRP	Agouti gene-Related Peptide
ANOVA	Analysis Of Variance
AP	Area Postrema
ARC	Arcuate Nucleus
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BLQ	Below Limit of Quantification
BMI	Body Mass Index
BOLD	blood-oxygen-level-dependent
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CART	Cocaine- and Amphetamine-Regulated Transcript
CCK	Cholecystokinin
CCK1 receptor	Cholecystokinin receptor 1
cDNA	Complementary DNA
CHO	Chinese Hamster Ovary
Cmax	Maximum drug concentration
CNS	Central Nervous System
C-terminal	Carboxy-terminal
db	*diabetes* gene
DIO	Diet-Induced Obesity
DMH	Dorsomedial nucleus
DPP-4	Dipeptidyl Peptidase IV
DVC	Dorsal Vagal Complex
ECD	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fmoc	Fluorenylmethoxycarbonyl
fMRI	Functional magnetic resonance imaging
fT_3	Free Triiodothyronine
fT_4	Free Thyroxine
GABA	Gamma-Aminobutyric Acid
GHRH or GRF	Growth Hormone-Releasing Hormone
GHS-R1a	Growth Hormone Secretagogue Receptor 1a
GIP	Gastric Inhibitory Peptide
GLP-1	Glucagon-Like Peptide-1
GLP-1R	Glucagon-Like Peptide-1 Receptor
GLP-2	Glucagon-Like Peptide-2
GPBAR1/TGR5	Bile acid receptor GPBAR1/TGR5
GPCR	G-protein-coupled receptor
GPR40	Free fatty acid receptor GPR40

GPR41	Short chain fatty acid receptor GPR41
GPR43	Short chain fatty acid receptor GPR43
GPR119	Bile acid receptor GPR119
GRP	glucagon-related peptide
GRPP	Glicentin-Related Polypeptide
H2	Histamine-2 receptor
HbA1c	Glycated Haemoglobin
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	High Pressure Liquid Chromatography
ICV	Intracerebroventricular
IP	Intraperitoneal
IP-1	Intervening Peptide-1
IP-2	Intervening Peptide-2
IP_3	Inositol 1,4,5-triphosphate
IUPAC	International Union of Pure and Applied Chemists
IV	Intravenous
K_d	Dissociation constant for enzyme of receptor-ligand complex
K_i	Dissociation constant for enzyme or receptor-inhibitor complex
LAGB	Laparoscopic adjustable gastric banding
LC/MS-MS	Liquid chromatography/tandem mass spectrometry
MALDI-ToF	Matrix assisted laser desorption/ionization-time of flight
MC1R	Melanocortin-1 Receptor
MC3R	Melanocortin-3 Receptor
MC4R	Melanocortin-4 Receptor
ME	Median Eminence
MEMRI	Manganese-enhanced magnetic resonance imaging
Mins	Minutes
MPGF	Major Proglucagon Fragment
MPO	Medial Preoptic area
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NPY	Neuropeptide Y
NS	Not Significant
N-terminal	Amino-terminal
nt	Nucleotide
NTS	Nucleus Tractus Solitarius
NYHA	New York Heart Association
ob	*obese* gene
Obes	Obestatin
Ob-R	Leptin receptor
OFC	Orbital frontal cortex
OVLT	Organum vasculosum laminae terminalis
OXM	Oxyntomodulin
PACAP-38 and -27	Pituitary adenylate cyclase activating peptides-38 and -27

PAM	Peptidylglycine alpha-Amidating Monooxygenase
PHM	Peptide Histidine Methionine
PK	Pharmacokinetics
PKA	Protein kinase A
PLC	Phospholipase C
PNET	Pancreatic Neuroendocrine Tumour
PNS	Peripheral Nervous System
POMC	Pro-opiomelanocortin
(h)PP	(human) Pancreatic Polypeptide
PVN	Paraventricular Nucleus
PYY	Peptide YY
QC	Quality Control
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RQ	Respiratory Quotient
RYGB	Roux-en-Y Gastric Bypass
SC	Subcutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
SCFA	Short Chain Fatty Acids
SFO	Subfornical organ
SGLT	Sodium-glucose co-transporter
SON	Supraoptic nucleus
STAT3	Signal Transducer and Activator of Transcription-3
T1R2/T1R3	Heterodimeric sweet taste receptor T1R2/T1R3
T_3	Triiodothyronine
T_4	Thyroxine
TFA	Trifluoroacetic Acid
t_{max}	Time of occurrence of maximum drug concentration
TNF-a	Tumour Necrosis Factor-a
TRH	Thyrotropin-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
UCP	Uncoupling Protein
UK	United Kingdom
USA	United States of America
VAS	Visual Analogue Scales
VCO_2	Rate of carbon dioxide production
VIP	Vasoactive Intestinal Peptide
VO_2	Rate of oxygen consumption
WHO	World Health Organisation
Y1	Neuropeptide Y1 receptor
Y2	Neuropeptide Y2 receptor
Y4	Neuropeptide Y4 receptor
Y5	Neuropeptide Y5 receptor
Y6	Neuropeptide Y6 receptor

1 INTRODUCTION

1.1 The clinical problem of obesity

Obesity is an increasing problem worldwide, and has become more prevalent by 75% since 1980 (Flegal, 2005). It is likely that the human species has evolved in response to limited food supplies with adaptations to increase food intake and to reduce energy expenditure, whenever possible. The "obesogenic" modern environment now affords us large quantities of cheap, high energy foods tailored to activate our hedonic centres, whilst limiting our daily energy expenditure through reduced opportunities for exercise and labour saving devices. Thus, the interaction between these two factors has conspired to produce the present obesity epidemic (Bloom, 2007).

Obesity is associated with various co-morbidities, chief amongst them cancer, cardiovascular disease, type 2 diabetes mellitus, stroke, hypertension and obstructive sleep apnoea. The health and economic burden of obesity is considerable, incurring direct health care costs and indirect economic cost due to illness (The Comptroller and Auditor General, 2001). Therefore there is a pressing need for effective, well-tolerated and safe treatments for obesity. In order to consider how novel treatments for obesity may be discovered, it is first necessary to understand the normal physiology of appetite regulation. There are two principal inter-connected systems for regulating energy balance. Firstly, central nervous system (CNS) circuits regulate appetite, food seeking behaviour and the hedonic aspects of eating (Murphy and Bloom, 2006). Secondly, peripheral signals such as those from the gut and adipose

tissue feed back signals of satiety and overall energy balance to the CNS (Murphy and Bloom, 2006).

1.2 Regulation of body weight by central appetite circuits

Regulation of energy balance involves a number of different processes, including:

- The homeostatic control of energy intake;

- The processing of gustatory, visual and olfactory sensation;

- Determination of the rewarding/emotional aspects of food;

- Higher cortical functions involved in motivation and impulse control;

- Control of energy expenditure through metabolic rate and activity.

These different processes need to be co-ordinated, and this occurs in the hypothalamus and the brainstem (**Figure 1**). These centres have extensive reciprocal connections with each other and influence each other's activity. The brainstem receives signals from the periphery via the area postrema (AP), where the blood brain barrier is not complete, and visceral vagal afferents carrying signals from the gut. The hypothalamus is influenced by higher centres, the brainstem and by peripheral humoral signals via the median eminence (ME), which also has an incomplete blood brain barrier.

The arcuate nucleus of the hypothalamus (ARC) is particularly vital to appetite regulation. It contains two populations of neurons with antagonistic actions. The first group of neurons in the lateral ARC co-expressing pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) inhibit food intake, i.e.

they are 'anorexigenic' (Elias et al., 1998). POMC is processed to produce alpha-melanocyte stimulating hormone (α-MSH) that in turn activates the melanocortin-4 receptor (MC4R). Conversely, another group of neurons, in the medial ARC, express both neuropeptide Y (NPY) and agouti gene related peptide (AgRP) (Broberger et al., 1998). These neurotransmitters stimulate food intake and energy seeking behavior, i.e. they are 'orexigenic'. In particular, AgRP's action is to antagonize α-MSH at the MC4R. The balance of activity between these two groups of neurons therefore regulates body weight via the paraventricular nucleus (PVN), which in turn influences higher centres in the cerebral cortex that regulate food seeking behaviour, and the pituitary-thyroid axis and the sympathetic nervous system to regulate the body's basal metabolic rate and resting energy expenditure (Murphy and Bloom, 2006).

The peripheral signals that regulate appetite, food intake and energy balance originate from the gut and adipose tissue. Two general categories of signal are recognized. Firstly, short-term signals which indicate the ingestion of a meal and which cause post-prandial satiation and meal termination. Examples of short-term signals that regulate acute food intake include the anorexigenic gut hormones glucagon-like peptide-1 (GLP-1) (Turton et al., 1996), peptide YY (PYY) (Batterham et al., 2002), oxyntomodulin (OXM) (Cohen et al., 2003) and pancreatic polypeptide (PP) (Batterham et al., 2003b). Characteristically, these gut hormones are found at relatively low levels in the circulation before meals, and are secreted at high levels after eating a meal. The only orexigenic (appetite increasing) gut hormone is ghrelin, which is characteristically secreted at high levels just before meals, and which declines rapidly after ingestion of a meal (Kojima et al., 1999; Wren et al., 2000). Secondly, long-term signals act to report the overall levels of energy stored in the

body, mainly in the form of adipose tissue. The prime example of this is leptin, which is produced by the adipose tissue and whose circulating levels are positively correlated with total body fat mass (Considine et al., 1996).

This thesis examines the physiology of the three specific gut hormones: PYY, GLP-1 and PP. The following sections summarise our current state of knowledge regarding these hormones.

Hypothalamus

PVN

NPY
AgRP

ARC

POMC
CART

Sympathetic nervous system

Thyroid axis

Cerebral cortex

HYPOTHALAMUS

Leptin

PP

AP

BRAINSTEM
NUCLEI

DNV NTS

GLP-1
PYY$_{3-36}$
OXM

Vagus
nerve

Ghrelin
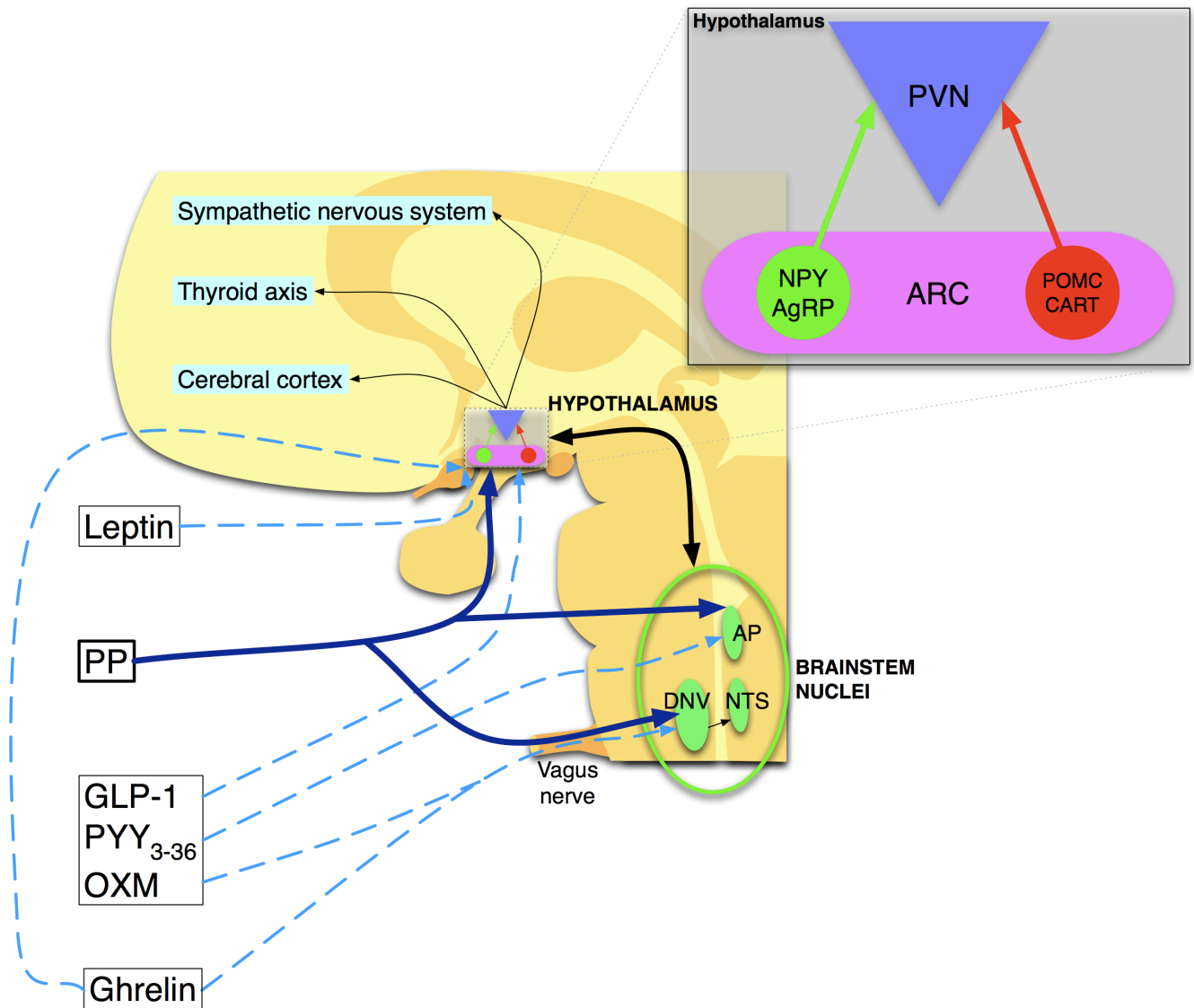
Figure 1: Appetite regulatory centres of the CNS. The two main centres in the CNS that regulate appetite, the brainstem nuclei (DNV – Dorsal Nucleus of the Vagus; NTS – Nucleus Tractus Solitarius; AP – Area Postrema) and the hypothalamus (ARC – arcuate nucleus; PVN – paraventricular nucleus) are indicated. These two centres enjoy extensive reciprocal connections with each other. Within the ARC there exist two antagonistic sets of neurones projecting to the PVN: orexigenic (green) neurones expressing NPY and AgRP, and anorexigenic (red) neurones expressing POMC and CART (top right hand of figure). The regulation of food intake and weight is determined by the balance of activities between these two sets of neurones, and their influence on the PVN. The PVN in turn influences higher centres in the cerebral cortex (to regulate food seeking behaviour), the thyroid axis and sympathetic nervous system (to regulate basal metabolism). Some peripheral influences on the brainstem nuclei and the hypothalamus are indicated (leptin, GLP-1, PYY$_{3-36}$, OXM, Ghrelin – the light blue dashed arrows indicate likely mechanisms of action). In particular, PP is thought to exert its effects by: (1) diffusion through areas where the blood-brain barrier is incomplete and activation of Y4 NPY receptors at the AP and perhaps the median eminence (ME) of the hypothalamus; and (2) activating visceral vagal nerve afferents (dark blue arrows). Adapted from Bloom et al., 2008.

1.3 Pancreatic polypeptide (PP)

PP was originally discovered as a contaminant of insulin preparations from chicken pancreas (Kimmel et al., 1975; Kimmel et al., 1968). PP was also independently isolated from bovine insulin preparations (Lin and Chance, 1974). The human PP was subsequently discovered when a specific radioimmunoassay was developed (Adrian et al., 1976a).

1.3.1 Structure of PP

PP is a 36 amino acid, C-terminally amidated peptide (**Figure 2**). It is a member of the PP-fold peptide family. This family encompasses NPY, PYY and PP (Berglund et al., 2003a). These peptides contain a common structural motif, the PP-fold, which consists of two anti-parallel helices, a type 2 proline helix and an alpha helix connected by a type 2 beta turn (Fuhlendorff et al., 1990; Wood et al., 1977). The two helices interact via hydrophobic residues, stabilising the fold (Glover et al., 1984).

The sequences of human and bovine PP are closely related and differ only by two residues (Boel et al., 1984; Kimmel et al., 1975). When the sequences of the PP orthologues from mammals, avians and reptiles are compared, there are eight conserved residues, i.e. Pro^5, Pro^8, Gly^9, Ala^{12}, Leu^{24}, Tyr^{27}, Arg^{33} and Arg^{35}, implying that these particular residues mediate important interactions/functions (Conlon et al., 1998).
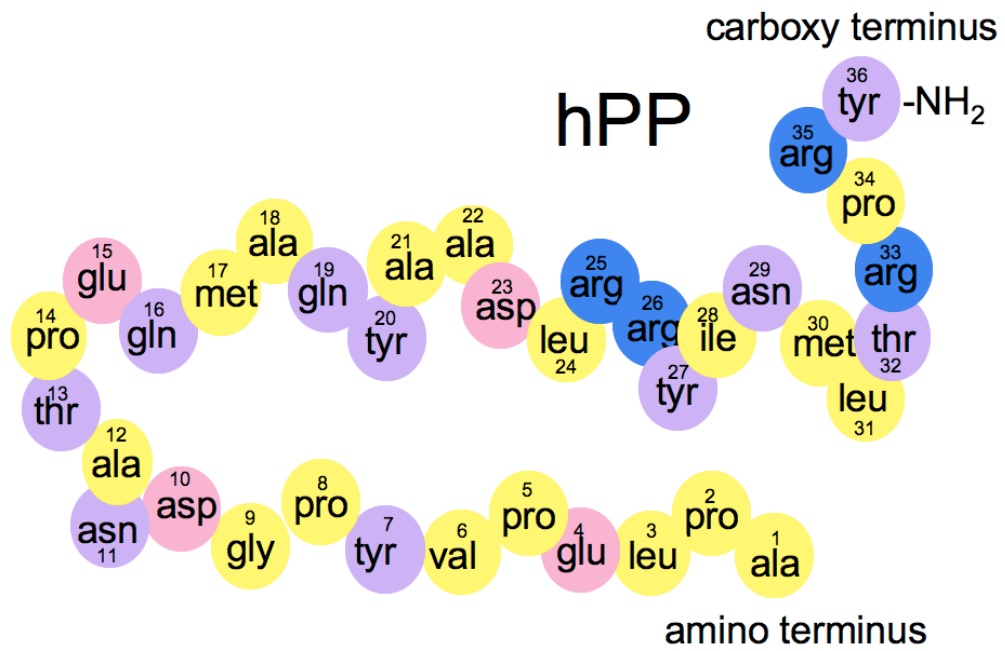
Figure 2: Primary sequence of human PP (hPP). Amino acid residues are abbreviated as per Appendix 7.1. NH$_2$=carboxy terminal amide group. Blue=positively charged side chains, Pink=negatively charged side chains, Lilac=polar uncharged side chains, Yellow=hydrophobic side chains. Numbers denote the residue numbers from the amino terminus.

1.3.2 Structure of the PP gene

The *H. sapiens* gene encoding the precursor preproPP (HGNC name: *PPY*) is located on chromosome 17, adjacent to the gene encoding the precursor of PYY (HGNC name: *PYY*). *PPY* appears to have come about as a tandem duplication of the *PYY* gene with or just before the emergence of tetrapods, approximately 4-500 million years ago (Conlon, 2002). In a second evolutionary event, the *PYY-PPY* gene cluster has been duplicated in the primate and ungulate lineages, after the divergence of the rodents, to create a *PYY2-PPY2* cluster on chromosome 17. The *PPY2* gene is a pseudogene as the gene does not encode a functional protein: there is a premature stop codon in the signal peptide (Conlon, 2002).

The cDNA for *PPY*, first isolated and sequenced by Boel and colleagues, consists of four constitutively spliced exons (Boel et al., 1984). Exon 1 encodes the sequence of the 5′ untranslated region (45 nt). Exons 2-4 encode the protein sequence. Exon 2 contains the signal peptide and most of PP except the C-terminal Tyr^{36}. Exon 3 encodes Tyr^{36} and the C-terminal PP 'icosapeptide' (Schwartz et al., 1984). Exon 4 encodes the C-terminal heptapeptide and the 3′ end of exon 4 contains a 3′ untranslated region, 124 nt (Leiter et al., 1985).

1.3.3 Processing of preproPP and PP

The precursor (preproPP) is a 95 amino acid peptide, which is processed to PP (residues 30-65 of preproPP). The N-terminal signal peptide is cleaved in the endoplasmic reticulum (residues 1-29 of preproPP) to release proPP (residues 30-95). Residues 66-68 (Gly-Lys-Arg – 'GKR') are the cleavage and amidation signal

(Bradbury et al., 1982; Bradbury and Smyth, 1987). Residues 69-87 encode the PP icosapeptide (Schwartz et al., 1984), which is followed by the C-terminal heptapeptide (residues 88-95).

From pulse-chase experiments, proPP is thought to be first cleaved at the 'dibasic cleavage site' Lys-Arg (residues 67,68) to release PP and the proPP C-terminal peptide (Schwartz and Tager, 1981). The proPP C-terminal peptide is subsequently processed at the 'monobasic cleavage site' to release the PP icosapeptide and the C-terminal heptapeptide (Wulff et al., 1993). Immunohistochemical studies show that the prohormone convertases PC1/3 and PC2 do not co-localise with PP in human islets, raising the possibility that other enzymes are responsible for the dibasic cleavage (Portela-Gomes et al., 2008).

C-terminal amidation of PP is required for PP bioactivity. A C-terminal amidation activity is commonly found within exocrine granules (von Zastrow et al., 1986). The enzyme peptidylglycine alpha-amidating monooxygenase (PAM) is known to be involved in the C-terminal amidation of similar peptides (Bradbury et al., 1982). However, immunohistochemical studies show that PAM does not co-localise with PP in human islets (Martinez et al., 1993), so the identity of the enzyme responsible for the C-terminal amidation remains unknown.

Multiple forms of PP immunoreactivity, separable by gel filtration chromatography, are found in circulation (Villanueva et al., 1977). Normal pancreatic tissue only produces one form of PP, which is indistinguishable from purified PP (Adrian et al.,

1976b; O'Hare et al., 1985). Therefore the circulating forms are likely to represent incompletely cleaved or breakdown products instead of representing the secretion of modified forms of PP from the pancreas.

Pharmacokinetic studies with bovine PP infusions into human volunteers reveal that PP is rapidly broken down, with an apparent terminal elimination half-life of 6.9 ± 0.3 min, a metabolic clearance rate of 5.1 ± 0.2 ml/kg/min and an apparent volume of distribution of 51 ± 3 ml/kg (Adrian et al., 1978b). This rapid breakdown is mediated by endogenous peptidases such as neprilysin (neutral endopeptidase 24.11) and dipeptidyl peptidase IV (DPP-4) (Baxter et al., 2009; Baxter et al., 2010).

1.3.4 Distribution and secretion of PP

PP is secreted by the PP-cells of the islets of Langerhans. The PP-cells tend to be localized in the periphery of the islets (Baetens et al., 1979), and are found in greater quantities in the head of the pancreas as opposed to the tail (Ekblad and Sundler, 2002; Larsson et al., 1975; Orci et al., 1978). In primates, the majority (93%) are found in the pancreas, but PP containing cells are also found in the distal gut. However, no detectable circulating PP levels are found in patients who have undergone pancreatectomy, indicating that the extra-pancreatic PP may not be physiologically important (Adrian et al., 1976b).

The mean fasting plasma level of PP in normal volunteers is 31.2 ± 6.2 pmol/L (Adrian et al., 1976b). PP secretion is stimulated by food, and this secretion is

biphasic, with an acute peak of secretion from 0–60 minutes and a more sustained peak from 60 to at least 360 minutes (Adrian et al., 1976b). PP secretion is proportional to the size of the meal eaten (Track et al., 1980). Post-prandial PP secretion appears to be under the control of the vagus nerve and in particular by cholinergic neurotransmission, such that vagotomy or atropine markedly reduce PP secretion (Schwartz et al., 1978).

Stimulation of PP secretion by food intake is triggered by enteral nutrient ingestion. Oral glucose is able to trigger a 50% rise in PP secretion (Sive et al., 1979). The intraduodenal instillation of triglyceride emulsions causes PP release, although the magnitude of release is not as large as with a complete meal. This phenomenon is inhibited by a lipase inhibitor, indicating it is dependent on digestion of triglycerides (Feinle-Bisset et al., 2005). PP release is not stimulated by parenteral infusions of glucose, amino acids or fat (Adrian et al., 1977).

Insulin-induced hypoglycaemia induces a sharp rise in PP secretion from the pancreas (Adrian et al., 1977). Similarly, tissue glucopenia induced by the non-metabolizable glucose analogue, 2-deoxyglucose, is able to stimulate PP release. The effect of hypoglycaemia on PP release appears to be at least partly mediated by cholinergic transmission as atropine reduces the amplitude of PP release (Hedo et al., 1978; Zulueta et al., 1982).

Other influences on PP secretion of less certain physiological relevance include:

Cholecystokinin (CCK): The infusion of caerulein, a cholecystokinin analogue, in humans can stimulate PP release (Adrian et al., 1977). The main CCK peptides found in man, CCK-8 and CCK-33, similarly stimulate secretion when infused into volunteers (Ahren et al., 1991).

Bombesin: Bombesin is a 14-residue peptide originally isolated from the skin of frogs, homologous to the mammalian peptides gastrin releasing peptide and neuromedin B (Gonzalez et al., 2008). In the isolated perfused dog pancreas, bombesin does not stimulate PP release (Adrian et al., 1978a). However, a bolus injection of bombesin into human subjects can cause PP secretion in response, albeit with variable effect (Lamers et al., 1984), and the physiological relevance of this observation is unknown.

Secretin, Gastric Inhibitory Peptide (GIP), Vasoactive Intestinal Peptide (VIP): Secretin, GIP and VIP are all related 'secretin family' gut hormones which stimulate PP release (Adrian et al., 1977; Adrian et al., 1978a; Ahren et al., 1991; Glaser et al., 1980).

Somatostatin: Although early studies showed that infusion of somatostatin was able to inhibit PP secretion in gastrectomized patients (Marco et al., 1977), later studies showed that there appears to be no direct effect of somatostatin-14 (the shorter, alternatively cleaved form of somatostatin) infusion on PP secretion from an isolated, perfused human pancreatic preparation (Kleinman et al., 1995). There was no effect when intraislet somatostatin was immuno-neutralized (Kleinman et al., 1995).

Somatostatin's effects may therefore be indirect, for example, due to a tonic inhibition of other factors inducing PP release.

Ghrelin and related peptides: The octanoylated form of ghrelin and obestatin (an alternative product of the processing of the proghrelin precursor) inhibit the secretion of PP by isolated rodent islets (Qader et al., 2008).

1.3.5 Receptors and signaling cascades for PP

PP-fold peptides bind to the neuropeptide Y family of seven-transmembrane-domain G-protein coupled receptors Y1, Y2, Y4, Y5 and Y6 (Berglund et al., 2003a). PP binds with highest affinity to the Y4 receptor with a K_d of 13.8 pM (Dumont et al., 2007; Lundell et al., 1995). PP also binds to Y1 and Y5 with lesser affinity (Berglund et al., 2003a). The Y4 receptor is widely distributed, with expression in the small and large intestine (Goumain et al., 1998) and in the CNS (Larsen and Kristensen, 2000; Whitcomb et al., 1997). CNS expression is found in the hypothalamus, the area postrema (AP) and a sub-region of the NTS, the subnucleus gelatinosus (Dumont et al., 2007; Larsen and Kristensen, 1997, 2000). The Y4 receptor exists in the cell membrane as homodimers, but these dissociate upon binding PP (Berglund et al., 2003b). The activated Y4 receptor inhibits adenylate cyclase via the G_i G-protein, therefore reducing intracellular cyclic AMP levels (Dumont et al., 2007; Lundell et al., 1996). Y4 also activates G_q, inositol triphosphate release and intracellular Ca^{2+} release (Misra et al., 2004).

The key residues involved in binding of PP to Y4 appear to be Pro^2, Pro^3, Arg^{33}, Arg^{35}, Tyr^{36}. Equally of importance is the C-terminal amide: elimination of this feature substantially reduces binding (Gehlert et al., 1996) and removes the physiological effects of bovine PP, for example the inhibition of pancreatic secretion (Lin et al., 1977).

A distinctly different specific PP-binding receptor activity has been found in preparations from rat liver, which binds PP with a lower affinity (K_d of 3.4-4.6 nM) (Nguyen et al., 1992), and which is up-regulated by PP treatment of animals (Seymour et al., 1996). The Y4 receptor is not expressed in the liver (Bard et al., 1995), implying that the hepatic PP receptor is a different receptor type, the exact nature of which is not known.

1.3.6 Biological actions of PP

1.3.6.1 Effects on appetite, energy expenditure and energy balance

A major physiological action of PP is its role as a post-prandial satiety hormone. Peripherally administered PP reduces food intake when given to mice (Malaisse-Lagae et al., 1977). Repeated IP PP injections in *ob/ob* mice reduce body weight gain, ameliorate insulin resistance and lower plasma lipid levels (Asakawa et al., 2003). Over-expression of PP in the pancreatic islets in transgenic mice reduces food intake and fat mass (Ueno et al., 1999).

A study in lean human volunteers showed that IV infusion of PP at 10 pmol/kg/min for 90 minutes which increased PP levels from a baseline of 15.5 ± 4.4 pmol/L to 258.5 ± 21.4 pmol/L (a supraphysiological concentration) reduced food intake by $21.8 \pm 5.7\%$, as assessed by consumption of a buffet lunch (Batterham et al., 2003b). Interestingly, despite the relatively short time of exposure to PP, there was a significant inhibition ($25.3 \pm 5.8\%$) of cumulative food intake during the 24 hours following infusion, indicating that the biological effect of the PP appears to outlast the elevation in PP levels induced by the infusion as the PP levels had essentially returned to baseline levels 120 minutes after termination of the infusion (Batterham et al., 2003b).

A follow-up study by the same group showed that a lower dose at 5 pmol/kg/min, when infused into lean human volunteers, achieved concentrations similar to post-prandial levels (mean peak of 184.4 ± 29.7 pmol/L). This was able to reduce food intake but to a lesser degree, by 11% (Jesudason et al., 2007). Unlike the earlier study, there was no significant inhibition of cumulative 24-hour food intake with this lower dose (Jesudason et al., 2007).

Schmidt et al. also administered IV PP at doses up to 2.25 pmol/kg/min in lean human volunteers (Schmidt et al., 2005). Interestingly, this lower-dose infusion achieved peak levels of 299 ± 23.7 pmol/L, higher than in Batterham's study (Batterham et al., 2003b). The study did not focus on food intake as an endpoint, but it was noted that PP infusion was not associated with significant reductions in hunger and appetite as assessed by visual analogue scales.

A possible site of PP's appetite-suppressive action is the AP, as Y4 receptors are particularly rich in this area, and as this area is outside the blood-brain barrier (Kojima et al., 2007; Larsen and Kristensen, 2000). IP PP injection activates the AP and nucleus tractus solitarius (NTS) as assessed by staining for the neuronal activation marker c-*fos* (Lin et al., 2009). Visceral vagal nerve afferents (which terminate in the AP) are another plausible site of action, as vagotomy abolishes the anorectic effect of PP in mice (Kojima et al., 2007).

The hypothalamus is also another possible site of action, as circulating PP may be able to access the hypothalamus through the incomplete blood-brain barrier at the ME. The PVN of the hypothalamus is known to express Y4 receptor mRNA as shown by *in situ* hybridization (Larsen and Kristensen, 2000). A conditional knockout of the Y4 receptor in the hypothalamus abolishes the activation of the alpha-MSH neurons by IP PP, suggesting that peripherally administered PP does indeed bind and activate hypothalamic Y4 receptor (Lin et al., 2009). IP PP activates c-*fos* and POMC expression in the anorexigenic neuronal group of the ARC that releases alpha-MSH, which is processed from POMC (**Figure 1** and Lin et al., 2009). Knockout of α-MSH's cognate receptor, MC4R, abolishes the anorexigenic effect of IP PP. The implication, therefore, is that peripheral PP binds to hypothalamic Y4 receptors, and that the anorexigenic effect is then mediated by α-MSH and the MC4 receptor.

In addition to its action on food intake, repeated IP injections of PP also stimulate sympathetic activity and oxygen consumption in animals, causing an increased energy

expenditure that would be predicted to augment weight loss (Asakawa et al., 2003). However, such an effect is not seen in the transgenic model of PP over-expression (Ueno et al., 1999). This may be due to differences in the temporal pattern of PP elevation as the repeated IP injections were given twice a day, whereas the transgenic model caused a more continuous over-expression of PP. Alternatively, this difference may be due to the fact that the IP injection study was conducted in *ob/ob* mice (Asakawa et al., 2003), whereas the transgenic over-expression study was conducted in mice with a different genetic background (Ueno et al., 1999). No studies in humans yet exist to confirm if PP does increase energy expenditure.

Finally, it is notable that centrally administered PP appears to have an opposite effect to peripherally administered PP, stimulating food intake in animals (Asakawa et al., 1999; Clark et al., 1984; Flynn et al., 1999; Inui et al., 1991). It is as yet unclear if this effect is mediated via central Y4 or other receptors. It is also unclear if this effect has any physiological significance.

1.3.6.2   Effects on pancreatic secretion and gall bladder function

PP inhibits pancreatic exocrine secretion (Lin et al., 1977) and the motor function of the biliary tree and gall bladder, as assessed by measurement of trypsin and bilirubin output respectively in duodenal juice samples in volunteers undergoing infusion of secretin and the CCK receptor agonist caerulein to stimulate pancreatic secretion and gall bladder contraction. The infusion of bovine PP to achieve typical post-prandial PP levels in this context inhibited trypsin output four-fold, and suppressed bilirubin output almost completely (Greenberg et al., 1978). However, this effect is indirect:

studies on isolated rat pancreatic acini demonstrate that bovine PP is unable to inhibit amylase secretion (Jung et al., 1987; Louie et al., 1985). Extrinsic denervation of the pancreas does not affect PP inhibitory activity, which suggests that intrinsic mechanisms are involved (DeMar et al., 1991; Kohler et al., 1991). The physiological relevance of this inhibition of pancreatic secretion is unknown. Studies utilizing immunoneutralization with anti-PP serum have shown contradictory results. One study showed that this treatment did not affect pancreatic secretion, but did partially inhibit the effects of exogenous PP (Konturek et al., 1987). However, another study showed that anti-PP serum did significantly increase interdigestive and postprandial pancreatic secretion (Shiratori et al., 1988). It is therefore still unclear whether the inhibitory effects of PP on pancreatic secretion are physiologically relevant.

1.3.6.3   Gastric effects

Bovine PP increases gastric acid secretion in dogs (Lin et al., 1977) but this effect appears to be absent in humans (Adrian et al., 1981; Parks et al., 1979). The published effects of PP on gastric emptying are conflicting. The study of Schmidt and colleagues, where human PP was infused IV into volunteers eating a radioactively labeled meal, showed that this treatment slowed gastric emptying of solid food but not water (Schmidt et al., 2005). However, their study used a dose that was sufficient to increase circulating levels to approximately 3-fold those seen after meals in the control group. Moreover, Adrian and colleagues' study did not show any slowing of gastric emptying with their IV infusion of human PP (Adrian et al., 1981). Thus, the physiological relevance of Schmidt and colleagues' observation is unclear.

1.3.6.4 Metabolic effects

The infusion of bovine PP does not affect the basal nor the post-prandial secretion of insulin (Adrian et al., 1980). Similarly an infusion of human PP did not affect the basal secretion of insulin (Batterham et al., 2003b). Both studies showed no significant effects of PP infusion on the secretion of glucagon, gastrin, GIP, secretin, oxyntomodulin, PYY, GLP-1, ghrelin or leptin (Adrian et al., 1980; Batterham et al., 2003b). Schmidt et al. did show a slight delay (16 minutes) to the peak of post-prandial insulin secretion at the highest dose of human PP employed, together with a sustained post-prandial rise in glucose. This may be explained by the delay in gastric emptying of solid food that they observed (Schmidt et al., 2005).

Can PP influence insulin sensitivity? The rate of glucose disposal during an euglycaemic-hyperinsulinaemic clamp study is significantly reduced by 44% after pancreatectomy in dogs, i.e. the procedure induces insulin resistance. The insulin sensitivity in these animals was restored to normal with 16 days treatment with a "pulsed" PP infusion. However, the glucose excursion in response to an oral glucose load was not significantly influenced by the infusion (Prillaman et al., 1992). This phenomenon was also studied in patients with chronic pancreatitis. Brunicardi and colleagues showed that an 8-hour bovine PP infusion restored insulin sensitivity in these patients. PP reduced patients' mean glucose levels in response to an oral glucose challenge in two of the five patients studied (Brunicardi et al., 1996). These effects may be mediated by the hepatic PP receptor, which appears to be over-expressed when rats are rendered PP-deficient using a model of chronic pancreatitis (Seymour et al., 1998). Chronic PP treatment of insulin-resistant and hyperlipidaemic *ob*/*ob* and fatty liver Shionogi-*ob*/*ob* mice causes the animals to lose weight by reducing food

intake. This lowered glucose levels slightly without altering insulin levels, suggesting that the weight loss caused a commensurate reduction in insulin resistance (Asakawa et al., 2003).

1.3.6.5   Pancreatic polypeptide and neuroendocrine tumours

High levels of plasma PP are frequently detected in patients with pancreatic neuroendocrine tumours (PNETs) (Polak et al., 1976). Some of these are explained by PP cell hyperplasia in the normal islets surrounding a PNET, and some due to incorporation of PP-secreting cells within the tumour itself. More rarely, some cases are caused by a primary PP-secreting PNET, a so-called 'PPoma' (Schwartz, 1979). PPomas are generally clinically silent until they become large enough to present with mass effects. Although watery diarrhea was initially thought to be part of the PPoma tumour syndrome (Larsson et al., 1976), it is now evident that this only occurs in 1 out of 3 patients (Kuo et al., 2008). Weight loss as a presenting symptom occurred in 50% of patients in a small series, consistent with a PP-mediated suppression of appetite (Strodel et al., 1984). In contrast to other PNET subtypes, metastatic/malignant PPomas are relatively uncommon (Kuo et al., 2008).

1.3.6.6   The relationship of obesity to pancreatic polypeptide

Given its post-prandial release and postulated role in reducing appetite after food intake, it is possible that obesity may be due, at least partially, to a reduction in PP secretion, therefore decreasing the post-prandial feedback inhibition of food intake. In an early study, the circulating levels of PP were measured in obese subjects and compared to lean subjects. Fasting PP levels were lower in obese subjects, and there

was a decreased secretion of PP to a protein-rich meal (Lassmann et al., 1980). Other groups have found similar decreases in the magnitude of PP responses to food intake, consistent with such a hypothesis (Holst et al., 1983; Koska et al., 2004; Lieverse et al., 1994). Conversely, other studies have found that there are no significant differences in PP levels (fasting or post-prandial) between lean and obese subjects (Jorde and Burhol, 1984; Wisen et al., 1992). Thus, there remains some controversy as to the exact relationship of circulating PP levels and obesity. These inconsistencies may perhaps be due to the fact that there are marked variations in PP secretion, due to diurnal variation, influenced by age (where increasing age is associated with increased PP levels), and characterized by large inter-individual variations (Johns et al., 2006), leading to divergences in measured PP levels. Moreover, another possible factor at play here is that PP levels positively correlate with visceral adiposity but not subcutaneous adiposity (Tong et al., 2007).

1.4 Peptide YY (PYY)

PYY was discovered in 1980 by Tatemoto and Mutt at the Karolinska Institute in Stockholm, Sweden (Tatemoto and Mutt, 1980). They isolated a novel peptide from porcine small intestine by developing a chemical assay method to identify the C-terminal amide structure, characteristic of many biologically active peptides including PP and GLP-1 (Tatemoto and Mutt, 1980). PYY gained its name as the primary sequence incorporates a high proportion of tyrosine residues, including one at both the N- and C-terminals.

1.4.1 Structure of PYY

PYY is a 36 amino acid peptide hormone that incorporates a C-terminal amide group, like PP and NPY, the other members of the 'PP fold' family, and these have a common hairpin-like U-shaped fold tertiary structure. The structure of PYY_{1-36} (Nygaard et al., 2006) is illustrated in **Figure 3**.
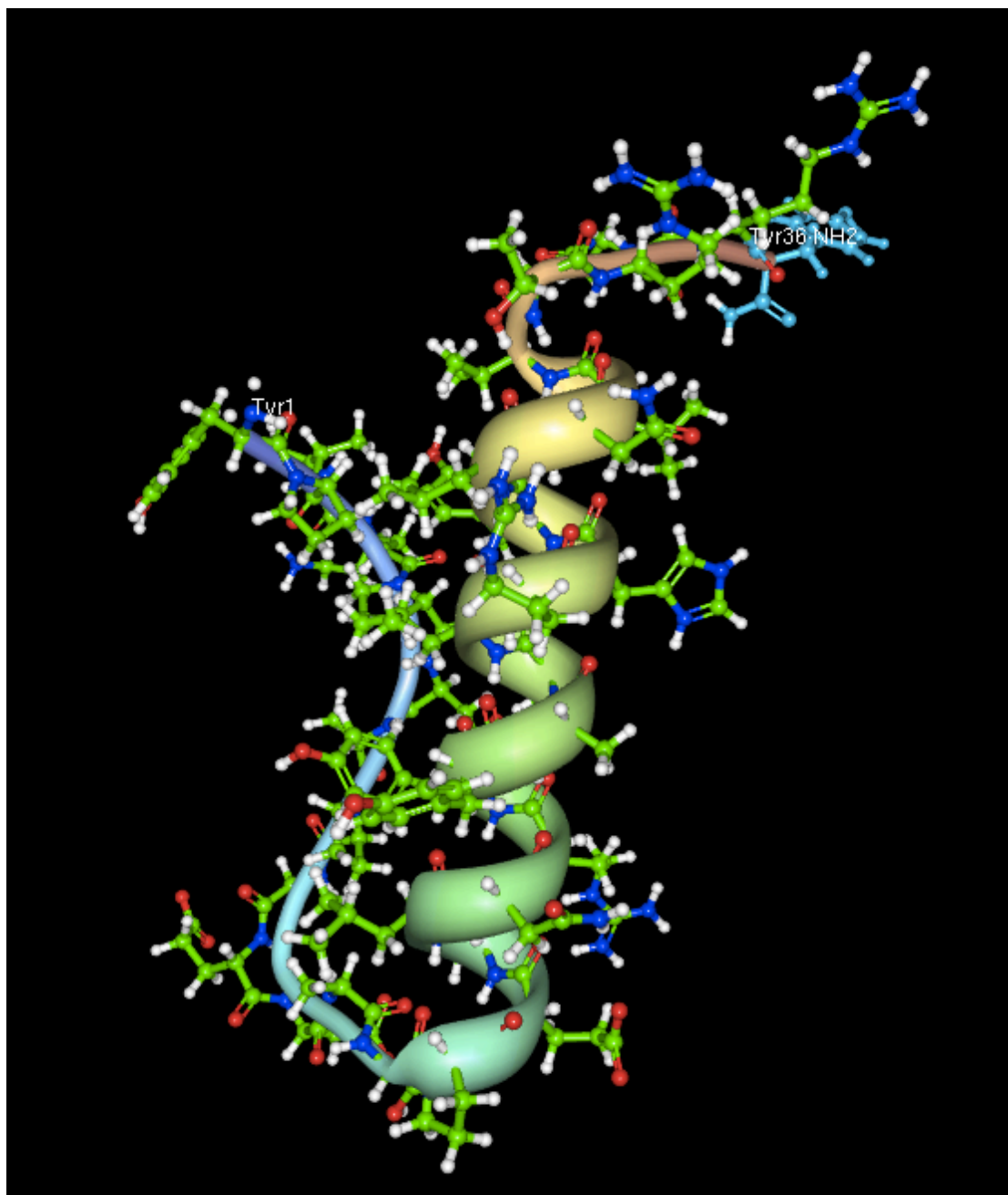
Figure 3: Structure of PYY_{1-36} from nuclear magnetic resonance analysis. The line of the backbone is shown as a ribbon diagram and the side chains are shown as ball-and-stick representations (green =

C, red = O, blue = N, white = H). Tyr^1 and Tyr^{36} (which is C-terminally amidated: NH_2) are labeled. Residues 15 to 32 adopt an alpha-helical conformation (shown by the ribbon helix). Structure from Nygaard et al. (2006) and generated using RCSB SimpleViewer.

1.4.2 Structure of the PYY gene

The gene encoding pre-proPYY (HGNC name: *PYY*) is found on Chromosome 17q21.1. *PYY* consists of four exons and three introns (Hort et al., 1995), and is in close proximity to *PPY*, whereas the gene encoding NPY (*NPY*) is on chromosome 7. These three peptides represent an example of a multiple gene duplication event (see Section 1.3.2). *PYY2* is a duplicate of *PYY* and is also a pseudogene, like *PPY2*.

As per *PPY*, exon 1 of *PYY* encodes the 5´ untranslated region and exon 2 encodes the signal peptide and the mature PYY peptide save the C-terminal Tyr residue. Exon 3 encodes the C-terminal Tyr followed by the 'GKR' cleavage and amidation signal, and 25 amino acids of the C-terminal extension peptide. Exon 4 encodes the remaining 7 residues of the C-terminal extension peptide and the 3´ untranslated region (Hort et al., 1995).

1.4.3 Processing of preproPYY and PYY

Pre-proPYY incorporates a signal peptide, the 36 amino acid mature PYY sequence, and a C-terminal extension peptide (Hort et al., 1995). Processing of pre-proPYY involves cleavage of the signal peptide, amidation of the C-terminal tyrosine, which is necessary for biological activity, and proteolytic cleavage by a prohormone convertase to give PYY$_{1-36}$, the 36 amino acid form of the peptide, which is secreted. As with PP, the specific enzyme responsible for C-terminal amidation is not known,

nor has there been any direct evidence that prohormone convertases 1/3 and 2 are involved in proPYY processing. Following secretion, the cell surface enzyme DPP-4 cleaves the N-terminal dipeptide from PYY_{1-36} to give PYY_{3-36}, the predominant circulating form of PYY (Eberlein et al., 1989; Grandt et al., 1993).

1.4.4 Distribution and secretion of PYY

PYY is found in the neuroendocrine L cells of the gut, which are located anywhere from the distal jejunum and ileum to the colon and rectum. These L cells are flask-shaped cells who project luminal villi via narrow apical processes, allowing the cell to sense enteral nutrient content. The largest numbers of L cells are found most distally (Bryant et al., 1983). PYY co-localises with glucagon-like peptide-1 (GLP-1), although the quantitative distribution of GLP-1 in the gut is different from that of PYY (Stanley et al., 2004). PYY is expressed in small amounts in the small intestine, but at progressively higher levels from the ascending colon though to the rectum (Adrian et al., 1985a), whereas GLP-1 is expressed more highly in the jejunum with smaller amounts in the colon, duodenum and caecum (Zhou et al., 2006).

PYY is secreted from the L cells in response to an oral nutrient load, with levels reaching a plateau within 1-2 hours after a meal and remaining elevated for up to 6 hours (Adrian et al., 1985a). The peak post-prandial levels of PYY are proportionate to the calorific content of meals (Le Roux et al., 2006b). In studies utilizing isocaloric meals, protein is the most potent macronutrient stimulus for PYY release, followed by fat and then carbohydrate (Batterham et al., 2006). Circulating PYY levels begin to rise within 15 minutes of a meal, which suggests that whilst PYY is released from the

distal gut, the mechanisms regulating its secretion may be located more proximally, implying the involvement of other neural or hormonal influences (Fu-Cheng et al., 1997). Although the vagus nerve is a prime suspect in mediating the release of PYY, the evidence is somewhat contradictory on its role. Truncal vagotomy was demonstrated by Fu-Cheng and colleagues to reduce PYY release (Fu-Cheng et al., 1997) whereas Zhang and colleagues showed that vagotomy (or cholinergic blockade) elevates basal and post-prandial release of PYY (Zhang et al., 1993).

Instillation of fat into the duodenum triggers PYY secretion, a process that requires intact lipase activity (Feinle-Bisset et al., 2005; Feltrin et al., 2006). Interestingly, intraduodenal amino acid or liver extract instillation fails to stimulate PYY release (Greeley et al., 1989a). PYY release is at least partially controlled by intestinal taste receptors on the L cells, in particular by the sweet taste receptor T1R2/T1R3. Blockade of T1R2/T1R3 by lactisole in human volunteers blunts the GLP-1/PYY response to intraduodenal glucose but not to a liquid mixed meal, suggesting that the post-prandial release of PYY is more importantly controlled by mechanisms other than direct sensing by T1R2/T1R3 (Gerspach et al., 2011). Other stimuli that directly stimulate PYY secretion from the colon are short chain fatty acids (SCFA) (Longo et al., 1991), the bile salt deoxycholate (Adrian et al., 1993; Ballantyne et al., 1989; Izukura et al., 1991), amino acids, liver extract and even saline (Greeley et al., 1989a) can.

SCFA sensing is of particular interest in this context. PYY concentrations are elevated for up to six hours post-prandially, and direct sensing of SCFA in the distal gut may

be the mechanism responsible for this prolonged elevation of peptide levels. The SCFA receptors GPR43 and GPR41 have been co-localized to the PYY-releasing enteroendocrine L cells of the distal gut (Karaki et al., 2006; Nohr et al., 2013; Tazoe et al., 2008). PYY (and GLP-1) mRNA expression in the distal gut is inducible by administering a diet high in resistant starch, which is known to increase the levels of SCFA in the distal gut by fermentation (Zhou et al., 2008).

Cholecystokinin (CCK), a hormone secreted from the duodenum in response to fat and protein ingestion, is a key factor mediating PYY release in response to these stimuli (Greeley et al., 1989b). In addition to regulating PYY release, CCK also regulates Y2 receptor expression on the vagus nerve, i.e. it regulates the sensitivity of the vagus to PYY's effects (Burdyga et al., 2008). In CCK receptor null mice, or on administration of a CCK receptor antagonist, PYY secretion is attenuated (Dockray, 2009). Intraduodenal fat administration does stimulate PYY secretion, but this effect can be blocked by a CCK1 receptor antagonist, demonstrating that CCK has a key role in mediating the PYY response to nutrient ingestion (Degen et al., 2007).

More recently, PYY has been detected in saliva and increased secretion of salivary PYY is seen after meals (Acosta et al., 2011). Salivary PYY partially derives from re-secretion of plasma PYY, but some is derived from direct expression and secretion of PYY by the taste cells in the taste buds of the tongue. Moreover, the Y2 receptor, which is activated by PYY, is expressed in the tongue epithelium. Using a viral vector, Acosta and colleagues induced hypersecretion of PYY in the saliva and this led to reductions of body weight and food intake in animal models of obesity

indicating that salivary PYY appears to be able to suppress food intake in a similar fashion to circulating PYY (Acosta et al., 2011).

1.4.5 Receptors and signaling cascades

As with PP, PYY binds to the NPY receptor family. Members of this receptor family, like the PP-fold peptides, appear to have arisen from a common ancestor gene through duplication (Larhammar et al., 1998). In mammals, five receptor subtypes have been cloned, namely Y1, Y2, Y4, Y5 and Y6 (Michel et al., 1998). In humans, PYY_{1-36} binds to all receptors in the Y family (Y1, Y2, Y4 and Y5). PYY_{3-36}, the predominant circulating form of PYY, is more selective for the Y2 and Y5 receptors, and has a lesser affinity for Y1 and Y4 receptors (Keire et al., 2000b).

1.4.5.1 Signalling Pathways

Y receptors are coupled to inhibitory, pertussis toxin-sensitive G proteins, and their activation results in inhibition of adenylate cyclase, the enzyme responsible for formation of cyclic AMP (cAMP) from ATP, modulation of calcium and potassium channels, and the mobilization of intracellular calcium (Michel et al., 1998). In addition to this activity, there is evidence that Y1, Y2 and Y4 are coupled to smooth muscle contraction via inositol 1,4,5-triphosphate (IP_3) dependent calcium release in rabbit gastric smooth muscle cells (Misra et al., 2004). Similarly, the Y1 receptor activates phospholipase C (PLC) and IP_3 mediated intracellular calcium release, triggering a positive inotropic effect in cardiac myocytes (Heredia et al., 2005).

1.4.5.2 Y1 Receptors

Y1 receptors have a wide tissue distribution. Y1 receptors are particularly found in the colon, kidney, adrenal gland, heart and placenta (Wharton et al., 1993). The majority of Y1 expression, however, is within blood vessels and nerves (Matsuda et al., 2002). Y1 receptor agonists have been shown to mediate vasoconstriction in renal and splenic arteries (Malmstrom, 1997). In the same study, the Y1 receptor antagonists SR 120107A and BIBP 3226 eliminated the prolonged vasoconstriction mediated by perivascular sympathetic nerves, suggesting an important role for the Y1 receptor in the sympathetic nervous system and blood pressure control.

Furthermore, Y1 receptors are detected in the human brain, and throughout the CNS (Migita et al., 2001). Of particular interest is the fact that they are present in the hypothalamic nuclei involved in the regulation of appetite and energy homeostasis, where they mediate the suppressive effects of NPY on appetite (Stanley et al., 2004). Y1 receptors bind intact PYY_{1-36}, but PYY_{3-36} does not bind well to this receptor (Keire et al., 2000a).

1.4.5.3 Y2 Receptors

Y2 receptors were first discovered when it was noted that a C-terminal fragment of PYY, PYY_{13-36}, was found to have reduced potency in vascular preparations compared to NPY and PYY_{1-36}, whilst remaining potent in vas deferens preparations. This was the first suggestion there was more than one NPY receptor subtype, and at that time the two identified subtypes were named Y1 (receptors with poor affinity for C-terminal fragments of NPY) and Y2 (receptors with good affinity for C-terminal

fragments) (Michel, 1991). Y2 receptors have limited homology with Y1 receptors, but a much higher affinity for PYY, particularly PYY_{3-36} (Michel et al., 1998). For Y1 receptor binding, the K_d for PYY_{1-36} is 0.42 nM, with a calculated K_i value of 1050 nM for PYY_{3-36}. At the Y2 receptor, the K_d for PYY_{1-36} is 0.03 nM with a K_i value for PYY_{3-36} of 0.11nM (Keire et al., 2000a). These values show that PYY_{3-36} binds poorly to Y1 receptors, as specified above, whilst PYY_{1-36} and PYY_{3-36} have roughly equivalent binding affinities for the Y2 receptor. Like Y1 receptors, Y2 receptors are found throughout the peripheral nervous system (PNS) as well as the CNS, particularly in the ARC. The ARC is closely related to the ME, which as previously mentioned possesses a blood brain barrier that is incomplete, a property that allows CNS sensing of peripheral, in this case hormonal, signals. In humans, Y2 receptor mRNA has been localized in the mucosa of the ileum and ascending colon, as well as in the muscular wall of the ileum and descending colon (Ferrier et al., 2002).

1.4.5.4 Y3 Receptors

This does not exist: the Y3 receptor was initially thought to be a subtype that had a higher affinity for NPY compared to PYY, but it has never subsequently been cloned, and no specific agonists or antagonists for this receptor subtype have been identified. As the 'Y3' designation had already been allocated to this potential receptor subtype, the designation has not been used again for subsequently discovered genuine NPY receptors (Michel et al., 1998).

1.4.5.5　Y4 Receptors

Y4 receptors are the principal receptor for PP (Michel et al., 1998). They have a relatively low affinity for PYY_{3-36}, and are discussed in further detail in Section 1.3.5.

1.4.5.6　Y5 Receptors

Y5 receptors, like Y1 and Y2, are found with a wide tissue distribution within the brain in murine studies (Nakamura et al., 1997). By Northern blot analysis, Borowsky et al. have shown that the Y5 receptor also has a wide tissue distribution in the human brain (Borowsky et al., 1998). The potency of PP fold peptides for the Y5 receptor is in the order of $NPY \geq PYY_{1-36} \approx PYY_{3-36} > PP$ (Michel et al., 1998).

1.4.5.7　Y6 Receptors

The Y6 receptor is functional in mice and rabbits (Larhammar et al., 1998), but the human Y6 gene on chromosome 5 appears to be a pseudogene, inactivated by a frame shift mutation (Gregor et al., 1996; Matsumoto et al., 1996). The Y6 receptor is absent entirely in the rat.

1.4.6　Biological Actions of PYY

1.4.6.1　Gastrointestinal Tract

PYY reduces gastric emptying, and slows intestinal transit time. This effect is termed the 'ileal brake' as it represents the ability of the distal small intestine (ileum) to inhibit gastric emptying in response to delivery of chyme to the ileum, a negative

feedback mechanism that prevents the ileum being overloaded with food from the stomach (Ballantyne, 2006). This phenomenon is reproduced by infusion of PYY_{3-36} to healthy volunteers (Ballantyne, 2006). In rats, specific PYY binding sites have been identified in the dorsal vagal complex (DVC), an area of the medullary brainstem that incorporates the AP, the NTS and the dorsal motor nucleus of the vagus. It is likely to be through these areas that PYY exerts control over the slowing of intestinal transit (Chen and Rogers, 1995).

In addition to its effects on gastrointestinal motility, PYY is also known to inhibit gastric acid release when administered peripherally (Guo et al., 1987a). An intravenous infusion of PYY_{1-36} inhibits the cephalic (anticipation and smell/taste of food) and gastric (stimulated by gastric distension and chemical effects of food in the stomach) phases but not the duodenal (stimulated by small intestine dilatation and chyme entering the duodenum) phases of gastric acid secretion. They also noted that PYY does not act to reduce acid secretion via a reduction in gastrin release or binding to its receptor (Guo et al., 1987b). However, when injected centrally into the DVC, PYY increased gastric acid secretion (Stanley et al., 2004). It appears that there are two opposing mechanisms in the CNS to regulate vagal tone and therefore gastric acid secretion: PYY binds to Y1 receptors in the AP and NTS subcomponents of the DVC and stimulates gastric acid secretion, whereas Y2 receptors inhibit gastric acid secretion. In the gastric mucosa itself, PYY_{1-36} activates Y1 receptors on enterochromaffin cells, which inhibits gastrin-stimulated histamine release, and therefore indirectly inhibits the component of gastric acid secretion from parietal cells stimulated by histamine-2 (H2) receptors (Yang, 2002).

PYY is also a potent inhibitor of secretin- and CCK-mediated pancreatic exocrine secretion (Ballantyne, 2006). PYY_{3-36} inhibition of pancreatic secretion is regulated for the most part via Y2 receptors on vagal afferents. The vagus nerve therefore appears to play multiple important roles in mediating the effects of PYY on the gastrointestinal system.

1.4.6.2 Inhibition of Appetite and Food Intake

In 2002, Batterham et al. investigated the effects of peripheral administration of PYY_{3-36} on food intake. They showed, in rats, that PYY_{3-36} had a potent inhibitory effect on food intake both when injected peripherally, and when injected centrally into the ARC, which expresses Y2 receptor abundantly (Batterham et al., 2002). This study also established that there was no reduction in food intake in response to the administration of PYY_{3-36} in Y2 receptor null mice, providing further evidence that PYY_{3-36} acts via Y2 receptors to mediate its effects on food intake and satiety (Batterham et al., 2002). The effects of PYY_{3-36} on food intake are reduced in both Y1 and Y5 null mice, implying that Y1 and Y5 also play a part in central control of appetite (Stanley et al., 2004), but it is agreed that the major role in this pathway is played by Y2 receptor. This notion is supported by the fact that a selective Y2 receptor antagonist, when injected into the ARC, blocks the anorexigenic effect of peripherally administered PYY_{3-36} (Abbott et al., 2005b).

In humans, a 90 minute IV infusion of 0.8 pmol/kg/min of PYY_{3-36} administered in order to mimic post-prandial concentrations caused a reduction in food intake by more than a third. These healthy subjects also had a significant reduction in the

subjective feeling of hunger as assessed by visual analogue scores (Batterham et al., 2002). This anorexigenic effect was later shown to hold true in obese subjects (Batterham et al., 2003a). There was no effect on gastric emptying with the PYY_{3-36} infusion, and there was no reported nausea or vomiting (Batterham et al., 2002). PYY_{3-36} infusion was, however, shown to reduce levels of the orexigenic hormone ghrelin i.e. the anorexigenic effect of PYY_{3-36} might be mediated both directly and indirectly (Batterham et al., 2003a).

Studies in *Pyy*-null mice show that PYY has a physiological role in the control of appetite. These mice are hyperphagic, with a greater cumulative food intake than controls, and an augmented re-feeding response after fasting (Batterham et al., 2006). When total body fat was measured by MRI scanning, these mice are considerably fatter compared with wild-type controls. Replacement treatment with PYY_{3-36} caused significant weight loss and reversal of the obesity phenotype (Batterham et al., 2006).

One important site that mediates PYY_{3-36}'s actions on food intake is the hypothalamus. PYY_{3-36} administered directly into the ARC has been demonstrated to have an anorexigenic effect similar to that induced by peripheral administration of PYY_{3-36} (Batterham et al., 2002). PYY_{3-36} inhibits the orexigenic NPY/AgRP neurons in the hypothalamus in the ARC via pre-synaptic Y2 receptors, suppressing appetite. This is supported by the observation that *Npy* mRNA expression in the hypothalamus is suppressed by PYY_{3-36} treatment (Batterham et al., 2002). The NPY/AgRP neurons inhibit POMC/CART neurons via a GABA-mediated tonic inhibition of the anorexigenic POMC/CART neurons. Consequently, inhibition of the NPY/AgRP

neurons leads to activation of the POMC/CART neurons. Indeed, Batterham et al.'s paper showed that PYY_{3-36} was able to activate POMC/CART neurons in electrophysiological studies (Batterham et al., 2002). However, PYY_{3-36} still is able to suppress food intake even in mice bearing knockouts for the POMC gene (Challis et al., 2004) and MC4R gene, both of which are known to mediate the effects of POMC/α-MSH on appetite (Halatchev et al., 2004). This therefore suggests that the principal CNS mechanism for PYY_{3-36}'s effects on food intake is the pre-synaptic inhibition of NPY/AgRP neurons, and that the activation of POMC/CART neurons is not necessary for food intake inhibition.

The vagus nerve is the other principal site of PYY_{3-36} action. Y2 receptors are expressed in the nodose ganglion of the vagus and are transported to afferent vagal terminals (Koda et al., 2005). PYY_{3-36} activation of these Y2 receptors serves to activate vagal efferents, which in turn activate neurons in the NTS. In turn, the NTS activates the hypothalamus via ascending pathways. This model is supported by the fact that subdiaphragmatic vagotomy (interrupting the vagal efferent fibres) and midbrain transection (interrupting ascending fibres from the NTS) are both able to abolish the anorexigenic effects of IV PYY_{3-36} (Koda et al., 2005). However, peripherally administered PYY_{3-36} is still able to activate POMC/CART neurons in the ARC even with vagotomy, consistent with Batterham et al.'s results, and suggesting that PYY_{3-36} is still able to access and activate the ARC despite the vagotomy (Koda et al., 2005). Therefore, PYY_{3-36} can suppress food intake via two sites of action, the hypothalamus and the vagus nerve.

In contrast to PYY_{1-36}, administration of PYY_{1-36} into the CNS of rats causes an increase in food intake, mediated via Y1 receptors (Ballantyne, 2006). Activation of the Y5 receptor, like Y1, has an orexigenic effect (Ballantyne, 2006). If PYY_{1-36} is given peripherally, this causes a reduction in food intake, likely due to the peripheral conversion of PYY_{1-36} to PYY_{3-36} by DPP-4 prior to the peptide crossing the blood brain barrier (Ballantyne, 2006).

1.4.6.3 Effects on Glucose Metabolism and Energy Expenditure

Van den Hoek et al. demonstrated in mice that PYY_{3-36} acutely augments insulin-mediated glucose disposal in euglycaemic clamp studies, i.e. it increases insulin sensitivity (van den Hoek et al., 2004). This group went on to further investigate the effect of PYY_{3-36} on glucose metabolism and energy expenditure (van den Hoek et al., 2007). They were unable to demonstrate an increase in energy expenditure, heat production, or physical activity in mice treated with PYY_{3-36}, but they did show that PYY_{3-36} treatment reduced the respiratory quotient (RQ), suggesting a shift from carbohydrate metabolism towards fat oxidation. The observed reduction in RQ was not attenuated by chronic administration of PYY_{3-36}, and the effect was sustained, in comparison to a pair fed group, suggesting that the reduction in RQ is not simply due to the anorexigenic effect of PYY_{3-36} (van den Hoek et al., 2007). Extending their previous studies in 2004 (van den Hoek et al., 2004), they went on to confirm that PYY_{3-36} augments chronic as well as acute insulin-mediated glucose disposal. In addition, this group showed that glucose uptake in adipose tissue was significantly increased following treatment with PYY_{3-36} (van den Hoek et al., 2007). In rodents,

therefore, PYY_{3-36} appears to have metabolic effects in terms of increasing the sensitivity to insulin action, at least when given in relatively supraphysiological doses.

Such effects have not yet, however, been clearly established in humans. Sloth et al. examined the effects of acute infusions of PYY_{1-36} and PYY_{3-36} in human volunteers and did not establish clear effects on energy expenditure, although they noted increased lipolysis and free fatty acid levels which may have been due to activation of the sympathetic nervous system (Sloth et al., 2007). This study also noted an increased post-prandial secretion of insulin after an ad libitum meal with PYY_{3-36} infusion (Sloth et al., 2007). The latter finding is discussed in more detail in the discussion to Chapter 5.

1.4.6.4   Neuroimaging Studies

Early studies using PYY_{3-36} looked at expression of c-*fos*, a marker of neuronal activation, in order to establish which brain areas were involved in PYY action. PYY_{3-36} has been shown to increase c-*fos* expression in the ARC, NTS, AP, amygdala and thalamus (Batterham et al., 2002; Blevins et al., 2008). More recently, functional neuroimaging techniques such as manganese-enhanced magnetic resonance imaging (MEMRI) are being employed to look at the global neurophysiology underlying these actions. Manganese in particular is able to enter the hypothalamus, and can be used as an indirect marker of hypothalamic neural activation (Hankir et al., 2011). Subcutaneous administration of PYY_{3-36} in rats produced a trend of decreased signal intensity in the hypothalamus during MEMRI. Such a reduction in signal intensity would suggest reduced neuronal activity, in this case of the orexigenic NPY/AgRP

hypothalamic neuronal population. There was no effect of PYY administration on signal intensity in the AP (Hankir et al., 2011).

The effect of PYY$_{3-36}$ on the metabolism of various brain regions has been assessed using whole-brain blood-oxygen-level-dependent (BOLD) functional magnetic resonance imaging (fMRI). An increase in BOLD fMRI signal in a region of interest implies an increase in metabolism and therefore neuronal activity. Batterham et al., in 2007, studied normal-weight, food-deprived subjects and measured changes in BOLD fMRI signal during infusions of PYY$_{3-36}$ or saline placebo. Importantly, the protocol did not involve any sensory food-related cues. When PYY$_{3-36}$ was administered to mimic the post-prandial state by increasing circulating PYY$_{3-36}$ levels to those seen after eating, the brain area most notably activated was the caudolateral orbital frontal cortex (OFC) (Batterham et al., 2007). The OFC is an area that is implicated in reward, possibly activated through projections from the hypothalamic nuclei involved in energy regulation and food intake. Loss of grey matter in this particular brain area in fronto-temporal lobar degeneration is implicated in the hyperphagia observed in this disease (Whitwell et al., 2007). Other areas of the brain that were activated by PYY$_{3-36}$ included the limbic system (insula and anterior cingulate cortices), ventral striatum (globus pallidus and putamen) and discrete regions in the frontal, parietal, temporal and cerebellar cortices, as well as the posterior hypothalamus (Batterham et al., 2007).

In the same study, Batterham et al. examined the BOLD signal change with time in the hypothalamus and the OFC, and correlated this data to the caloric intakes of the

subjects when they were given an ad libitum study meal at the end of the scanning period. It was also found that when saline was infused, the change in signal in hypothalamus positively correlated with caloric intakes, but there was little correlation of the change in signal in the OFC with caloric intakes. PYY_{3-36} infusion changed this pattern. Under these conditions, the change in the BOLD signal from the OFC negatively correlated with the caloric intakes, i.e. it became a stronger predictor of caloric intake. At the same time, the change in the signal from the hypothalamus did not correlate with caloric intake (Batterham et al., 2007). The administration of PYY_{3-36} to achieve circulating levels similar to those seen after eating, thus mimicking the fed state, therefore appears to modulate the activity pattern of brain regions. It was speculated that the presence of PYY_{3-36} switches the brain area that controls food intake from the area involved in homeostatic responses to food intake (i.e. the hypothalamus) to the area implicated in hedonic responses to food intake (i.e. the OFC) (Batterham et al., 2007).

In another study, De Silva and colleagues examined brain activity as measured by BOLD fMRI in subjects given PYY_{3-36} (De Silva et al., 2011). They pre-selected six regions of interest (amygdala, caudate, insula, nucleus accumbens, OFC, and putamen) previously implicated in responses to food reward and to gut hormone infusions. For example, in another study, the same areas were shown to be activated by the orexigenic hormone ghrelin (Malik et al., 2008). In a distinctly different protocol to that of Batterham et al. (Batterham et al., 2007), De Silva et al.'s study measured the change in BOLD signal when subjects were presented with food images versus non-food images. It was found, during the control study, that these areas were more activated by food compared to non-food images. Following feeding, the

activation of these areas in response to food cues was less marked. Infusion of PYY_{3-36} or GLP-1 inhibited the activation of these brain areas by food images, similar to that observed after feeding. When PYY_{3-36} was combined with GLP-1, the inhibition became even more marked (De Silva et al., 2011). Although this study did use a different fMRI protocol, the clear implication of both studies is that PYY_{3-36} (and other gut hormones such as ghrelin and GLP-1) modify the activity of key areas of the brain that are involved in the processing of hedonic responses to eating, in a similar fashion to that observed after feeding.

1.4.7 Pathophysiology of PYY and potential uses of PYY in disease

1.4.7.1 PYY levels in obesity

In rodent models of diet-induced obesity, obesity is associated with lower fasting PYY concentrations and blunted post-prandial PYY secretion are also seen in rodent models of diet-induced obesity (Le Roux et al., 2006b; Xu et al., 2011). Similarly, in obese humans, Le Roux et al. demonstrated a significantly lower fasting PYY concentration in obese subjects compared to normal weight controls (Le Roux et al., 2006b). Furthermore, they also demonstrated a blunted post-prandial PYY response, with obese people needing to eat double the amount of calories to achieve post-prandial levels equivalent to those of normal weight controls, and importantly, the lower post-prandial PYY levels achieved in obese subjects was matched by a reduced feeling of satiety (Le Roux et al., 2006b). This blunting of post-prandial PYY secretion is likely to be the consequence rather than the cause of obesity, because mice randomized to a high-fat diet show reduced PYY levels compared to genetically similar mice randomized to a low-fat diet, i.e. implying that the reduced PYY

secretion could possibly maintain obesity but is not likely to initiate the obesity in the first place (Le Roux et al., 2006b).

In the rodent diet-induced obesity models, there was also a reduced suppression of food intake after administration of exogenous PYY_{3-36} (Xu et al., 2011). Interestingly, obese humans remain sensitive to the appetite-suppressive effects of PYY_{3-36}, achieving a similar reduction in food intake during an IV infusion to non-obese controls (Batterham et al., 2003a).

1.4.7.2 PYY levels after Gastric Bypass surgery

Bariatric surgery procedures have become more popular as the only class of obesity treatments that has been shown to confer significant and sustained weight loss, reductions in mortality, and, strikingly, early and maintained remissions in diabetes mellitus in many patients (Mingrone et al., 2012; Pournaras et al., 2012; Sjostrom et al., 2007). Several hypotheses have been advanced to explain these observations, including the notion that diversion of nutrients to the lower bowel after gastro-intestinal bypass is responsible for inducing the enhanced secretion of L-cell gut hormones such as GLP-1, PYY and oxyntomodulin, the so-called 'hindgut hypothesis' (Thaler and Cummings, 2009). In turn, the elevated gut hormones are responsible for suppressing food intake, and enhancing carbohydrate metabolism. Consistent with this model, post-prandial levels of PYY and GLP-1 are seen to be elevated as early as two days following bypass surgery, both in humans and in experimental animal models (Borg et al., 2006; Le Roux et al., 2006a). If an anti-PYY antibody is used to neutralize PYY, the reduction in food intake induced by the

bypass surgery is abrogated (Le Roux et al., 2006a) suggesting that the rise in PYY levels is indeed key to suppressing food intake after bypass.

Further support for the 'hindgut hypothesis' model comes from studies on a type of bariatric surgery called 'ileal transposition' where a segment of ileum (containing the L-cells that secrete GLP-1 and PYY) is transposed to the upper jejunum with no gastric restriction or malabsorption. These L-cells are therefore exposed at an early stage to the nutrients arriving in the upper jejunum. Ileal transposition induces marked increases in post-prandial GLP-1 and PYY secretion (Chelikani et al., 2010) and improves glycaemia in animal models of obesity and diabetes (Patriti et al., 2007).

1.4.7.3   PYY in other diseases

Certain disease states are known to be associated with raised levels of PYY. Elevated levels are seen in coeliac disease, active Crohn's disease, and in patients with ileal resection (Stanley et al., 2004). PYY levels may be raised in these individuals due to malabsorption, therefore causing an increased flux of luminal nutrients in the ileum gut, and triggering increased PYY secretion. Circulating levels are also raised in patients with cirrhosis, a recognized anorectic state (Valentini et al., 2011). The number of cells expressing PYY in the colon is increased in diabetic gastroparesis, a condition associated with delayed gastric emptying and abnormalities of intestinal transit time (Stanley et al., 2004). Lastly, feed-intolerance in critically ill patients is associated with higher basal and nutrient-stimulated PYY and CCK levels (Nguyen et al., 2006). All these observations do suggest that the anorexia observed in these states are, at least partially, mediated by elevations in PYY.

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

1.5.1 Discovery and structure of the preproglucagon gene

The preproglucagon gene, encoding the precursor for glucagon, oxyntomodulin, GLP-1 and GLP-2, was originally cloned by Habener's laboratory in Massachusetts General Hospital from anglerfish, whose endocrine pancreas is segregated anatomically from the exocrine pancreas, allowing for easy isolation and cloning of islet mRNAs (Lund, 2005). It was found that anglerfish carry two separate non-allelic genes encoding preproglucagon. Sequence analysis of the proglucagon-I precursor showed that there was an N-terminal glucagon peptide and a C-terminal glucagon-related peptide (GRP), preceded by a characteristic Lys-Arg cleavage site. GRP bears homology to glucagon, GIP, secretin and VIP. Cloning of the orthologous proglucagon cDNA from mammals (Bell et al., 1983a; Bell et al., 1983b) showed that these precursors include three peptides arranged in tandem, i.e. glucagon, GLP-1 and a second related peptide, GLP-2. GLP-1 is the orthologous peptide to the GRP originally characterized by Lund et al. (Lund et al., 1981).

The preproglucagon gene consists of six exons (**Figure 5**): exons 1 and 2 encode the 5′ untranslated region plus the signal peptide. Exons 3, 4, 5 consecutively encode glucagon, GLP-1 and GLP-2 (with adjacent sequences termed intervening peptides-1 and -2). Exon 6 encodes the 3′ untranslated region of the mRNA. The modular arrangement of exons 3-5 encoding the three separate peptides is likely to reflect exon

duplication from an ancestral exon encoding glucagon alone (Campbell and Scanes, 1992).

Gene duplication and divergence from the ancestral glucagon gene has led to the generation of a superfamily of glucagon-related peptides. Members include GLP-1 and GLP-2, glucagon, oxyntomodulin (in essence, glucagon plus an 8 residue C-terminal extension known as IP-1), secretin, peptide histidine-methionine amide (PHM), GH-releasing hormone (GHRH or GRF), pituitary adenylate cyclase activating peptide (PACAP), and VIP (**Figure 4**). Also included are a small family of peptides isolated from the venom of the Gila monster (*Heloderma suspectum*) and its relative, the Mexican beaded lizard (*Heloderma horridum*): exendins 3 and 4, helospectins 1 and 2, and helodermin. Exendin-4 (also known as exenatide) is notably an agonist of the GLP-1 receptor, and it is used clinically as a treatment for type 2 diabetes (Todd and Bloom, 2007).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Glucagon	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T																
OXM	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	K	N	N	I	A								
GLP-1₇₋₃₇	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	G														
GLP-1₇₋₃₆amide	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	am														
GLP-2	H	A	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	W	L	I	Q	T	K	I	T	D												
Secretin	H	S	D	G	T	F	T	S	E	L	S	R	L	R	E	G	A	R	L	Q	R	L	L	Q	G	L	V	am																	
PHM	H	A	D	G	V	F	T	S	D	F	S	K	L	L	G	Q	L	S	A	K	K	Y	L	E	S	L	M	am																	
GHRH	Y	A	D	A	I	F	T	N	S	Y	R	K	V	L	G	Q	L	S	A	R	K	L	L	Q	D	I	M	S	R	Q	Q	G	E	S	N	Q	E	R	G	A	R	A	R	L	am
PACAP-38	H	S	D	G	I	F	T	D	S	Y	S	R	Y	R	K	Q	M	A	V	K	K	Y	L	A	A	V	L	G	K	R	Y	K	Q	R	V	K	N	K	am						
Exendin-4	H	G	E	G	T	F	T	S	D	L	S	K	Q	M	E	E	E	A	V	R	L	F	I	E	W	L	K	N	G	G	P	S	S	G	A	P	P	P	S	am					

Figure 4: Alignment of selected glucagon-related peptides showing conserved residues (shaded green). Standard IUPAC one-letter amino acid codes used. am=C-terminal amide group. OXM = oxyntomodulin, PHM = peptide histionine-methionine, PACAP-38 = 38 residue form of pituitary adenylate cyclase activating peptide, GHRH = GH releasing hormone.

Figure 5: Structure of preproglucagon gene, mRNA and peptide. (A) The gene is labeled to show exons E1-E5, introns IA-E; the mRNA is labeled to show regions coding for untranslated regions (UN-TX), signal peptide (S), N-terminal peptide (N), glucagon (Gluc), GLP-1, GLP-2, and intervening peptides 1 and 2 (IP-1 and IP-2). (B) The differential post-translational processing of the proglucagon peptide. In the pancreas, this generates glicentin-related pancreatic polypeptide (GRPP), glucagon and the major proglucagon fragment comprising the sequences of GLP-1, GLP-2 and IP-2. In the intestine/brain, this generates oxyntomodulin, comprising glucagon plus a C-terminal extension (IP-1), GLP-1 and GLP-2. From Kieffer and Habener (1999).

1.5.2 Structure and processing of GLP-1 peptide

Proglucagon is post-translationally processed by prohormone convertases to generate glucagon in the alpha cells of the islets of Langerhans, and GLP-1, GLP-2 and oxyntomodulin in the intestinal L cells and the brain (**Figure 5**). Prohormone convertases 1/3 are involved in generating GLP-1 (Rouille et al., 1997) and prohormone convertase 2 generates glucagon (Rouille et al., 1994). Initial experiments with full-length GLP-1$_{1-37}$ showed absent or weak bioactivity (Ghiglione et al., 1984). Further N-terminal processing of GLP-1 generates bioactive GLP-1, which consists of the peptides GLP-1$_{7-37}$ − 32 residues − and GLP-1$_{7-36amide}$ − 31 residues, C-terminally amidated (Mojsov et al., 1986). Similar to glucagon, GLP-1 tends to adopt an alpha-helical conformation through most of its mid-portion (residues 7-27) with the N-terminal and C-terminal portions being relatively unstructured (**Figure 6**).

Once secreted, GLP-1$_{7-36amide}$ is broken down by the enzyme DPP-4 to GLP-1$_{9-36amide}$ which is conventionally thought to be biologically inactive (Mentlein et al., 1993). Inhibition of DPP-4 with drugs such as sitagliptin and vildagliptin, therefore, is used as a strategy to increase active GLP-1 levels for the treatment of diabetes mellitus type 2 (Raz et al., 2006). The peptidase neprilysin (neutral endopeptidase 24.11) is also responsible for breaking down GLP-1 (Plamboeck et al., 2005).
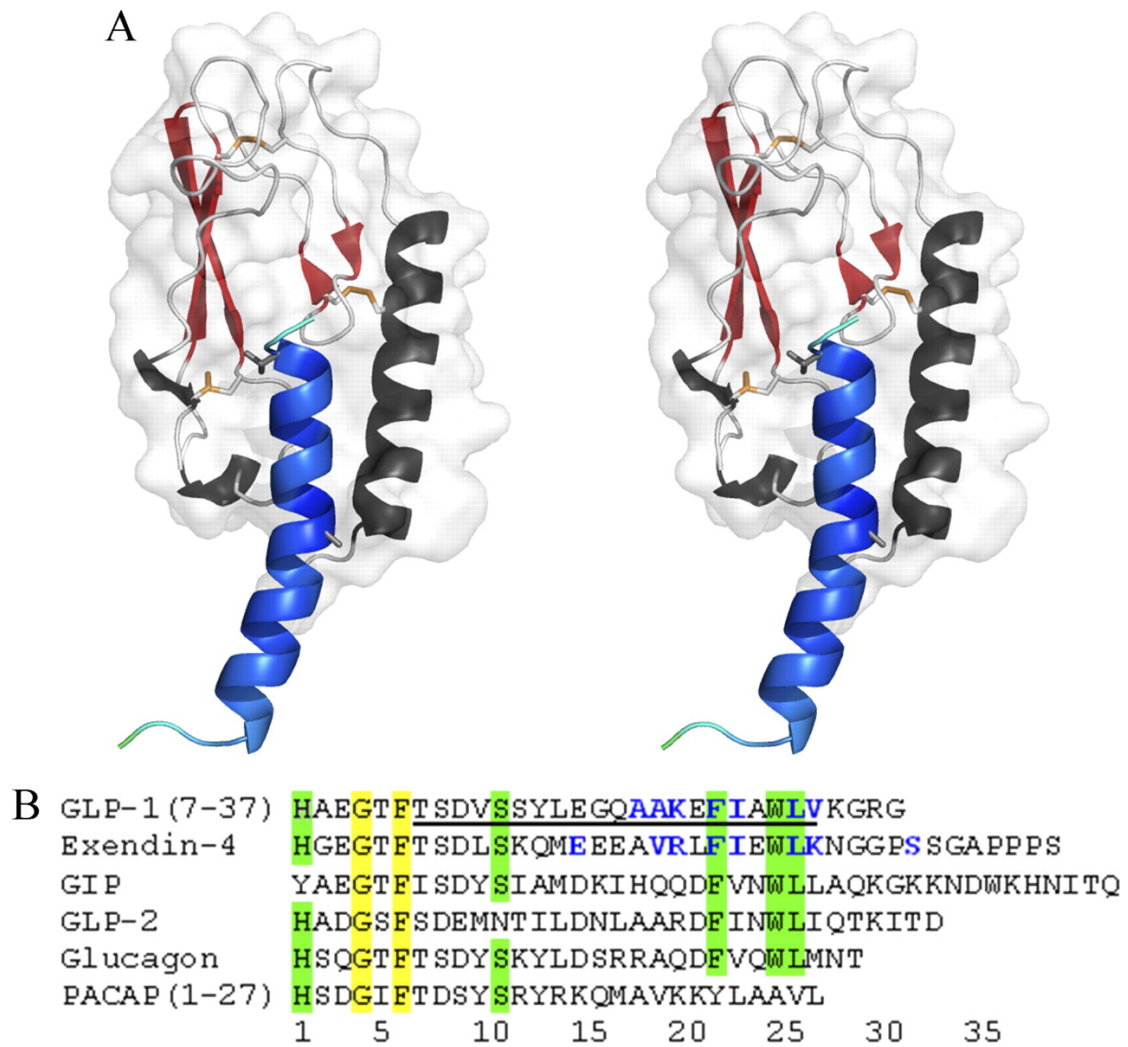
Figure 6: Structure of GLP-1 bound to the extracellular domain of the GLP-1 receptor. (A) GLP-1 is indicated as a blue ribbon diagram, superimposed on a light grey space-filling diagram that represents the GLP-1 receptor extracellular diagram. (B) Sequence comparison of GLP-1 and related orthologues. The green-shaded residues are partially conserved and the yellow-shaded residues fully conserved between the shown orthologues. The residues 7-27 of GLP-1$_{7-37}$, which form the alpha helix, are underlined. From Underwood et al. (2010).

1.5.3 Distribution and secretion of GLP-1

1.5.3.1   Intestinal distribution of GLP-1

GLP-1 is found principally in the intestine, within the L neuroendocrine cells and, as mentioned before, co-localised with PYY. There is a differential expression of these two peptides, with GLP-1 being found more proximally in the jejunum with smaller amounts in the colon, duodenum and caecum (Zhou et al., 2006). PYY is found in larger quantities more distally (Adrian et al., 1985a).

1.5.3.2   CNS distribution of GLP-1

GLP-1 expressing neurons are particularly prevalent in the caudal portion of the NTS, where they serve to integrate signals from the vagus and glossopharyngeal nerves, and project to various fore, mid and brainstem centres involved in the control of appetite and cardiac output (Larsen et al., 1997a). GLP-1 immunopositive fibres are also found in the hypothalamus, especially in the PVN and the ventral diffuse part of the dorsomedial (DMH) hypothalamic nucleus. The ARC is less densely innervated by GLP-1 immunopositive fibres in comparison to PVN and DMH. Consistent with this, GLP-1 receptor expression is also found in hypothalamic nuclei: in particular, the supraoptic (SON), ARC, PVN and DMH nuclei. Additionally, GLP-1 binding sites are found in sensory circumventricular organs including the subfornical organ (SFO), organum vasculosum laminae terminalis (OVLT) and AP (Tang-Christensen et al., 2001).

1.5.3.3 Regulation of GLP-1 release in the intestine

GLP-1 levels are lowest when fasting, and release is triggered by eating. There is a biphasic release of GLP-1 to eating, with an acute phase (up to 30-60 min) triggered by neural and hormonal signals, and a chronic phase (1-2 h) triggered by direct nutrient sensing by L cells (Herrmann et al., 1995). Carbohydrates, fats and proteins all stimulate GLP-1 release (Carr et al., 2008; Herrmann et al., 1995). There is a synergistic GLP-1 response to mixed meals: a 47% greater GLP-1 response is obtained with a mixed meal compared to sum of responses to the individual macronutrients (Ahlkvist et al., 2012).

The key player in the acute phase of release is the vagus nerve, as vagotomy abolishes intraduodenal fat-stimulated GLP-1 release. GIP, released from K cells in the duodenum, stimulates GLP-1 secretion but indirectly via the vagus nerve as selective vagotomy of hepatic branches also abolishes GLP-1 secretion (Rocca and Brubaker, 1999).

The chronic phase of release is mediated by direct sensing of nutrients in the enteral lumen. At low concentrations, glucose which is transported into L cells via sodium-glucose co-transporters (SGLT1 and 3), is metabolized and causes a rise in the ATP/ADP ratio, which in turn triggers membrane depolarization by K_{ATP} channel closure and therefore secretion of the GLP-1 containing granules at the base of the L cells. This first mechanism is reminiscent of the mechanism that operates to trigger insulin release in the beta cells. A second mechanism that comes into play at higher concentrations of sugars involves the influx of co-transported Na^+ ions via SGLT 1/3

which generates an inward current large enough to trigger membrane depolarization (Gribble et al., 2003). Fatty acids are directly sensed via the G-protein coupled receptors GPR40 (Edfalk et al., 2008), and bile acids likewise via GPR119 (Lauffer et al., 2009) and GPBAR1/TGR5 (Parker et al., 2012). These G-protein coupled receptors stimulate adenylate cyclase, cAMP production and consequent membrane depolarization, voltage-gated Ca^{2+} entry and degranulation. The mechanisms responsible for GLP-1 secretion in response to protein involve the direct sensing of peptones (oligopeptides) or amino acids by peptide transporter-1 and the calcium-sensing receptor in L cells (Diakogiannaki et al., 2013).

1.5.4 Receptors and signaling cascades

GLP-1 acts on ß-cells by binding to the GLP-1 receptor (GLP-1R), a seven trans-membrane domain, G-protein-coupled receptor (GPCR) (Thorens et al., 1993) in the secretin-like class B family. The GLP-1R is expressed in many tissues, including pancreatic islets, lung, CNS and PNS, stomach, kidney and heart (Thorens et al., 1993; Wei and Mojsov, 1995). The crystal structure of GLP-1 bound to the ECD of the GLP-1R has recently been solved (Underwood et al., 2010), although a full agonist-bound structure has yet to be published. However, a predicted model of GLP-1R bound to exendin-4, based on computer modeling and the crystal structure of the ECD, has recently been published (Kirkpatrick et al., 2012). Similar to the interaction of glucagon with its receptor, this model predicts that N-terminal residues of GLP-1 (or analogues such as exendin-4) interact with the 7TM domain whereas the C-terminal residues interact with the extracellular domain (ECD) of GLP-1R which is a

feature shared with other class B GPCRs (Kirkpatrick et al., 2012; Underwood et al., 2010).
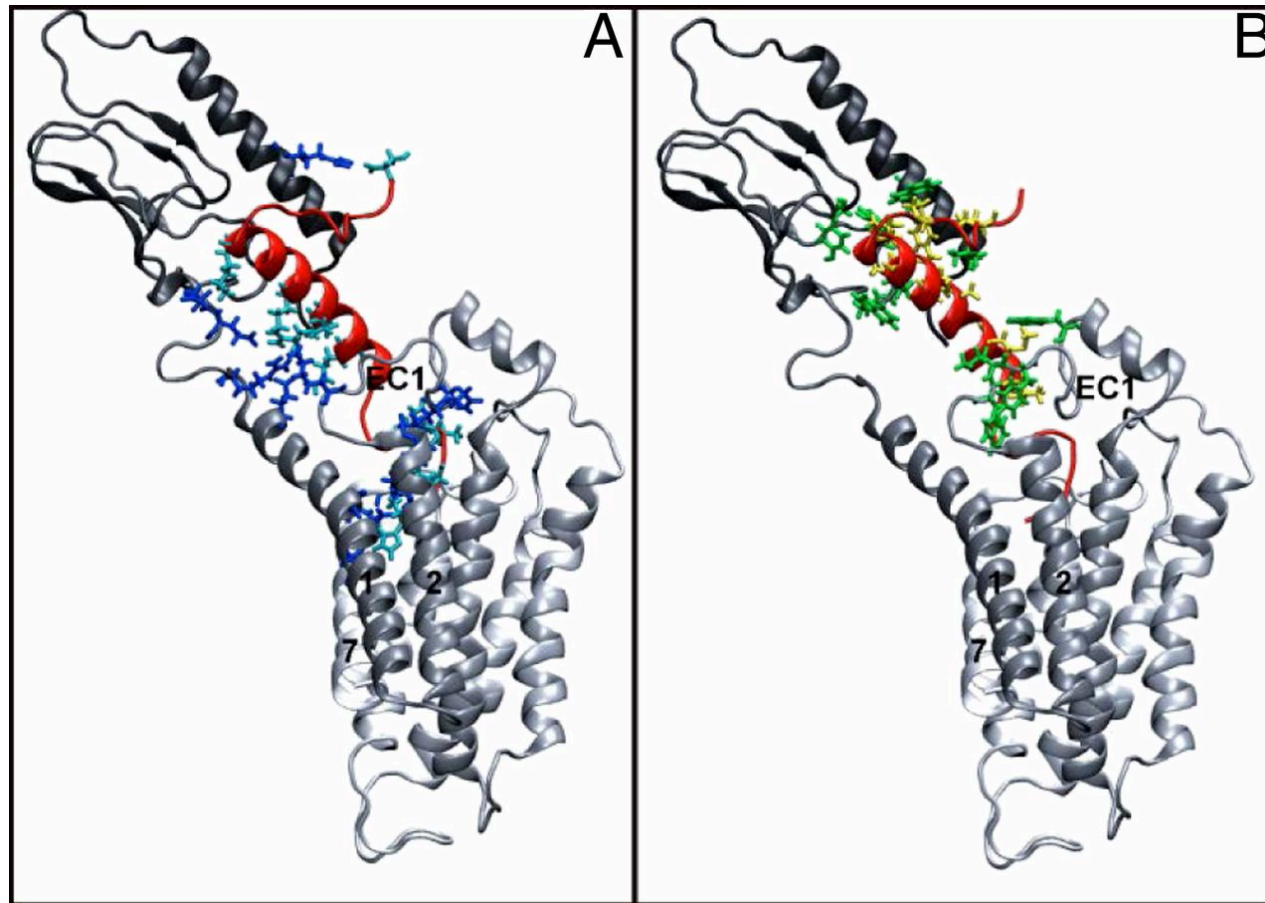
Figure 7: Predicted structure of the GLP-1 receptor agonist exendin-4 bound to the GLP-1 receptor extracellular domain (ECD). Exendin 4 is shown as a ribbon diagram (red) with its N terminus situated in the middle of each diagram and the C terminus extending to the top center of the diagram. The GLP-1 receptor is shown as a ribbon diagram with a colour transition applied from white to black as the protein goes from the N terminus to the C terminus, i.e. the ECD is the structure in black in the top left corner. (A) shows the hydrophilic interactions between blue protein residues and ligand residues in turquoise. (B) shows the hydrophobic interactions between green protein residues and ligand residues in yellow. From Kirkpatrick et al. (2012).

Ligand binding stimulates adenylate cyclase activity (Lu et al., 1993; Thorens et al., 1993). This stimulation of adenylate cyclase activity and cAMP production is mediated via the G_s G-protein, leading to activation of protein kinase A (PKA) and the cAMP-regulated guanine nucleotide exchange factor Epac 2 (Holz, 2004). An alternative pathway for stimulated cAMP production by GLP-1R activation involves recruitment of the scaffold protein ß-arrestin and downstream activation of ERK, CREB, and increased IRS-2 expression (Sonoda et al., 2008).

For many years, there has been speculation that GLP-1 binds to a distinctly different receptor in the liver than the one characterized in ß-cells. Villaneuva-Penacarrillo and colleagues showed that there was a specific GLP-1$_{7-36amide}$ binding activity in rat hepatic membranes which strikingly did not activate adenylate cyclase with GLP-1 treatment (Villanueva-Penacarrillo et al., 1995). The nature of this hepatic GLP-1 receptor is unclear. Although the presence or absence of the canonical GLP-1R in the liver was the subject of controversy for some time, a recent study has confirmed that GLP-1R is expressed in the liver (Svegliati-Baroni et al., 2011). It is therefore possible that the hepatic GLP-1 receptor is indeed GLP-1R, but perhaps activating an alternative pathway not involving adenylate cyclase and cAMP generation.

1.5.5 Biological actions of GLP-1

1.5.5.1 Incretin effect on insulin secretion and actions on glucagon secretion

GLP-1 functions as an incretin hormone, that is as a physiological, glucose-dependent insulin secretagogue, the action of which is to potentiate post-prandial insulin release (Kreymann et al., 1987). It stimulates ß cell proliferation and differentiation, insulin

gene expression and insulin secretion (Drucker et al., 1987; Stoffers et al., 2000) and is capable of normalizing the blunted insulin responses to glucose seen in type 2 diabetic patients (Rachman et al., 1996). GLP-1 is the more physiologically influential incretin hormone compared to GIP as antagonism of GLP-1 action causes suppression of insulin secretion and an increased glucose excursion in response to oral glucose whereas GIP antagonism has no such effect (Baggio et al., 2000). In addition to its insulintropic effect, GLP-1 also suppresses glucagon release in a glucose-dependent fashion from alpha cells (Nauck et al., 1993b), likely via somatostatinergic mechanisms (de Heer et al., 2008). The net effect of GLP-1 secretion is to lower blood glucose levels via increasing glucose uptake and suppressing hepatic glucose output.

Some recent data indicates that GLP-1 may operate directly on the liver without involving insulin or glucagon. Seghieri and colleagues utilized a "pancreatic clamp" where somatostatin was used to suppress the native production of insulin and glucagon, and insulin and glucagon infusions were used to fix the respective levels of the hormones. Under these conditions, GLP-1$_{7-37amide}$ was able to suppress hepatic glucose output directly, without invoking indirect effects of GLP-1 on insulin or glucagon secretion (Seghieri et al., 2013). Even more interestingly, Habener's laboratory has also published studies that show that GLP-1$_{9-36amide}$, the breakdown product of GLP-1$_{7-36amide}$ after DPP-4 processing, is able to suppress hepatic glucose output in an insulin-like manner, and that this is not suppressed by GLP-1R blockade with exendin$_{9-39amide}$ (Elahi et al., 2008; Tomas et al., 2010). The effects of GLP-1 on carbohydrate metabolism on the liver, therefore, appear to be independent of the GLP-1R and may involve the as yet uncharacterized hepatic GLP-1 receptor.

1.5.5.2 Appetite reduction

GLP-1 inhibits food intake in rats, after either central or peripheral administration (Tang-Christensen et al., 2001; Turton et al., 1996). In humans, acute IV GLP-1 infusion reduces energy intake at a subsequent *ad libitum* meal (Flint et al., 1998), while chronic SC infusion to patients with type 2 diabetes mellitus results in weight loss and improved glycaemic control (Zander et al., 2002). The satiating effect of GLP-1 is partly due to delayed gastric emptying (Willms et al., 1996). It is also due to direct effects in the CNS, since peripherally administered GLP-1 causes neuronal activation in the ARC (Abbott et al., 2005a), while central administration causes neuronal activation in the PVN and SON of the hypothalamus, the NTS and AP (Larsen et al., 1997b; Rowland et al., 1997). Although Ma and co-workers published evidence that GLP-1 activates POMC neurons in the ARC, implying that it exerts anorexigenic actions via α-MSH and MC4R, this paper was later retracted (Ma et al., 2007). Indeed, in experiments where GLP-1 was given intracerebroventricularly to rodents, it was shown that the MC4R antagonist AgRP(83-132) did not inhibit GLP-1's anorexigenic action, implying that GLP-1 exerts its effects via MC4R independent mechanisms (Edwards et al., 2000).

In addition to the central effects of GLP-1, it is also possible that GLP-1 may act via the vagus nerve, since GLP-1R gene expression has been demonstrated in the nodose ganglion of the vagus nerve (Nakagawa et al., 2004). Furthermore, the effect of peripherally administered GLP-1 on both energy intake and activation of ARC neurones is attenuated by either bilateral sub-diaphragmatic truncal vagotomy or

bilateral transections of the brainstem-hypothalamus pathway (Abbott et al., 2005a), suggesting that GLP-1's anorexigenic effects are mediated by the vagus nerve and connections from the DVC to the hypothalamus.

1.5.5.3 Cardiovascular actions

As alluded to above, the GLP-1 receptor is present on various tissues such as the lung, kidney, lymphocytes, blood vessels, and heart (Bullock et al., 1996; Thorens et al., 1993). The cardiovascular system has emerged as a key physiological target of GLP-1 action (Ussher and Drucker, 2012). Known effects of GLP-1 on the cardiovascular system include vasodilatation (Nystrom et al., 2004), a pressor effect in rodents (Barragan et al., 1994), and a direct inhibition of chylomicron secretion by the intestine (Hsieh et al., 2010), which may be responsible for suppressing the post-prandial rise in triglyceride levels (Meier et al., 2006). Some of these actions may however be species-specific: the pressor effect that is noted in rodents does not occur when human volunteers are infused with IV GLP-1, even with relatively high doses such as 1.2-2.4 pmol/kg/min (Bharucha et al., 2008).

1.5.5.4 Control of adipose tissue metabolism

Although it is well known that glucagon can activate adipose tissue metabolism, the effects of GLP-1 on adipose tissue are much less well known. Intracerebroventicular infusion of GLP-1 for 48 h has been shown to reduce fat mass in rodents (Nogueiras et al., 2009). This phenomenon is not explained by the anorectic effects of GLP-1 as pair-fed mice, i.e. control mice that are fed the exact same amount of food that the animals treated with GLP-1 eat, do not lose fat mass (Lockie et al., 2012). Using

multifibre recording from sympathetic nerve fibres innervating white adipose tissue, it was shown that GLP-1 activated sympathetic nervous system activity (Lockie et al., 2012); moreover, transgenic mice possessing triple knockouts of the genes encoding the β1, β2 and β3-adrenergic receptors did not respond to GLP-1 with a loss of fat mass, implying that GLP-1 exerts its effects via the sympathetic nervous system and activation of both white and brown adipose tissue metabolism (Lockie et al., 2012; Nogueiras et al., 2009).

1.5.6 Pathophysiology of GLP-1 and potential uses of GLP-1 in disease

1.5.6.1 GLP-1 secreting tumours

Rarely, neuroendocrine tumours have been found that secrete GLP-1 inappropriately with consequent effects on carbohydrate metabolism. In one case, a pelvic neuroendocrine tumour that co-secreted GLP-1 and somatostatin was described as a cause of profound reactive hypoglycaemia in response to oral and IV glucose challenges (Todd et al., 2003). In another case, a patient presented with a pancreatic neuroendocrine tumour that co-secreted glucagon and GLP-1. Interestingly, the initial biochemical picture was that of diabetes, presumably due to the hyperglucagonaemia, but this was later followed by spontaneous fasting hypoglycaemia, presumably due to the insulinotropic effect of the GLP-1 causing beta-cell hyperplasia and autonomous secretion of insulin (Roberts et al., 2012). These cases therefore suggest that GLP-1 secreting tumour can present with hypoglycaemia, and this may either occur on fasting or post-prandially.

1.5.6.2   GLP-1 physiology in type 2 diabetes mellitus

In type 2 diabetes mellitus (T2DM) patients, a reduction in the post-prandial GLP-1 response may well contribute to the dysfunction of insulin secretion noted in T2DM (Vilsboll et al., 2001). The action of endogenous GLP-1 in patients with T2DM is still of importance in regulating insulin secretion as antagonism of the GLP-1 receptor with exendin$_{9-39}$ suppresses post-prandial insulin secretion (Salehi et al., 2010). Patients with T2DM continue to be sensitive to the incretin effects of exogenous GLP-1, unlike GIP, where the incretin effect is significantly blunted in these patients (Nauck et al., 1993a). Infusion of GLP-1 rapidly normalizes glycaemia in patients with T2DM (Gutzwiller et al., 1999; Nauck et al., 1993b) and these observations are the basis for the use of GLP-1 analogues as clinical treatments for T2DM.

To make such treatment practical, DPP-4-resistant GLP-1 analogues have been developed with extended half-lives and longer durations of action. The oldest drug in this class is exenatide (exendin-4, Amylin/Lilly, now BMS). Exenatide was originally isolated from the saliva of the Gila monster lizard. It shares a 53% homology with human GLP-1 and is given as a subcutaneous injection. Exenatide improves HbA1c by 0.8-1.11% and reduces weight by 1.6 to 2.8 kg (Todd and Bloom, 2007). The most common side effect with exenatide is that of nausea and vomiting. The nausea is common in the first few weeks of treatment and gradually decreases with time.

Exenatide is usually given twice a day. A once-weekly formulation of exenatide, exenatide LAR (Amylin/Lilly/Alkermes, now BMS), has been recently been introduced. This encapsulates exenatide in poly(lactic-co-glycolic) microspheres,

leading to extended release. Exenatide LAR is superior to exenatide in terms of reduction in HbA1c (1.9% versus 1.5% respectively) and similar in terms of body weight reduction (3.7 and 3.6 kg respectively). Exenatide LAR has a reduced potential for nausea compared to exenatide but skin reactions are more prevalent (Drucker et al., 2008).

Liraglutide (Novo Nordisk) is 97% identical to native GLP-1, but contains an amino acid substitution (K34R) to allow a palmitate fatty acid group to be linked via a γ–glutamic acid spacer. The fatty acid group binds to albumin, increasing the half-life of liraglutide to 13 hours. It is given by subcutaneous injection once daily (0.6, 1.2 or 1.8 mg). Liraglutide (1.8 mg od) appears to be slightly superior to exenatide (10 µg bd) in a 26-week head-to-head trial, funded by Novo Nordisk, where the liraglutide group had a significantly better HbA1c reduction relative to exenatide (1.12% vs 0.79%) (Buse et al., 2009). There was also a minor but significant reduction in the incidence of nausea with liraglutide treatment compared to exenatide. There was otherwise a similar side effect profile and magnitude of weight loss (Buse et al., 2009).

After exenatide and liraglutide, other companies have followed with newer GLP-1 analogues. Lixisenatide (Sanofi-Aventis) is a recently approved once daily analogue based on exendin-4, but with the addition of six lysine residues. It appears to be similar in hypoglycaemic efficacy to exenatide but causes less nausea (Rosenstock et al., 2013b). Taspoglutide (Roche/Ipsen) was another once weekly analogue but development was stopped due to significant side-effects in Phase III trials (Rosenstock et al., 2013a). Other once-weekly GLP-1 analogues in development

include albiglutide (GlaxoSmithKline), dulaglutide (Lilly) and semaglutide (Novo Nordisk).

A drawback with all GLP-1 analogues so far is that they require injection. As an alternative, DPP-4 inhibitors such as sitagliptin (Raz et al., 2006) and vildagliptin (Ahren et al., 2005) have been developed as orally active treatments for type 2 diabetes mellitus. By inhibiting DPP-4, native GLP-1 levels are boosted, leading to improvements in glycaemia (Herman et al., 2006). A key and distinct difference, however, from GLP-1 analogues is that the DPP-4 inhibitors are weight neutral (Raz et al., 2006).

1.5.6.3   Increase in post-prandial GLP-1 levels in patients undergoing bariatric surgery.

One of the notable effects of bariatric surgery in patients with T2DM is the rapid resolution of hyperglycaemia after surgery, which persists long-term in 40% of patients undergoing Roux-en-Y gastric bypass (RYGB) (Cummings, 2009; Pournaras et al., 2012; Pournaras et al., 2010). Multiple explanations exist for this phenomenon. One such explanation is the so-called 'lower intestinal hypothesis' which holds that nutrients are delivered quickly to the lower bowel through the bypasses created in surgery, triggering the release of gut hormones such as GLP-1 and PYY from the L cells (Cummings, 2009). A prediction of this hypothesis is that bypass operations such as RYGB are associated with increased post-prandial secretion of GLP-1 (and PYY), whereas restrictive operations that reduce stomach volume such as laparoscopic adjustable gastric banding (LAGB) will not be associated with such

increases in GLP-1 and PYY secretion. Indeed, this contrasting pattern of secretion is seen and results in augmented insulin release in RYGB (Le Roux et al., 2006a; Vidal et al., 2009).

1.5.6.4 Association of GLP-1 agonist therapy with pancreatitis and possible pancreatic carcinoma

The attractive combination of weight loss with solid improvements in glucose levels has led to the rapid take-up of GLP-1 analogues into clinical practice. However, as these therapies are still relatively novel, long-term safety data are limited. Rodent studies have shown that liraglutide induces C-cell proliferation (Bjerre Knudsen et al., 2010). It is unclear whether these risks apply to humans and rodents may be peculiar in developing these tumours. Further, the drug exposures to liraglutide in the animal testing were much higher than in humans.

GLP-1 analogue therapy is also linked with pancreatitis and pancreatic cancer. GLP-1 analogue therapy appears to be associated with hyperplasia of both the exocrine pancreas and the endocrine pancreas, particularly alpha cells (Butler et al., 2013). However, industry-funded retrospective studies of large insurance databases showed no significant association of pancreatitis with exenatide therapy (Wenten et al., 2012) nor with liraglutide therapy (Funch et al., 2013). A recent review by the European Medicines Agency has concluded that there is no present evidence supporting these concerns (European Medicines Agency, 2013).

1.5.6.5 GLP-1 in hypertension

Despite the pressor effect of GLP-1 when this is given acutely, GLP-1 agonists, when given chronically, appear to reduce blood pressure in animal models of hypertension (Hirata et al., 2009). Analyses of data arising from the large-scale pivotal trials of GLP-1 analogues are supportive: for example, the DURATION-1 trial of exenatide LAR reported significant reductions in systolic BP of 3.8 to 6.2 mmHg (Buse et al., 2010), and similar findings have been reported with liraglutide when added to pre-existing anti-diabetic therapy (Gallwitz et al., 2010). Although some of these effects may be attributed to the weight loss induced by GLP-1 analogue therapy, the major part of the reduction in BP is seen within two weeks of initiation, whereas the full effects of body weight take eight weeks to be manifest, suggesting that the effects on blood pressure are in large part direct (Gallwitz et al., 2010). GLP-1 has direct effects on renal haemodynamics and the expression of the Na^+/H^+ exchanger isoform 3 (NHE3) in the proximal tubule, leading to diuresis and natriuresis in animal models (Crajoinas et al., 2011). Infusion of GLP-1 in healthy volunteers also induces natriuresis and reduces the glomerular hyperfiltration seen in obese subjects (Gutzwiller et al., 2004). In another study, Skov and colleagues confirmed that GLP-1 is capable of inducing natriuresis. Unlike the earlier study from Gutzwiller et al., their study purported to show a drop in angiotensin II level of 19%, although aldosterone and renin levels were not altered; the physiological significance of this observation is therefore obscure (Skov et al., 2013).

1.5.6.6 Cardioprotective effect of GLP-1

Some animal studies have demonstrated cardioprotective effects of GLP-1 when given in experimental models of ischaemic heart disease. For example, GLP-1 reduces infarct size after 30 mins occlusion of the left anterior descending artery in rats (Bose et al., 2005). However, this is not always a consistent finding as other studies do not show any salutary effects on infarct size, for example when GLP-1 was given in a porcine model of myocardial ischaemia (Kavianipour et al., 2003). Nevertheless, these pre-clinical data have not discouraged clinical investigators from studying the effect of GLP-1 and GLP-1 analogues in heart disease. A 72-hour GLP-1 infusion, when given to patients admitted for primary angioplasty for acute myocardial infarction and left ventricular dysfunction, improved left ventricular function (Nikolaidis et al., 2004). In another study, 172 patients admitted for primary angioplasty for acute ST segment elevation myocardial infarction were randomized to infusions either of saline placebo or exendin-4. It was shown that the exendin-4 group had reduced infarct sizes relative to the myocardial area at risk as assessed by cardiac MRI studies, but no significant differences in left ventricular function or 30-day event rates were observed (Lonborg et al., 2012). These data are certainly suggestive of benefit but a randomized controlled trial is required to definitively prove benefit.

GLP-1 has also been trialled in chronic heart failure. In a small non-randomized trial in 12 patients with NYHA Class III/IV heart failure, continuous subcutaneous infusion of GLP-1 of 2.5 pmol/kg/min over 5 weeks improves left ventricular ejection fraction, maximal O_2 uptake and the distance covered in a 6 minute walk test compared to a placebo infusion (Sokos et al., 2006). In a double-blind cross-over trial of a 48-hour GLP-1 infusion of 0.7 pmol/kg/min in 20 patients with NYHA Class

II/III heart failure, left ventricular ejection fraction and cardiac index were not improved, and the patients experienced a higher incidence of hypoglycaemia. It was also noted that the infusion caused small increases in heart rate and diastolic blood pressure (Halbirk et al., 2010). The dissimilarity of design between the two trials makes conclusions difficult to reach and the role of GLP-1 in heart failure is still to be elucidated.

1.5.6.7   Anti-atherogenic effects of GLP-1

The anti-atherogenic effects of GLP-1 can be divided into two classes: effects on lipid metabolism, and direct effects on the atherosclerotic pathophysiological process. Short-term infusions of GLP-1 have been shown to favourably inhibit the post-prandial rise in triglyceride and free fatty acids (Meier et al., 2006). GLP-1 infusions (Nagashima et al., 2011) and similarly liraglutide (Gaspari et al., 2013) have been shown to inhibit the development of atherosclerosis in hyperlipidaemic $Apoe^{-/-}$ knockout mice, although this effect occurs only in early onset atherosclerosis of a low burden; high burden, late stage disease did not respond to treatment (Gaspari et al., 2013). This phenomenon may well be related to direct effects of GLP-1 agonism on monocyte adhesion to atherosclerotic lesions and to reductions in inflammation as evidenced by reductions in the expression of pro-inflammatory markers such as TNF-α and monocyte chemoattractant protein-1 with exendin-4 treatment (Arakawa et al., 2010). This pre-clinical data, therefore, suggests that GLP-1 agonist and perhaps DPP-4 inhibitor treatment may well reduce cardiovascular events. However, clinical data to support this is currently lacking. One large clinical trial comparing saxagliptin (a DPP-4 inhibitor) with a placebo controlled group showed no difference in

cardiovascular event rates between the two groups when tested in 16,492 patients at risk for cardiovascular disease (Scirica et al., 2013).

1.6 Prospects for an effective treatment for obesity

The history of treatments for obesity is littered with failures. Non-surgical treatments for obesity have included the medications orlistat, sibutramine and rimonabant. Orlistat causes modest weight loss and its use is limited due to poor tolerance (Rucker et al., 2007). Sibutramine has recently been withdrawn from the European market, as a recent study has suggested an increased incidence of cardiovascular events (Sharma et al., 2009). Similarly, rimonabant was withdrawn due to an associated increase in anxiety and depression (Christensen et al., 2007; Rucker et al., 2007). Therefore it is clear that new strategies are urgently needed to tackle obesity. The gut hormones involved in appetite regulation have become attractive targets for the development of drugs that aim to cause effective weight loss with minimal side effects.

Bariatric surgery offers an instructive paradigm for understanding how to devise an effective treatment of obesity. It is currently the most successful treatment for this condition, causing durable loss of weight, proven reductions in cardiovascular events and overall mortality, and a sustained and prolonged remission of diabetes in many patients who enter surgery with Type 2 diabetes (Buchwald et al., 2009; Pournaras et al., 2012; Sjostrom et al., 2007). There are three major forms of surgery: restrictive, bypass and combined (**Figure 8**). Restrictive bariatric surgical techniques reduce the capacity of the stomach to receive a meal, and therefore food intake: examples include gastric banding, vertical banded gastrectomy, and sleeve gastrectomy. The

popularity of restrictive procedures stems from the relatively simple surgical techniques required and the possibility of reversibility (e.g. by removal of a gastric band). Bypass procedures are more complex surgically and act to divert the food ingested to a more distal point in the gastro-intestinal tract. Examples include duodenal-jejunal bypass and biliopancreatic diversion. Combination procedures, which combine both restrictive and bypass components, include Roux-en-Y gastric bypass, and biliopancreatic diversion plus duodenal switch (Buchwald et al., 2004). Bypass and combination procedures are associated with larger magnitudes of weight loss compared to restrictive procedures alone such as gastric banding (Buchwald et al., 2009; Sjostrom et al., 2007).

How does bariatric surgery work? One major hypothesis is that the surgery exerts its effects by influencing the secretion of gut hormones. Patients who have undergone bariatric surgery have been observed to have significantly different gut hormone profiles (**Figure 8**) and demonstrate chronic and sustained elevations of key gut hormones that are known to regulate appetite (PYY, GLP-1, OXM) and carbohydrate metabolism (GLP-1, OXM), suggesting many of the improvements in appetite suppression and carbohydrate metabolism achieved by bariatric surgery are likely to be due to these hormonal alterations (Clements et al., 2004; Korner et al., 2007; Le Roux et al., 2006a; Pournaras and Le Roux, 2009; Rubino et al., 2004). As previously mentioned, this enhanced secretion of L-cell gut hormones is thought to be induced by the diversion of nutrients to the lower bowel through the bypass – the 'hindgut hypothesis' (Thaler and Cummings, 2009).

Even with its proven utility, bariatric surgery cannot be a universal solution for obesity. It carries some serious shortcomings, which include: (1) the requirement for specialist surgeons and facilities, which restricts the number of procedures that can be done and increases costs; (2) a peri-operative mortality rate of 0.08–1.1% (Buchwald et al., 2004; Chang et al., 2013); (3) its relative irreversibility, particularly for the bypass and combination procedures. A safe and effective medical therapy for obesity and diabetes is still desperately needed.

The knowledge, therefore, that bariatric surgery appears to work, in major part, by elevating gut hormone levels offers a therapeutic opportunity for the treatment of diabetes and obesity. The central hypothesis of gut hormone therapy for obesity is as follows: simulation of the elevation of gut hormones seen after bariatric surgery will bring one or more of the following metabolic benefits:

1. Appetite suppression and consequent weight loss.
2. Improvements in glycaemic control, for example by improving insulin secretion or sensitivity.
3. Increases in energy expenditure that would synergize with the appetite-suppressive effects to induce even more weight loss.
4. Amelioration of obesity's complications. This can be direct (e.g. the abovementioned effects of GLP-1 on blood pressure, cardioprotection, atherosclerosis) or indirect, via the weight loss induced by therapy.
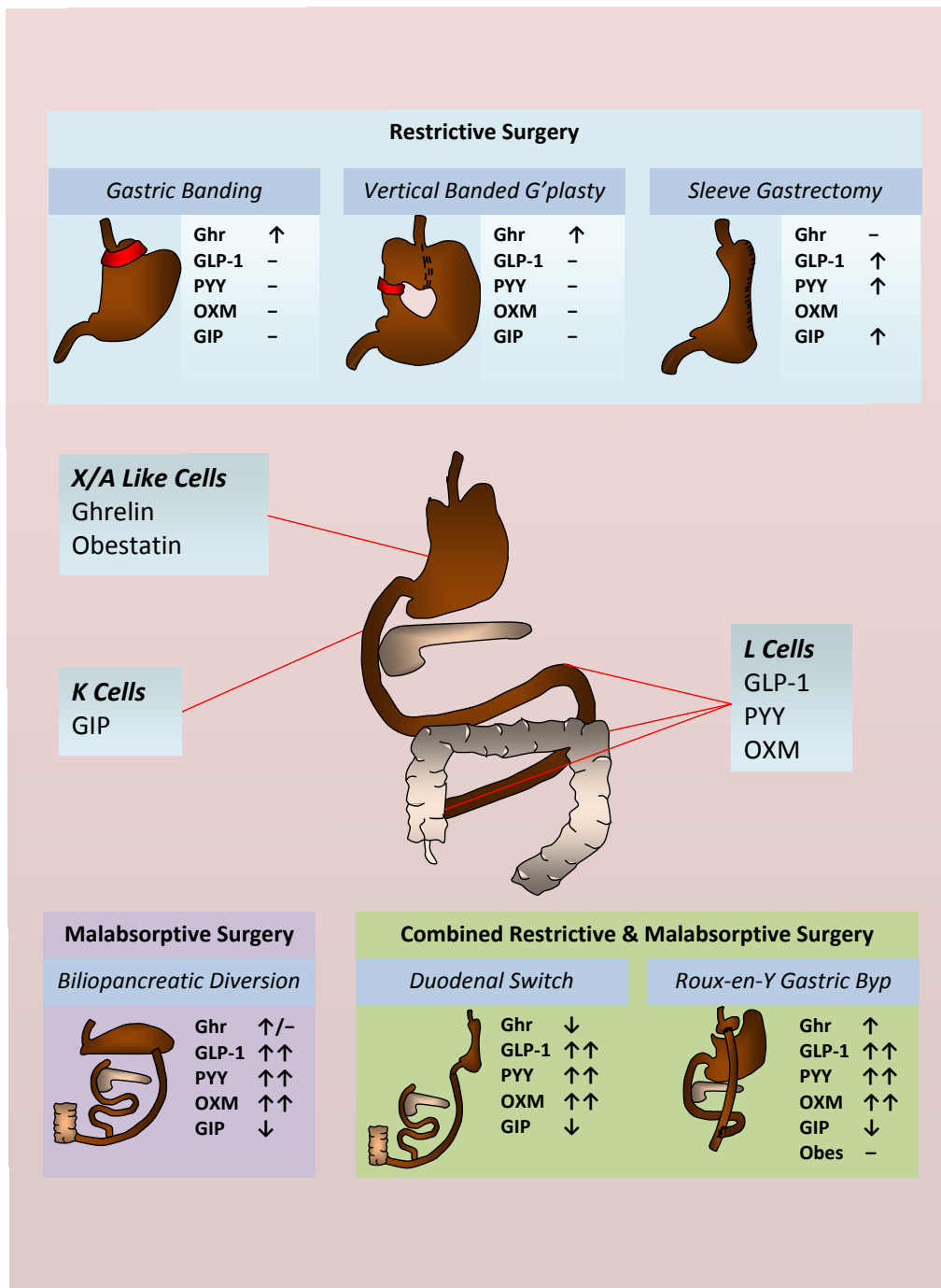
Figure 8: Comparison of bariatric procedures and their effects on gut hormone levels. *Centre of figure:* The neuroendocrine cells that secrete the various gut hormones are indicated in the boxes, which are connected to the principal areas of the gastrointestinal tract that contain these cells. X/A like cells are located in the fundus of the stomach and secrete ghrelin (Ghr) and obestatin (Obes). K cells are located in the proximal small bowel and release gastric inhibitory peptide (GIP). The L cells found more distally account for the release of glucagon-like peptide-1 (GLP-1), peptide tyrosine-tyrosine (PYY) and oxyntomodulin (OXM). The different forms of bariatric surgery have been classified

depending on whether they are restrictive (*top of figure*), malabsorptive or combined (*bottom of figure*). The changes in gut hormone levels, where known, are indicated next to each kind of procedure. The main form of restrictive surgery (*top of figure*), laparoscopic gastric banding, bands the upper part of the stomach, restricting the amount of food that can enter. The band can be adjusted by means of a subcutaneous fluid filled reservoir. This offers a significant advantage over vertical banded gastroplasty, that involves placing a non- adjustable band at the base of the stomach in addition to reducing stomach size by the use of staples to form a small pouch. A more recently developed bariatric procedure is the sleeve gastrectomy that involves exclusion of the stomach fundus. This may be used as definitive surgery or the first step prior to a combination procedure. The malabsorptive procedures (*bottom of figure)* include biliopancreatic diversion that results in food passing through a smaller stomach pouch which is in direct continuity with the distal bowel. The *duodenal switch* combines the restrictive sleeve gastrectomy with the biliopancreatic diversion to reduce the size of the stomach and preserve the pylorus. Finally, Roux-en-Y gastric bypass involves connecting the distal bowel to a surgically altered smaller stomach pouch. Adapted from Tharakan et al. (2011).

1.7 Aim of Studies

This thesis will describe investigations into the physiology of three gut hormones, PP, GLP-1 and PYY. The development of an analogue of PP as a potential therapy for obesity will also be investigated. Specifically, these studies have examined:

1. The effects of PP on food intake when injected SC, as a more-practicable alternative to IV infusion;

2. The effects of an analogue of PP, PP 1420, on food intake when combined with the anti-diabetic drug metformin;

3. The safety, tolerability and pharmacokinetics of PP 1420 when tested for the first time in humans in a Phase 1 clinical trial;

4. The effects of a combination of PYY and GLP-1 on carbohydrate metabolism.

The presented studies have addressed the following specific hypotheses.

1.8 Hypotheses

I hypothesise that:

1. PP reduces food intake when injected SC.

2. PP analogue PP 1420 reduces food intake in animal models.

3. PP 1420 is safe and well-tolerated.

4. PP 1420 exhibits extended pharmacokinetics compared to endogenous PP itself.

5. PYY enhances insulin secretion and/or insulin sensitivity when combined with GLP-1.

2 EFFECTS OF SUBCUTANEOUS INJECTIONS OF HUMAN SEQUENCE PANCREATIC POLYPEPTIDE COMPARED TO INTRAVENOUS INFUSIONS IN OBESE HUMAN VOLUNTEERS.

2.1 Introduction and basis for the present study

To date, studies looking at the effect of PP on appetite and food intake in humans have used IV infusions, and no study so far has looked at the effect of PP when injected subcutaneously (SC). If PP or a PP analogue is to be developed as a treatment for obesity, IV administration will be impractical. A SC injection would be more acceptable, as patients taking insulin for diabetes commonly administer SC injections. Therefore, I wished to investigate whether PP, if administered SC, could reduce food intake.

Moreover, the studies to date have looked at the effect of PP on lean human volunteers (Batterham et al., 2003b; Jesudason et al., 2007; Schmidt et al., 2005) and no study so far has been performed on overweight/obese volunteers. It is important to establish the efficacy of PP in overweight/obese humans, as there may be resistance to its effects in such people. Such a situation would be similar to leptin, where obese humans exhibit resistance to its anorexigenic effects (Munzberg and Myers, 2005). Before proceeding to clinical development of PP or an analogue thereof, we wished to

investigate the effects of SC and IV PP on food intake in overweight or obese volunteers.

2.2 Hypotheses and Aims

2.2.1 Specific Hypothesis

Human sequence PP (hPP), administered SC, will acutely reduce food intake in overweight and obese human volunteers.

2.2.2 Specific Aims

1. To evaluate the effects of hPP, using a standardized IV infusion at 10 pmol/kg/min, in overweight and obese human subjects on:

- Food intake
- Subjective measures of satiety, meal palatability and nausea.

2. To evaluate and compare the effects of ascending SC doses of PP on the abovementioned parameters.

3. To measure the dose that causes nausea in overweight and obese human volunteers.

2.3 Materials & Methods

2.3.1 Peptide

hPP was synthesized by Bachem (St Helens, Merseyside, UK) using 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis. The peptide was purified using reverse phase HPLC. Purity was confirmed using matrix assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectrometry. Under aseptic conditions, the peptide was then dissolved in sterile 0.9% saline (Bayer, Haywards Heath, UK), dispensed into sterile glass vials and lyophilized. To serve as placebo controls, the same volumes of sterile 0.9% saline were dispensed into glass vials and lyophilized.

To verify the peptide content, a randomly selected vial of PP was analysed for amino acid content by Alta Bioscience (Birmingham UK). Bioactivity of the peptide was confirmed by injection into fasted mice and measurement of food intake reduction. At the end of the study, the peptide sequence was independently confirmed by Edman degradation peptide sequencing (Cambridge Peptides, Cambridge, UK).

In order to assure that there was no endotoxin contamination, another randomly selected vial of peptide was sent for *Limulus* amoebocyte lysate testing (Associates of Cape Cod, Liverpool, UK). The peptide was confirmed to be sterile after being cultured for 7 days (Dept of Microbiology, Hammersmith Hospital, London). Toxicity studies, using a weight-adjusted dose at least 10 times the maximum intended for human administration, was carried out in mice. This dose was administered by

intraperitoneal injection to 10 mice, and 0.9% saline administered to another group of 10 mice. The mice were then observed for any abnormal behaviour. After 48 hours, the animals were killed using a Schedule I-approved method. Their lungs, heart, brain, liver, kidneys, stomach, small intestine, pancreas and spleen were dissected out and sent for examination for evidence of pathological toxicity by an independent histopathologist. No evidence of pathological toxicity was found with the tested PP.

2.3.2 Subjects

Overweight and obese volunteers were recruited by advertisement. The inclusion criteria were: age 18 years or older, stable body weight for the preceding three months, body mass index (BMI) 25–40 kg/m^2. The exclusion criteria were: significant physical or psychiatric illness, substance abuse, regular medication other than contraceptives, and current pregnancy. A qualified physician screened and examined the volunteers to determine their suitability for the study. Screening tests included a full blood count, urea and electrolytes, liver function tests, bone profile, thyroid function and 12-lead electrocardiogram. The SCOFF questionnaire (Morgan et al., 1999) was used to screen potential volunteers for disordered eating habits; the Dutch Eating Behaviour Questionnaire (van Strien et al., 1986) was used to screen volunteers for evidence of restrained eating. Potential volunteers were also asked to complete a three-day food diary to determine their usual eating habits. They were also given a sample of the study meal and asked to rate its palatability using a nine-point hedonic scale in order to exclude potential volunteers who either did not like the meal or liked it too much, both situations which might bias the determination of food intake during the study.

2.3.3 Protocol

The study was designed as an ascending-dose study of SC PP. The endpoints of the ascending SC dose study were: reduction in food intake by 10% from the control arm, and nausea, as assessed by a visual analogue score ≥80 mm. Three volunteers were given each SC dose. If the endpoints were not met, the dose was escalated and given to the next three volunteers. If, at a particular SC dose, an endpoint was met by the 3 volunteers, it was planned that dose escalation would be stopped, and another 3 volunteers would be given the same dose. In the event the dose escalation was stopped at 1200 nmol, even without meeting the pre-specified endpoints because of resource limitations, and because it became apparent that very high circulating PP levels were being generated at that dose level.

Study visits were spaced at least 72 hours apart. Female volunteers were asked to use contraception if necessary, and a urine ß-hCG test was performed before each study visit commenced to ensure that they were not pregnant. Volunteers were asked to take a standard diet, to abstain from alcohol and to avoid strenuous exercise for 24 hours before each visit. They were asked to fast and to drink only water from 2100h the night before each visit.

The first visit was an unblinded sham study employing IV 0.9% saline control and SC 0.9% saline control, in order to acclimatise the volunteer to the environment and procedures. Subsequent studies were double-blinded and randomized. Subsequently,

volunteers attended for four study visits, during which they received in random order the following treatments:

Treatment arm	IV infusion (t=0 to +90 min)	SC injection (at t=0)
IV PP	PP (10 pmol/kg/min)	0.9% saline
SC PP 1st dose	0.9% saline	PP (1st dose)
SC PP 2nd dose	0.9% saline	PP (2nd dose)
Saline control	0.9% saline	0.9% saline

The SC doses tested were as follows: 75 nmol (n=5), 150 (n=4), 300 (n=4), 600 (n=2), 1200 (n=3).

On arrival, a peripheral venous cannula was inserted into each forearm, one to allow infusion of PP or saline, the other to allow sampling of blood. The volunteers were then allowed to relax for 15 minutes before the IV infusion was started and the SC injection given. No time cues were allowed in the study room. All volunteers asked to relax by watching films or reading.

The IV infusion vehicle consisted of 5% (v/v) Gelofusine (B. Braun Medical, Sheffield, UK) with 95% (v/v) 0.9% saline, in order to reduce adsorption of the peptide to infusion lines and syringes. 50 ml vehicle was used to dissolve the contents of randomized vials of placebo and PP, and the mixture was drawn up into 50 ml syringes. A Graseby 3100 syringe driver was programmed to deliver the contents of the syringes over 90 minutes, and the infusion was started at t=0 minutes.

The SC injection vehicle was 0.9% saline alone. 200 μl of this vehicle was used to dissolve the contents of randomized vials of placebo and PP, and this was drawn up in

a 1 ml U100 26G insulin syringe (Becton Dickinson, Oxford, UK) before subcutaneous injection in the anterior abdominal wall at t=0 minutes.

Blood samples were collected at the following time points: t=-15, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180 and 210 minutes. These were collected into lithium heparin tubes (International Scientific Supplies Ltd, Bradford, UK) with 0.2 ml aprotinin (Trasylol, Bayer Schering Pharma, Germany) containing 2000 kallikrein inhibitor units. After inversion to mix the blood with the tube contents, samples were immediately placed on ice and centrifuged at 4°C. Plasma was separated immediately, transferred to 1.5 ml Eppendorf microfuge tubes in four aliquots and stored at -20°C before analysis.

Visual analogue scores (VAS) were completed by volunteers immediately before each blood sample was taken. These measured hunger, satiety, prospective food consumption and nausea. The VAS consisted of 100 mm lines with text expressing the most positive and the most negative rating anchored at either end of the line.

One hour after the end of the infusion, subjects were offered a buffet meal that was provided to excess, such that all appetites would be satisfied. The volunteers were provided with drinking water, freely available. Both food and water weights were measured pre and post-meal. Energy intake was calculated from the weight of the meal consumed. At the end of the meal, volunteers were asked to complete a VAS for the palatability of the meal.

Volunteers remained in the study room until 210 minutes after the infusion was started. They were then allowed to go home, but continued to record their food intake in diaries for a period of 24 hours after the buffet meal. The food diaries were analysed for energy intake using Dietplan software (Forestfield Software, West Sussex, UK) by a nutritionist blinded to treatment allocation.

The protocol was approved by Hammersmith, Queen Charlotte's and Chelsea Research Ethics Committee (ref. no 2003/6542). All study volunteers gave written informed consent, and the study was performed in accordance with the Declaration of Helsinki.

2.3.4 Radioimmunoassay (RIA)

Human plasma PP concentrations were measured using a specific and sensitive in-house RIA (Adrian et al., 1976b). An antiserum against human PP was produced in rabbits. It detects human PP fully but does not cross-react with PYY, NPY or any other known gastrointestinal hormone. ^{125}I-labelled PP was prepared by the iodogen method by Professor Mohammad Ghatei (Wood et al., 1981) and purified by HPLC. The specific activity of the ^{125}I-human PP label was 54 Bq/fmol.

The assay was performed in phosphate buffer with 0.3% BSA. Plasma samples were assayed in 100 μl and 20 μl aliquots in duplicate, in a total volume of 700 μl, containing rabbit anti-human PP (1:5000) and 1500 cpm/tube of iodinated PP. The assay was incubated for four days at 4°C before separation by charcoal absorption.

All samples were assayed in duplicate and within a single assay, thus eliminating inter-assay variation. Standard quality control samples were run with each assay. The plasma PP RIA could detect changes of ± 2.8 pmol/L (95% confidence interval) with an intra-assay coefficient of variation of 11.3%.

2.3.5 Statistical analysis

Combined data are presented as the mean $\pm$ standard error of the mean (S.E.M.), except where indicated. Analyses were performed using GraphPad Prism 4.03c software (GraphPad Software, San Diego, USA). A one-tailed Student's t-test was used to compare the energy intake between treatments. Least squares linear regression was used to analyse the relationships between BMI and the peak plasma PP concentration or reduction in energy intake. VAS scores were adjusted for baseline, and differences compared at each time point by paired non-parametric Wilcoxon signed rank test. The threshold of statistical significance was conventionally set at $p<0.05$.

2.4 Results

2.4.1 Recruitment of volunteers

A total of 23 volunteers were studied, with 7 men and 16 women. The mean age was 36.3 ± 1.9 years (range 22–54). The mean BMI was 31.8 ± 0.7 kg/m^2 (range 25.3–37.3). Of 23 volunteers, 2 participants (1 female, 1 male) withdrew from the study for

personal reasons. Data from one participant was excluded due to investigator error in allocation of randomized vials. One volunteer was withdrawn from the study after the second visit because of difficulties in sourcing the study food. Two volunteers were excluded because of protocol violations involving standardization of food intake before a study visit. Therefore, 17 volunteers were included in the final statistical analysis.

2.4.2 Adverse effects

One volunteer experienced a short period of moderate abdominal pain during a study visit that subsided completely without intervention. Another volunteer complained of a metallic taste in the mouth, but this occurred in study visits when both placebo and PP were given. No other adverse effects were reported.

2.4.3 Circulating plasma PP levels during the saline control visit

Fasting PP levels during the saline (SC and IV) control visit were 24.7 ± 2.2 pmol/L. They did not change significantly after SC injection of saline and during the IV infusion of saline, as expected, the means at the timepoints from 15 to 150 mins varying from 26.7 to 31.8 pmol/L. Upon consumption of the test meal at 150 mins, PP rose to normal post-prandial levels (Adrian et al., 1976b), achieving a peak of 204.9 ± 23.4 pmol/L at 180 mins.

2.4.4 Circulating plasma PP levels during the IV PP (10 pmol/kg/min) plus SC saline visit

The fasting plasma PP level was 20.2 ± 2.3 pmol/L immediately before commencement of the IV infusion. IV infusion of PP caused a rise to a concentration peak (C_{max}) of 1203.3 ± 127.6 pmol/L at a time to peak level (t_{max}) of 45 minutes. After termination of the infusion at 90 mins, the PP levels fell rapidly to 98.93 ± 8.22 pmol/L at 150 mins. After consumption of the test meal at 150 mins, PP levels peaked again at 191.77 ± 13.87 pmol/L at 180 mins.

2.4.5 Circulating plasma PP levels after SC PP doses

Table 1 summarises the mean peak concentrations achieved by each dose of PP, injected SC at time 0 mins. The t_{max} for SC injections was 30 mins with the exception of the 600 nmol dose at 90 mins. **Figure 9** shows the profile of plasma PP levels after SC injections, compared to IV infusion.

SC dose (nmol)	Number of volunteers	C_{max} (pmol/L)	t_{max} (min)
75	5	431.8 ± 6.4	30
150	4	1080.9 ± 54.0	30
300	4	1496.8 ± 291.6	30
600	2	2130.6 ± 24.0	90
1200	3	5879.8 ± 622.7	30

Table 1: Peak plasma levels (C_{max}) of PP (mean ± S.E.M.) and time to peak levels (t_{max}) developed after SC injection of PP.
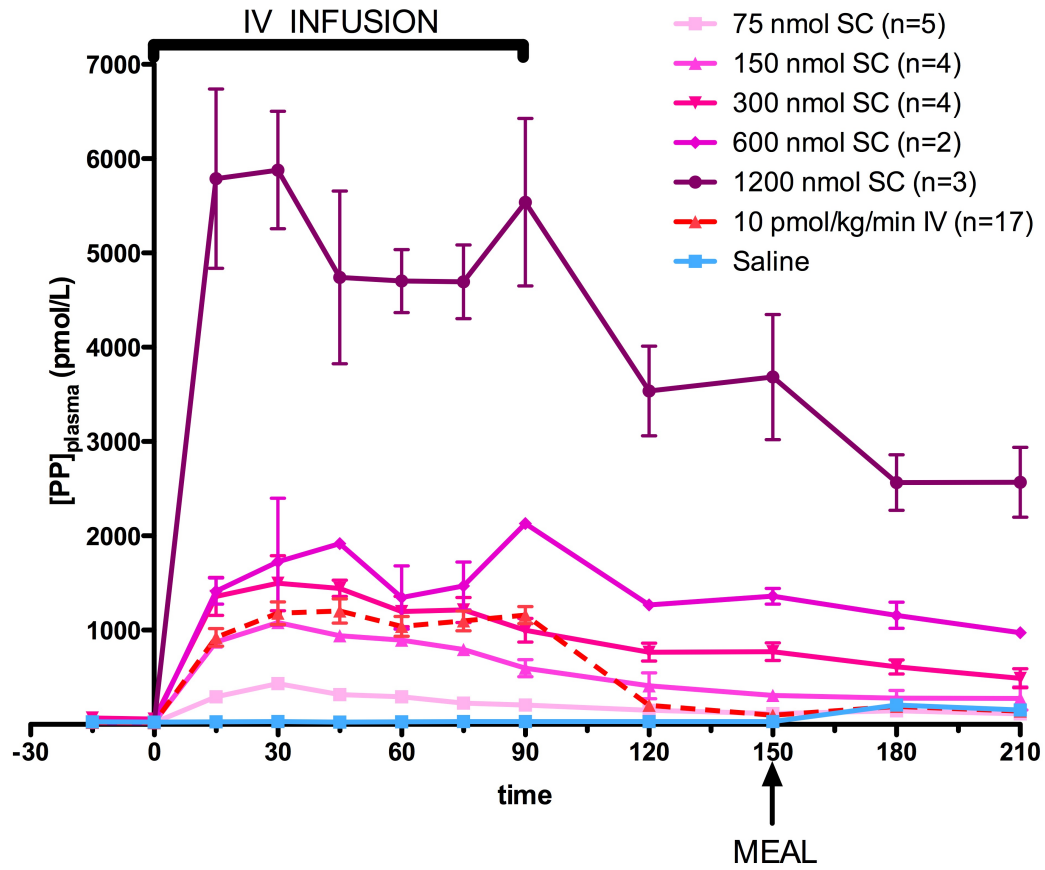
Figure 9: Plasma PP levels during IV infusion compared to SC injections at doses of 75–1200 nmol. The plasma PP level in pmol/L is plotted on the Y axis against time in minutes on the X axis. Each profile shows the mean PP levels achieved following IV or SC administration of PP (error bars show S.E.M.). The IV infusion time is indicated by the black bar. A test meal was given at 150 mins.

2.4.6 Linearity of PP concentration Area Under the Curve (AUC) with SC PP dose

The AUC from 0 to 210 mins (AUC_{0-210}) was calculated for each individual, and averaged for each SC dose. **Figure 10** shows the AUC achieved with each SC dose, plotted against the dose. This demonstrates that there is a strong linear relationship of the AUC to the dose injected, i.e. there is dose proportionality of PP exposure as judged by the AUC_{0-210}.
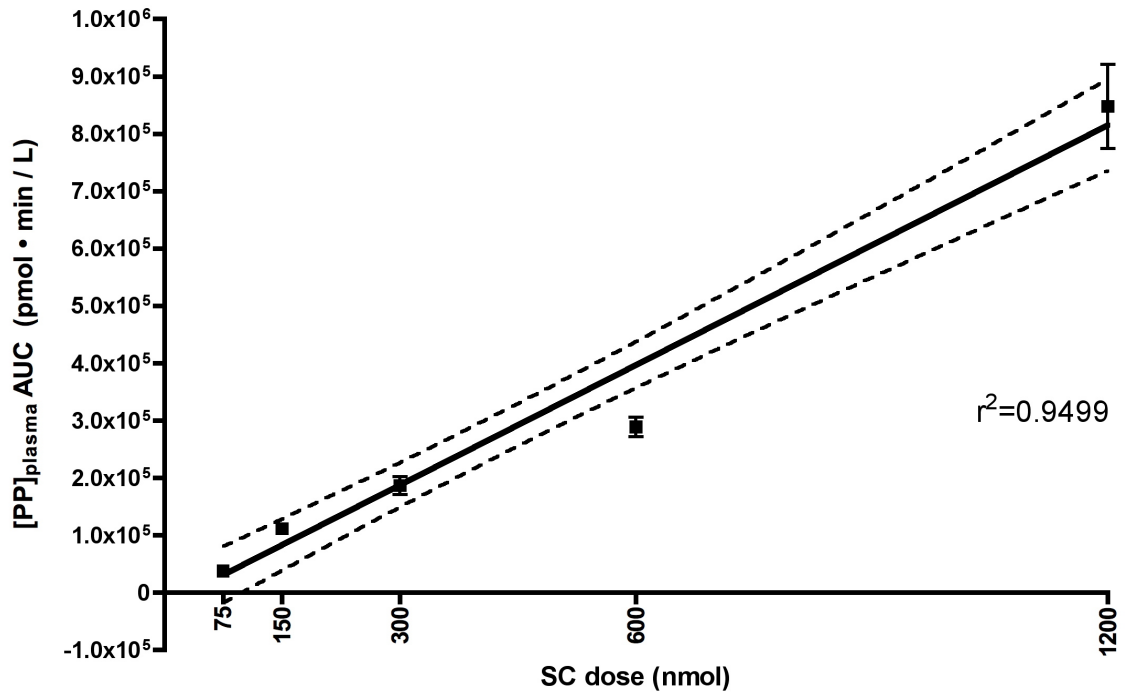
Figure 10: Relationship of [PP]$_{plasma}$ AUC with SC dose given. The individual AUCs for each dose from time 0 to 210 min were calculated, and the mean ± S.E.M. of these values plotted on the Y axis against the SC dose given on the X axis. A least-squares linear regression line was calculated (solid line) and the 95% confidence intervals for the regression line are indicated by the dotted lines. The r^2 coefficient of determination is 0.9499, indicating that the linear regression line fits the data points very well.

2.4.7 Effect of an IV infusion or SC injection of PP on acute food intake

The effect of PP on acute food intake was measured during the test meal. The mean food intake during the saline (IV and SC) control arm was 3196 ± 429.1 kJ, compared to the IV PP 10 pmol/kg/min (plus 0.9% saline SC) arm where the food intake was 2923 ± 430 kJ (mean reduction of 9.4 ± 3.7%, p=0.0073 – **Figure 11**).

The effect of SC PP at various doses on food intake was also investigated. Most doses did not have a significant effect on reduction of food intake. The mean energy intakes for SC PP at 150, 300, 600 and 1200 nmol doses respectively were 3374 ± 342.2 (n=3), 3430 ± 390.7 (n=4), 6922 ± 2856 (n=2), and 3188 ± 315.8 kJ (n=3). The exception was SC PP at 75 nmol, where the mean food intake measured was 1851 ± 339.8 kJ (n=5: mean reduction of 20.3 ± 8.3% versus saline IV and SC arm, p=0.0468 – **Figure 12**). The individual comparisons of food intake at the saline and 75 nmol visits show that there were reductions of food intake in four volunteers out of the five who received this dose (**Figure 13**).
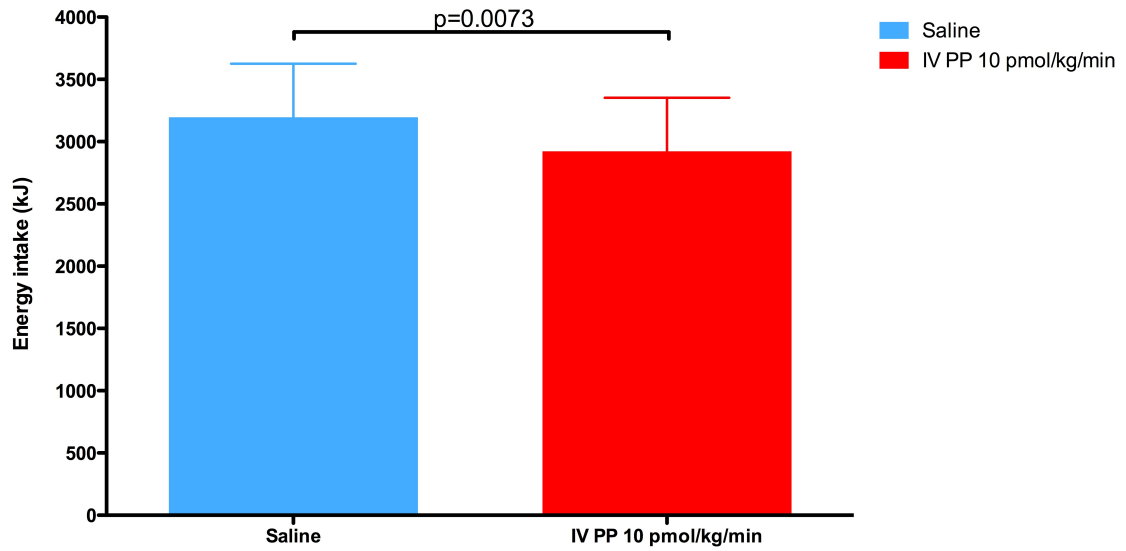
Figure 11: Effect of IV PP 10 pmol/kg/min on food intake. The mean acute food intake, measured during test meal and expressed in kJ, is plotted for 17 volunteers (error bars express S.E.M.). Paired one-tail Student's t-test p=0.0073 for difference between means.
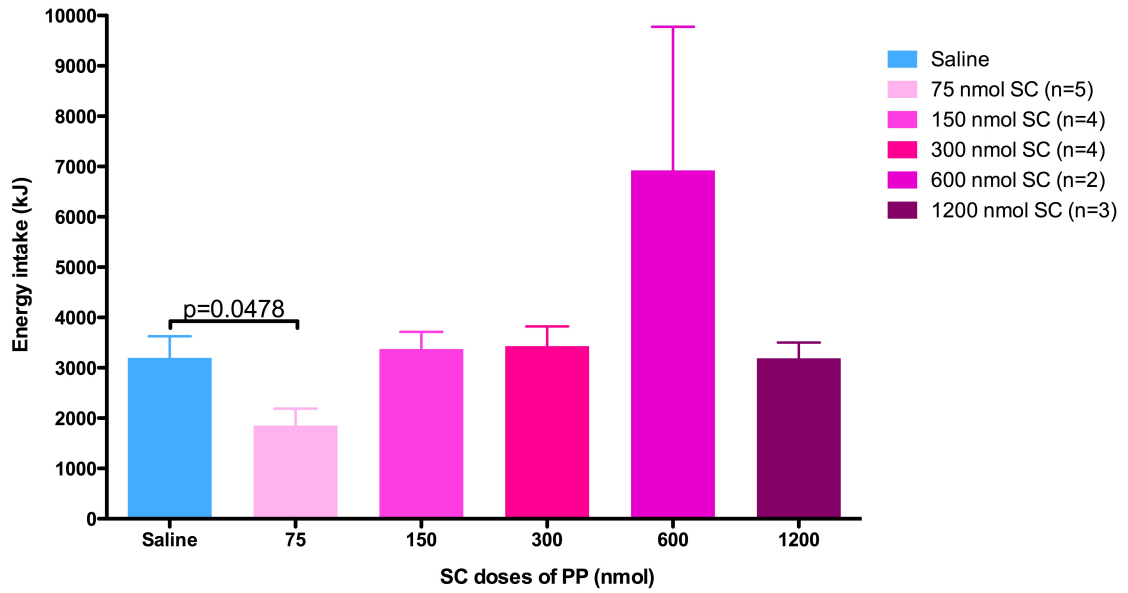
Figure 12: Effect of SC PP at escalating doses on acute food intake. Mean acute food intake, measured during test meal, and expressed in kJ is plotted against each SC dose in nmol (error bars express S.E.M.). Paired one-tail Student t-test p=0.0478 for difference between means of the saline control and the SC PP 75 nmol dose.
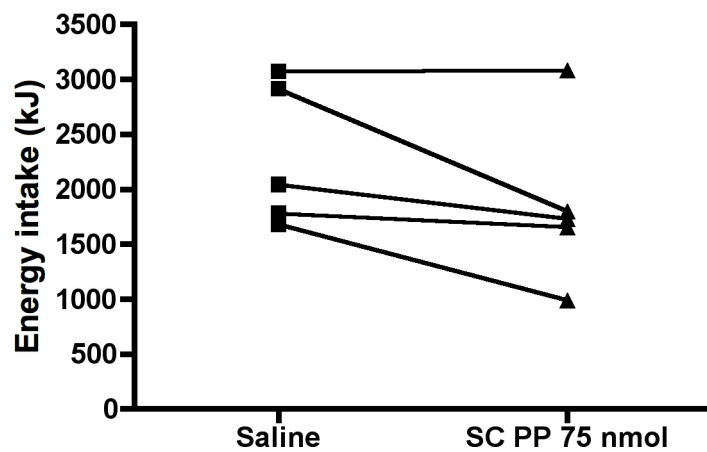
Figure 13: Individual comparisons of acute food intake during the saline control arm versus the 75 nmol SC PP dose. Each square represents a volunteer's food intake during the saline (IV and SC) control arm, and is linked by a line to the same volunteer's food intake during the 75 nmol SC (plus 0.9% saline IV) arm (triangles).

2.4.8 Effect of SC injection of PP on subjective satiety and meal palatability

Subjective satiety parameters were measured using VAS (**Figure 14**). No statistically significant differences were seen in these parameters with increasing SC PP dose or with IV PP (10 pmol/kg/min). Interestingly, even with increasing SC PP doses and the development of circulating PP levels approximately 30 times normal post-prandial levels (with the 1200 nmol SC PP dose) no participant at any time developed significant nausea as assessed by VAS score ("How sick do you feel?").
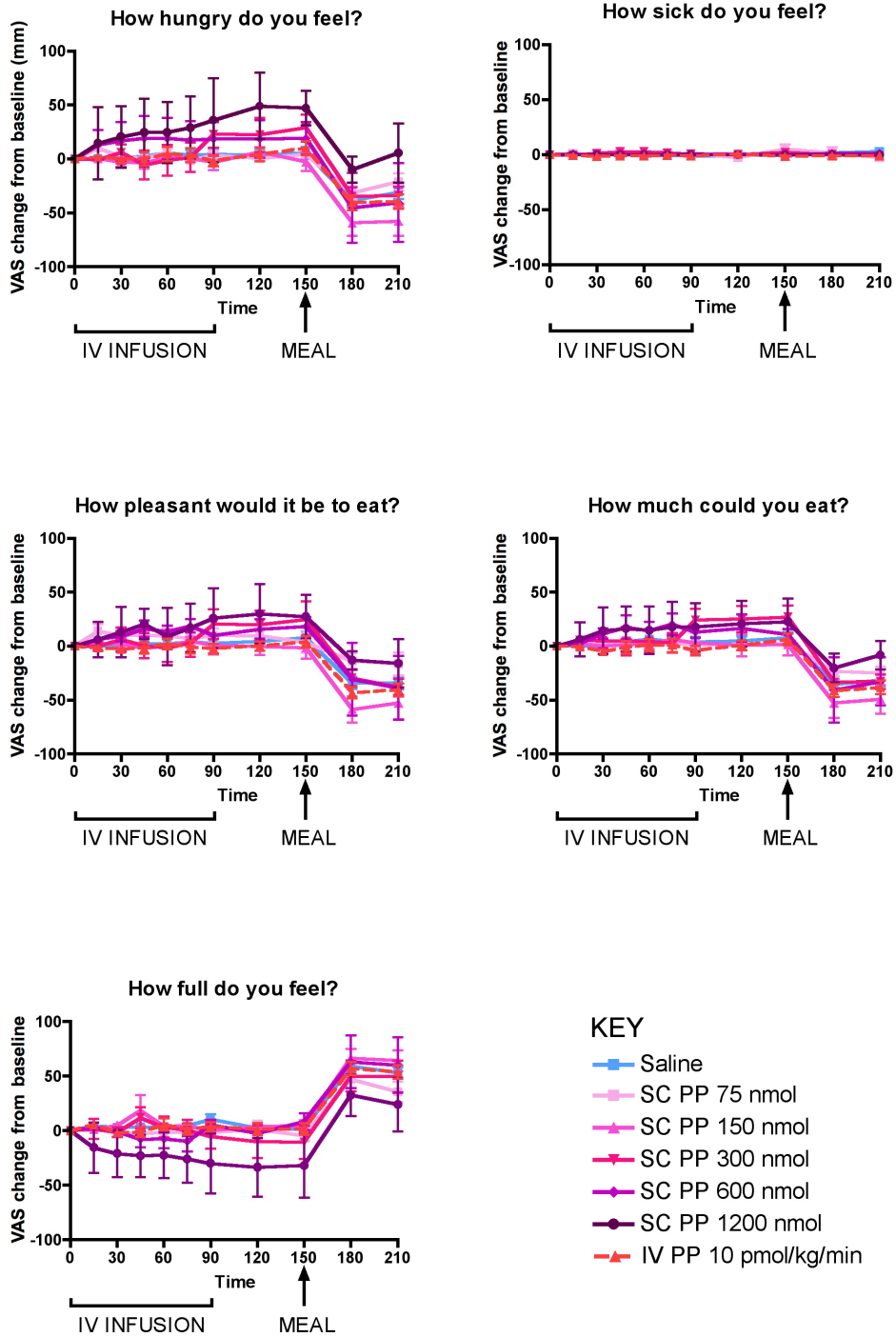
Figure 14: Effect of IV PP infusion or SC PP injection on subjective parameters of satiety. Baseline-corrected VAS scores (mm) plotted at each timepoint (error bars indicate S.E.M.).

2.5 Discussion

In the study presented, IV PP, at a dose of 10 pmol/kg/min, produced a small but significant reduction in acute food intake of 9.4% in overweight/obese human volunteers. This effect is smaller than when similar doses were infused into lean subjects in the Batterham (Batterham et al., 2003b) and Jesudason (Jesudason et al., 2007) studies. These found reductions of food intake of 22% with a 10 pmol/kg/min infusion, and 11% with a 5 pmol/kg/min infusion, respectively. This is despite the fact that the current study achieved peak concentrations of PP (~1200 pmol/L) that were much higher than Batterham's study (~260 pmol/L) despite using the same weight-adjusted IV infusion rate.

Even at the very high concentrations of PP achieved, it is notable that no volunteer reported any nausea, which is the usual side effect of giving supraphysiological levels of gut hormones to human volunteers, e.g. PYY_{3-36} (le Roux et al., 2008), GLP-1 (Ritzel et al., 1995), CCK-8 (Greenough et al., 1998) and OXM (K. Wynne, personal communication).

Some conjectures may be made to explain these findings and these are discussed in the following sections.

2.5.1 Conjecture 1: There is inter-assay variation between this study and the earlier studies

Some insight into this may be obtained by the comparison of comparable measurements in my study, Batterham's study (Batterham et al., 2003b) and Jesudason's study (Jesudason et al., 2007). The fasting level of PP in my study was 18.2 ± 2.2 pmol/L. In Batterham's study, the fasting level was documented at 15.5 ± 1.4 pmol/L (Batterham et al., 2003b) and in Jesudason's study, the fasting level was 17.2 ± 2.3 pmol/L (Jesudason et al., 2007). Thus, there is little evidence for inter-assay variation given these measurements.

Although there are differences in the peak post-prandial levels in the saline control arms between the studies (my study: 202.2 ± 27.4 pmol/L; Jesudason: 83.1 ± 18.9 pmol/L; Batterham 88.9 ± 17.2 pmol/L) the interpretation of these levels is complicated by the variable food intakes of the volunteers in each study (my study: 3196 ± 429 kJ; Batterham: $\sim 4800 \pm 300$ kJ; Jesudason: 2730 ± 180 kJ), which would necessarily influence the post-prandial PP levels. Moreover, quality control samples were run routinely on the RIAs used for this study and these showed no evidence of inter-assay variation of the magnitude necessary to explain the discrepancy. This conjecture, therefore, does not explain the discrepancy between PP levels.

2.5.2 Conjecture 2: The high PP levels seen on RIA reflect high levels of biologically inactive fragments

The RIA used in this study utilized a polyclonal antibody and as such can not reliably differentiate between full-length PP and biologically inactive PP fragments. In order to investigate this possibility, I therefore went on to evaluate the existence of PP breakdown products in volunteer plasma. However, the HPLC analysis was complicated by the fact that two standard pre-analytical purification steps introduced artefacts, perhaps by causing breakdown of the PP. FPLC analysis suffered from poor resolution and was therefore also considered unsuitable to resolve breakdown products. At present, this conjecture must remain unproven.

2.5.3 Conjecture 3: Overweight or obese people are intrinsically less sensitive to the effects of PP on appetite and food intake

This is similar to the situation with leptin, where obese people appear to be less sensitive to its anorexigenic actions (Myers et al., 2008). However, the conclusions that can be drawn with regards to this from the present study are limited, since the study did not directly compare the effects of PP in the overweight/obese volunteers with a cohort of volunteers with normal BMI. The design of the current study is also slightly different to Batterham's study in that I assessed acute food intake with a test meal at 60 minutes after the end of the infusion compared 120 minutes for Batterham's study (Batterham et al., 2003b). It is therefore possible that we might have seen a greater reduction in food intake if this was dependent on the delay between the infusion and the test meal. The present study design is the same as Jesudason's study in this respect (Jesudason et al., 2007). Another limitation that

should be mentioned here is that the present study, being designed as an ascending dose study, resulted in a relatively small number of volunteers being tested at each dose level. Thus, if the subjects were less sensitive to the effects of PP, the study may have been statistically underpowered to detect such effects.

2.5.4 Conjecture 4: The effects of PP are dose-dependent.

This conjecture postulates that peak levels of 150–1500 pmol/L are anorexigenic, but higher levels do not reduce food intake. SC PP produced a significant reduction in food intake of approximately 20.3% when given at a dose of 75 nmol. However, higher doses did not cause significant reductions in food intake. The 75 nmol dose achieved a peak PP level of 431.8 pmol/L. **Figure 15** shows the relationship between reductions in food intake and PP level achieved, which suggests that the optimum food intake reduction may be achieved at levels of between 250–500 pmol/L or thereabouts.

This phenomenon of dose-dependent effects might possibly be due to:

(1) Receptor desensitization, e.g. by internalization. This explanation is less likely, as a study with the Y4 receptor in Chinese hamster ovary cells indicated that this receptor is resistant to internalization and desensitization after exposure to high levels of PP for 24 hours (Voisin et al., 2000).

(2) Counter-regulation due to activation of orexigenic appetite circuits opposing the anorexigenic effects of Y4 receptor agonism. This phenomenon would be

116

expected to manifest itself as a regression to the baseline weight during chronic administration of PP, an 'escape phenomenon'. However, the study of Asakawa and colleagues showed that PP, given twice a day to *ob/ob* mice over a period of 14 days, caused a stable weight loss compared to saline-treated controls, making this explanation less likely (Asakawa et al., 2003). It should also be noted that the PP analogue 1420, when given chronically for up to 69 days to animals causes a stable weight loss compared to saline-treated controls with little evidence of escape (Chapter 3). Therefore, this explanation does not seem likely.

(3) PP activates orexigenic appetite circuits at higher levels. As previously noted in the Introduction, central administration of PP has a stimulatory effect on food intake in animals. It is therefore possible that peripheral administration of high doses of PP causes high levels of PP in the CNS, stimulating food intake and neutralising its anorexigenic effects. This is a distinct possibility, although there is no evidence of such an effect with high-dose administration of PP analogues to animals (Chapter 3).

2.5.5 Possible future work

Overall, I believe that the best explanation for the results I have obtained lies between Conjectures 2 and 4. Future studies to differentiate between these possibilities would need to incorporate the following features.

To test conjecture 2, a high-sensitivity assay capable of differentiating the fragments of PP which could be generated by breakdown from full-length PP is required. A method based on LC/MS-MS would be suitable for this task. Such assays for peptides of the required sensitivity are only just becoming possible (see Section 4.3.5).

To test conjecture 3, it will be necessary to directly compare the effects of IV PP on lean and overweight/obese volunteers using the same standardized study protocol.

To test conjecture 4, a new clinical study would be required, with a low-range dose titration (e.g. between 1-100 nmol per dose) to establish the optimal dose and circulating PP level to reduce appetite.
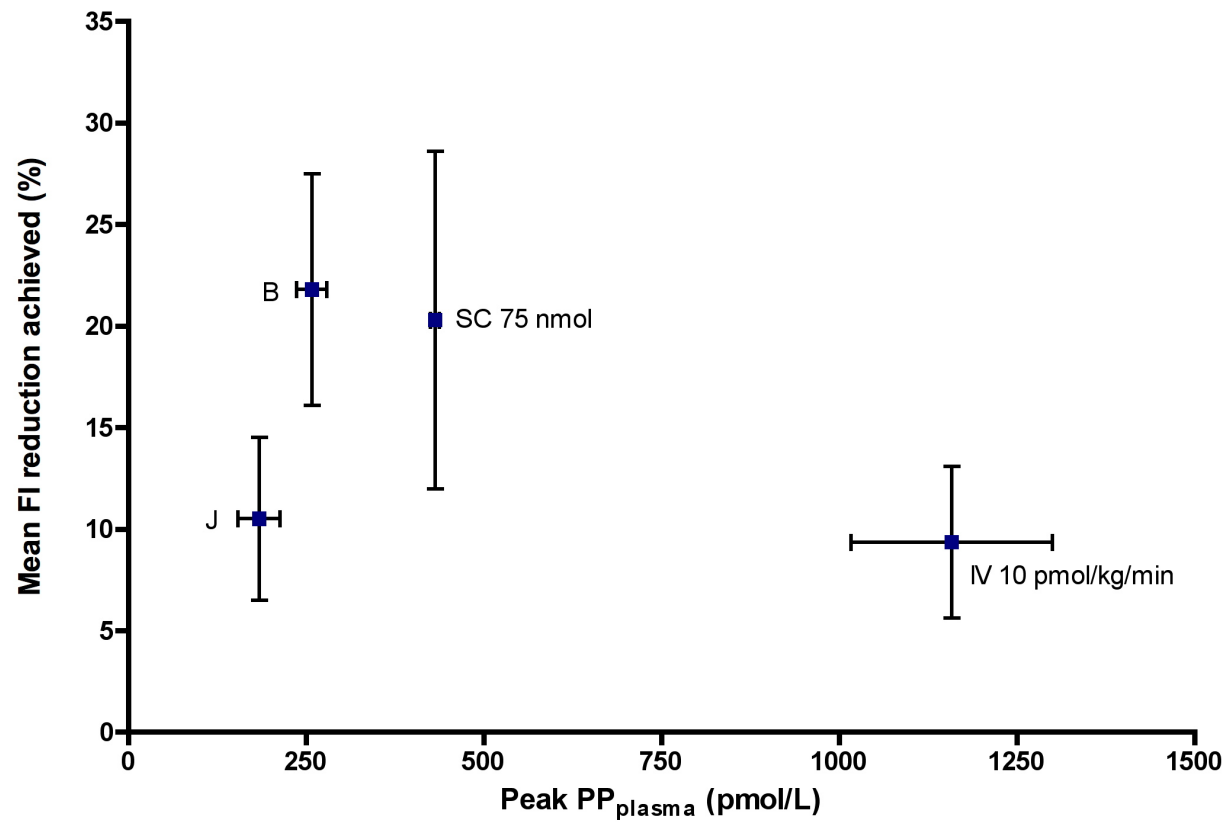
Figure 15: Relationship of peak PP level (X axis) to percentage reduction in food intake (Y axis). Y and X error bars indicate S.E.M. J = Jesudason study (5 pmol/kg/min, lean subjects). B = Batterham study (10 pmol/kg/min, lean subjects). SC and IV doses indicated from present study.

3 PRE-CLINICAL STUDIES ON AN ANALOGUE OF PANCREATIC POLYPEPTIDE, PP 1420

3.1 Introduction

The half-life in circulation of hPP is short at seven minutes (Adrian et al., 1978b), and previous studies on its physiological effects have overcome this problem by using prolonged, continuous IV infusions to establish physiologically effective circulating levels of PP (Batterham et al., 2003b; Jesudason et al., 2007). However, this technique is clearly not practicable as a day-to-day treatment. As with other peptide-based treatments such as insulin and GLP-1, the simplest and most practical method of hPP administration for therapy is SC injection. The data in Chapter 2 suggested that SC hPP injections could deliver sustained PP levels for up to 210 minutes. Ideally, a treatment for obesity would be injected once a day as this frequency of dosing has been shown to be acceptable to patients on a sustained basis, e.g. for GLP-1 analogues such as liraglutide. Extension of hPP's short half-life by changes in the peptide sequence to enhance peptidase resistance would therefore enable such once-daily injection.

To overcome the short half-life problem, the Department of Investigative Medicine has developed peptidase-resistant analogues of hPP with the following properties: longer half-lives, selective Y4 receptor binding activity, and powerful appetite-suppressive properties in validated pre-clinical models of obesity. One of these analogues, PP 1420, has an amino acid sequence that is similar to that of hPP, with

one additional amino acid compared with the hPP sequence (a glycine residue located at position 0) and substitutions of five other residues of PP within the 37-residue peptide. The peptide is C-terminally amidated and contains only standard L-amino acids. The chemical name for PP 1420 is:

L-Glycyl-L-alanyl-L-prolyl-L-leucyl-L-glutamyl-L-prolyl-L-valyl-L-tyrosyl-L-prolyl-L-glycyl-L-aspargyl-L-asparginyl-L-alanyl-L-threonyl-L-prolyl-L-glutamyl-L-glutaminyl-L-lysyl-L-alanyl-L-lysyl-L-tyrosyl-L-alanyl-L-alanyl-L-glutamyl-L-leucyl-L-arginyl-L-arginyl-L-tyrosyl-L-isoleucyl-L-aspargyl-L-arginyl-L-leucyl-L-threonyl-L-arginyl-L-prolyl-L-arginyl-L-tyrosinamide, Hydrochloride salt.

Or in standard IUPAC three-letter code (where ·NH$_2$ denotes the C-terminal amidation):

Gly-Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asn-Ala-Thr-Pro-Glu-Gln-Lys-Ala-Lys-Tyr-Ala-Ala-Glu-Leu-Arg-Arg-Tyr-Ile-Asp-Arg-Leu-Thr-Arg-Pro-Arg-Tyr·NH$_2$

PP 1420 has a potential indication in the treatment of obesity and its common association, type 2 diabetes. In man, the oral drug metformin, a biguanide, is commonly used as a first line drug for type 2 diabetes in the obese (Kahn et al., 2006). Metformin is preferred in this group as it is effective in lowering blood glucose and provokes less weight gain than other oral hypoglycaemics such as sulphonylureas or thiazolidinediones (Kahn et al., 2006). We wished to test the effect of the following combinations: PP 1420 alone, PP 1420 plus metformin on food intake and body weight in mice. Enhancement of weight loss by the addition of PP 1420 to metformin

might establish another indication for PP 1420 in patients with type 2 diabetes mellitus and obesity.

3.2 Hypotheses and Aims

3.2.1 Specific Hypothesis

PP 1420 has additive effects with metformin on food intake.

3.2.2 Specific Aims

To establish if PP 1420 administered in combination with metformin causes enhanced weight loss over and above that seen with PP 1420 alone.

3.3 Materials & Methods

3.3.1 Receptor Binding Affinity Studies

3.3.1.1 Preparation of Membranes for Cells

Membrane preparations were made from human embryonic kidney 293 (HEK 293T) cell lines over-expressing human Y4 receptor from cell lines between passages 15–35. The human Y4 receptor cDNA clone was purchased from cDNA Research Centre, Missouri University of Science and Technology, Missouri, USA (catalogue number: NPYR400000). Cells were harvested when they were 70–80% confluent and the membrane purified by centrifugation. Protein concentrations of membranes were assessed using a Biuret protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Waltham, Massachusetts, USA).

3.4　　　Transfection of Cells

Twenty-four hours prior to transfection, cells were sub-cultured and plated at a density of 20,000 cells per 60 mm plate in standard media [Dulbecco's Modified Essential Medium supplemented with foetal bovine serum at 10% (v/v), penicillin (100 IU/mL) and streptomycin (100 IU/mL) – Life Technologies, Paisley, Scotland]. Transfections were completed when the cells were 40% confluent.

Complexes of polyethyleneimine (PEI) and DNA were made with PEI (average MW ~ 25kDa) (Abdallah et al., 1996). A 0.1 M stock solution of ~25 kDa PEI pH 7.0 was prepared and filtered through a 0.2 μm filter prior to use. The plasmid DNA was prepared such that each plate was transfected with 6 μg DNA in 5% (w/v) glucose (the 10% glucose solution was also sterilised by filtration through a 0.2 μm filter). The cells were co-transfected with plasmid NPYR400000 and pcDNA3.1+ (plasmid containing neomycin resistance gene, Life Technologies, Paisley, Scotland) and nine nitrogen equivalents of PEI. Four plates were transfected with each receptor plasmid and pcDNA3.1 and two plates with receptor plasmid only (controls). Polyethyleneimine solution was slowly added to the DNA and glucose solution, vortexed for 30 seconds and allowed to stand at room temperature for 10 minutes before use. The PEI/DNA/glucose mix was slowly added to the cells and incubated for 3 h under standard conditions (37°C, 5% CO_2), after which the medium was removed and replaced with standard medium. The cells were maintained under standard conditions. Forty-eight hours later, the medium was supplemented with 800 μg/mL Geneticin, and replaced every 48 h with fresh Geneticin until all control cells were dead – usually 10 days after the start of treatment. Remaining cells from

each plate were then transferred to T-10 flasks (one plate to one flask) and maintained under standard conditions.

3.5 Iodination of Peptides

3.5.1 Direct Iodogen Method

The direct iodination method used was as previously described (Owji et al., 1996). Peptide (15 μg) in 10 μl of phosphate buffer (0.2 M) pH 7.2 was reacted with ^{125}I-Na (37 MBq) (PerkinElmer, Massachusetts, USA) and 1,3,4,6,-tetrachloro-3α, 6α-diphenylglycoluril (10 μg) (Iodogen reagent, Thermo Scientific, Massachusetts, USA) for 4 minutes at 22°C. The ^{125}I-peptide was purified by reversed-phase high performance liquid chromatography using an acetonitrile (AcN)/H_2O/0.05% trifluoroacetic acid (TFA) gradient.

3.6 Human and Mouse Y4 Receptor Binding Assay

Receptor binding assays were conducted in siliconised microtubes (1.5 mL) (Sigma T-4816) using a basal buffer of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 20 mM) pH 7.4, $CaCl_2.2H_2O$ (5 mM), $MgCl_2.8H_2O$ (1 mM), and bovine serum albumin (1%) at 4°C. Diprotin A (0.1 mM), phenylmethanesulphonylfluoride (PMSF) (0.2 mM) and phosphoramidon peptidase inhibitors (10 μM) were added fresh prior to each experiment. Complete buffers were kept at 4°C and used within 90 minutes.

The following protocol was used:

1. Concentrations of peptide were tested in duplicate or triplicate.

2. Basal buffer was added to the tubes.

3. ^{125}I-hPP label made up, 1000 cps/50 μL (500 Bq, 100 pM), 50 μL required per reaction tube and label added using repeater.

4. Freeze dried peptide was diluted in assay buffer. Peptide was added to the reaction tube.

5. Membrane was diluted to the required concentration and added to the reaction tube using a repeater.

6. Tubes were vortexed and left at room temperature for 90 minutes.

7. The centrifuge was pre-cooled to 4°C.

8. After 90 minutes, tubes were centrifuged for 3 minutes at 15000 rpm, 4°C. Supernatant was discarded, tubes were washed with 500 μL buffer (as specified above but protease inhibitors were not required).

9. While tubes were spinning, a background count of γ-counting equipment was started.

10. The pellet was disrupted and re-centrifuged as above. Supernatant was discarded and the pellet was counted in the γ-counter for 240 seconds.

11. The percentage specific binding was calculated by counting the number of observed reactions that contained unlabelled peptides, expressed as a percentage of total binding.

3.6.1 Chronic food intake study in diet-induced obese (DIO) mice

This study investigated the effect of PP 1420 on body weight and food intake in diet-induced obese male C57BL/6J mice.

3.6.1.1 Animal Supply and Acclimatisation

Diet-induced obese male C57BL/6J mice (aged 29 weeks) were purchased from Charles River, Margate, UK). The animals were allowed to acclimatise for at least 5 days after being purchased from Charles River.

3.6.1.2 Animal Housing, Diet and Water Supply

Animals were single housed from 7 weeks and throughout the study. Cages were changed when appropriate; approximately every 2 weeks. Obesity was induced by feeding the animals 45% energy from fat (Irradiated 58V8-55629, Test Diet) from 7 weeks of age. Animals were maintained on this diet throughout the study. Access to food was restricted to 19:20–08:00, however the animals had access to water at all times. Animals fasted at 08:00 on the first day of the study. PP 1420 was administered at 16:00 and animals were re-fed at 17:00. Body weight and food intake was measured daily at 08:00. Recovery and change in body weight following treatment was also monitored.

3.6.1.3 PP 1420 administration

One group of mice (n=7; mean body weight: 46.9 g; range: 44.9–50.4 g) received an s.c. injection of PP 1420 at a dose of 282 µg/kg for 69 days. Another group (n=7; mean body weight: 46.5 g; range: 45.2–48.8 g) received PP 1420 s.c. at a dose of 855 µg/kg for 69 days. Control mice (n=8; mean body weight: 46.7 g; range: 41.8–51.0 g) received an injection of vehicle (sterile 5% v/v water, 95% v/v 0.9% w/v sodium chloride) daily for 69 days. Body weight and food intake was measured daily. Vehicle or PP 1420 was administered on a daily basis (1600-1615 h) by subcutaneous injection.

3.6.2 Glucose Tolerance Studies

At the end of the chronic food intake study, the mice underwent a glucose tolerance test. They were fasted overnight following the last dose on day 69, and the glucose tolerance test was performed the following morning. The glucose tolerance test was performed by administering an IP dose of glucose (2 g/kg as 20% dextrose solution), with blood samples for glucose taken at 0, 15, 30, 45, 60, 90 and 120 minutes after the glucose dose from the tail vein. Glucose levels were measured in these samples using an Abbott Optium Xceed glucose meter utilising a glucose dehydrogenase amperometric method (Abbott Diabetes Care, Maidenhead, UK). The data were analysed using a two-way ANOVA with repeated measures (Prism 5.0d, GraphPad Software, San Diego, CA, USA).

3.6.3 Co-administration of PP 1420 with metformin in DIO male mice

Single housed male C57BL/6 mice (Charles River, Margate, UK), were fed a high fat diet (60% kcal by fat) *ad libitum* for 10 weeks to induce a diet-induced obesity (average body weight 36.0 g). Mice were acclimatized to injections and handling for 1 week before experimentation began. Mice were divided up into the following weight matched groups (n=7-8 per group):

Group	SC	IP
1	Saline 0.9% w/v	Saline 0.9% w/v
2	PP 1420 (64 µg/kg)	Saline 0.9% w/v
3	Saline 0.9% w/v	Metformin (62.5 mg/kg)
4	PP 1420 (64 µg/kg)	Metformin (62.5 mg/kg)
5	Saline 0.9% w/v	Metformin (125 mg/kg)
6	PP 1420 (64 µg/kg)	Metformin (125 mg/kg)

PP 1420, or saline, was administered at 1600h for 4 days, as subcutaneous injections at the indicated doses. I employed a lower dose than previously tested in the chronic food intake study (64 µg/kg), as this dose was known to cause some food intake reduction but at a lower magnitude, and as I wished to observe any additive effects between these two drugs. Metformin (Sigma Aldrich, Poole, UK), or saline, was administered IP at the indicated doses. Food intake was measured at the following time-points: 1, 2, 4, 8, 24, 48, 72 and 96 hours. Body weight was measured immediately prior to dosing on a daily basis. Fasting blood glucose was measured using a handheld glucose meter (Optium Xceed glucose meter, Abbott, Maidenhead, UK) from tail bleeds on day 4, after the mice had been fasted overnight.

Statistical analysis of the food intake and body weight data was carried out using a general estimating equations procedure (SPSS 17, IBM, Armonk, NY, USA) followed by a Mann-Whitney U test to assess the timepoint at which a significant difference was reached.

3.7 Results

3.7.1 Receptor Binding Affinity Studies

To confirm that PP 1420 retained high affinity binding to the Y4 receptor, a receptor binding affinity study was carried out. This relies on measuring the specific binding of radiolabelled ^{125}I-hPP to cell membrane preparations from HEK 293 cells over-expressing the Y4 receptor. The displacement of ^{125}I-hPP binding by unlabeled 'cold' PP analogue can therefore be used to estimate the affinity of the analogue for the Y4 receptor. The displacement curve of ^{125}I-hPP binding to the human Y4 receptor versus concentration of PP 1420 is shown in **Figure 16**. This showed that the IC_{50}, i.e. the concentration of PP 1420 that was able to reduce ^{125}I-hPP specific binding by 50% was 0.1 nM, confirming that PP 1420 is a high-affinity ligand for Y4 receptor with the same affinity as hPP (Bard et al., 1995).

The affinity of PP 1420 for mouse Y4 receptor (IC_{50} 0.045 nM) was similar to the affinity of PP 1420 for human Y4 receptor. This demonstrated that binding of PP 1420 to the Y4 receptor is not species-specific and supported the use of rodents for the pharmacodynamic study (**Figure 17**).

The most likely target for PP 1420 cross-reactivity is considered to be the Y2 receptor, which is normally bound by PYY_{3-36}. Therefore, the specificity of PP 1420 binding to the human Y2 receptor was investigated. The ability of PP 1420 to displace $^{125}I\text{-}PYY_{1-36}$ from the Y2 receptor was measured (**Figure 18**). The study demonstrated that PP 1420 had a lower affinity for human Y2 receptor by three orders of magnitude with an IC_{50} of 336 nM, compared to an IC_{50} of 0.33 nM for PYY_{3-36}.
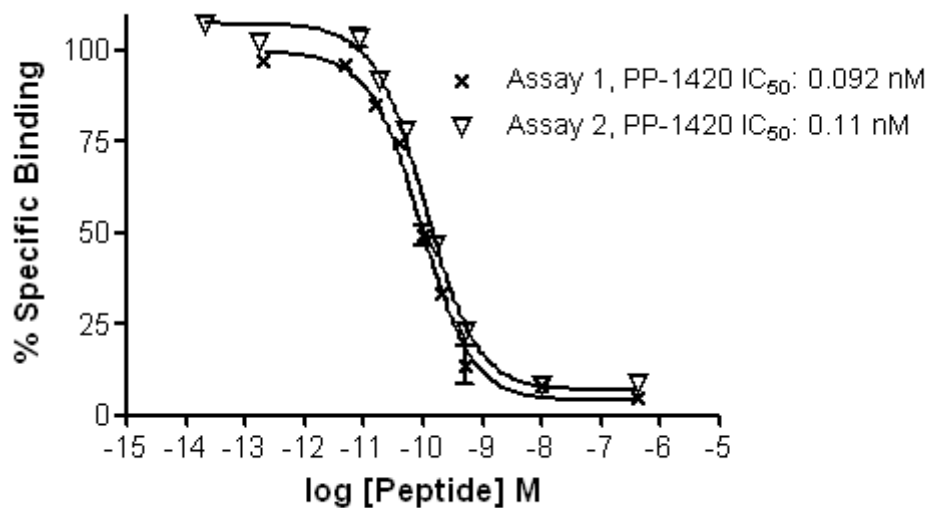
Figure 16: Displacement curve of ^{125}I-hPP binding to the human Y4 receptor as a function of added PP 1420 (Y axis: percentage specific binding; X axis: PP 1420 concentration in M, note logarithmic scale). Two repeat assays were performed. Mean IC_{50} 0.1 nM.
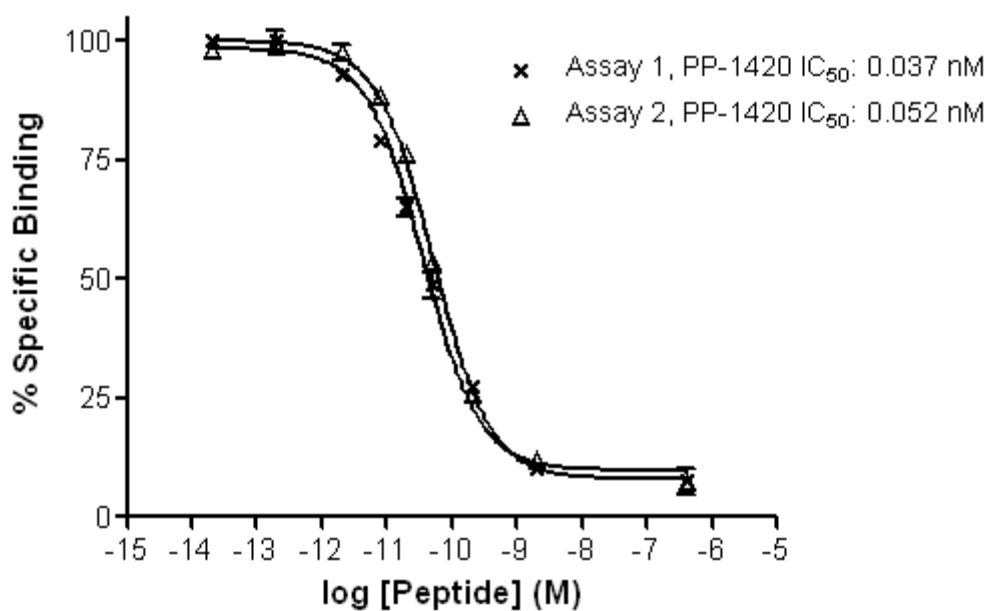
Figure 17: Displacement curve of ^{125}I-hPP binding to the mouse Y4 receptor as a function of added PP 1420 (Y axis: percentage specific binding; X axis: PP 1420 concentration in M, note logarithmic scale). Two repeat assays were performed, Assay 1 and Assay 2: mean IC_{50} = 0.045 nM.
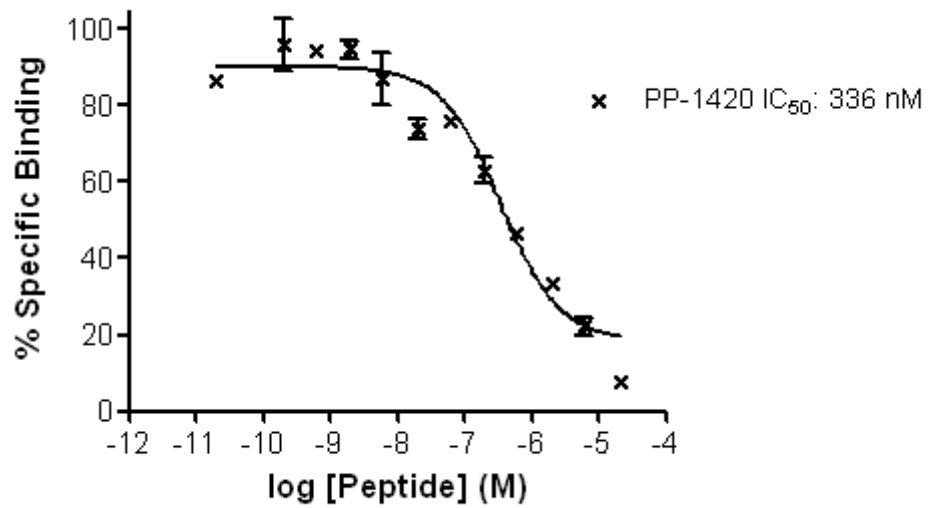
Figure 18: Displacement curve of ^{125}I-PYY$_{1-36}$ binding to the human Y2 receptor as a function of added PP 1420 (Y axis: percentage specific binding; X axis: PP 1420 concentration in M, note logarithmic scale). Mean IC$_{50}$ = 336 nM.

3.7.2 Chronic food intake study in diet-induced obese (DIO) mice

PP 1420 had, in the selection process, been shown to inhibit food intake over 24-48 hours. To confirm that the bioactivity of PP 1420 in suppressing food intake was long-lasting, with no evidence of an 'escape phenomenon' (i.e. loss of food intake inhibition with continued treatment) we measured its effect on reducing food intake and body weight in DIO mice when given chronically over 69 days (Asakawa et al., 1999). When given to these mice, I observed a trend of decreasing mean body weight over time with both the 282 μg/kg and the 855 μg/kg dose of PP 1420. **Figure 19** shows a summary of mean body weight from baseline expressed as a percentage of the control. Interestingly, the magnitude of weight loss appeared to be greater in the 282 μg/kg group than the 855 μg/kg group throughout most of the study, although the two groups had converged by the end of the study. There was high variability within the groups, and the differences in body weight compared with the control group were not statistically significant.

Greater weight loss was observed during the first week of the study. I speculate that this due to fluid loss and stress of the procedure encountered at the start of the study as in this particular study, the animals were not habituated to the study procedure before commencing. After the first 21 days continual weight loss compared with control was observed following dosing with both 282 μg/kg and 855 μg/kg PP 1420.
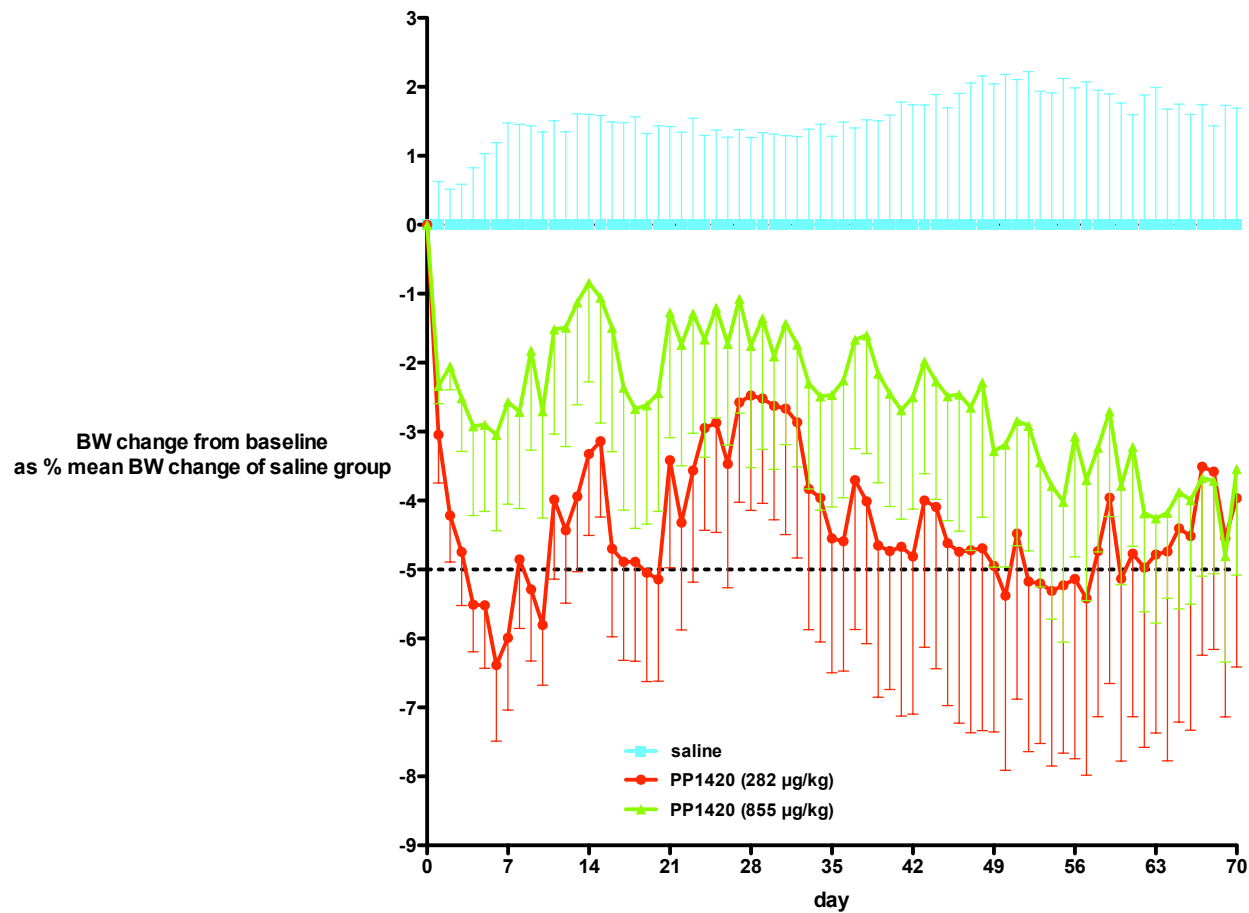
Figure 19: Mean daily body weight change from baseline during 69 days' treatment with PP 1420, expressed as a percentage of saline control dosing (Y-axis: means plotted, error bars show standard error of the mean; bw=body weight) versus day since commencement of injection (X-axis). Generalised estimating equation analysis showed no significant difference in body weight change between groups.

There was a trend to decrease in mean cumulative food intake with time for both the 282 μg/kg and the 855 μg/kg dose of PP 1420. The decrease in food intake was greater from Day 28 than at earlier time points, and dosing with the 855 μg/kg dose caused a greater reduction in food intake than dosing with the 282 μg/kg dose by the end of the study. However, no statistically significant differences compared with control were observed due to a large degree of intra-group variation. Mean cumulative food intake corrected for the control is shown in **Figure 20**.
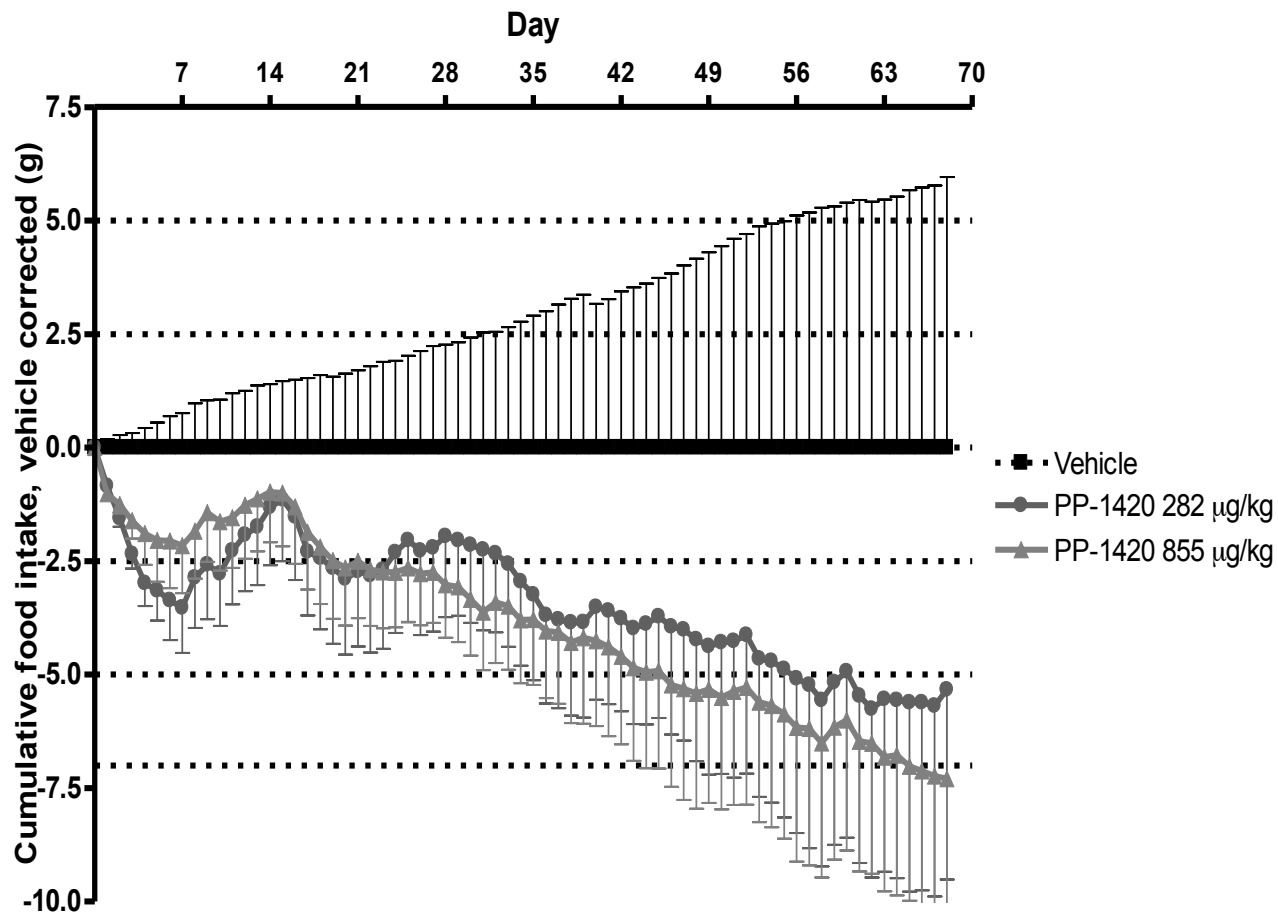
Figure 20: Mean cumulative food intake during 69 days' treatment with PP 1420, corrected for the control (Y-axis: means plotted, error bars show standard error of the mean) versus day since commencement of injection (X-axis). Generalised estimating equation analysis showed no significant difference in body weight change between groups.

3.7.3 Glucose Tolerance Studies

At the end of the food intake/body weight study in Section 3.7.2, I assessed the effect of chronic treatment with PP 1420 (vs vehicle) on glucose levels after an IP glucose tolerance test in mice. All mice, regardless of PP 1420 dose, showed the expected increase in blood glucose following the injection with glucose, with the level of glucose decreasing steadily over time. PP 1420, given at both doses, did not change blood glucose compared to control (**Figure 21**).
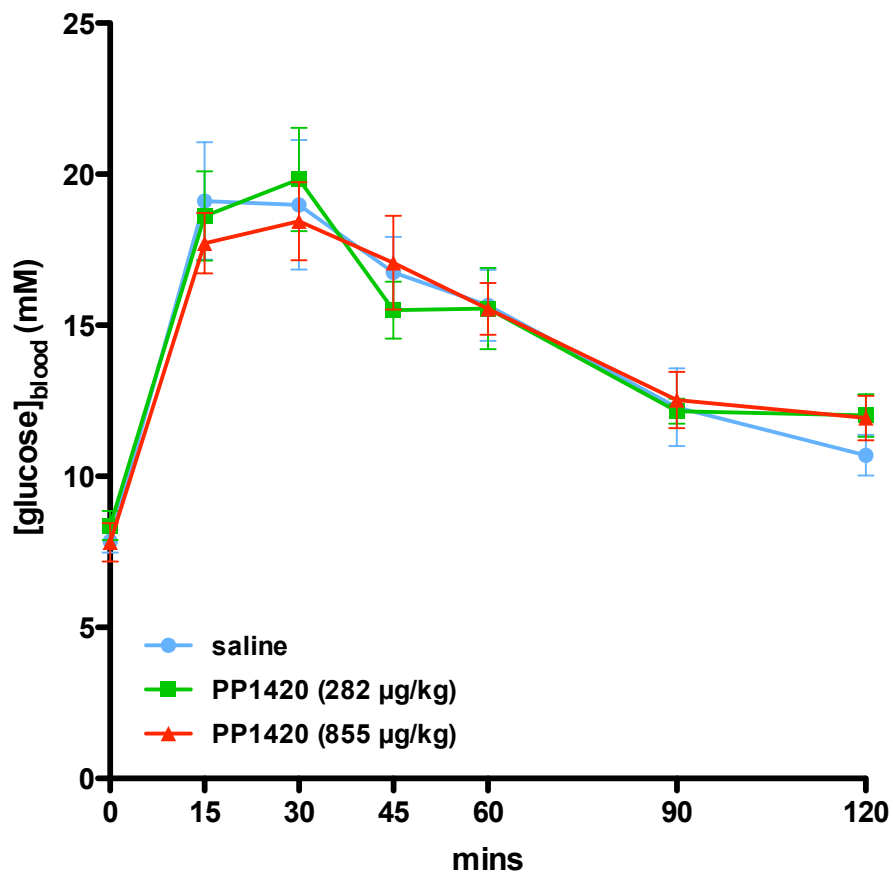
Figure 21: Mean blood glucose during the glucose tolerance test, following dosing with PP 1420 (282 µg/kg, 855 µg/kg) or saline control. Means plotted. Error bars show standard error of the mean. Two-way ANOVA analysis showed no significant difference in glucose levels between groups (p=0.9916).

3.7.4 Co-administration of PP 1420 with metformin in DIO male mice

3.7.4.1 *Effects of treatments on food intake*

Metformin treatment did not significantly reduce food intake compared to the saline control, although there appeared to be a non-significant trend towards a dose-dependent reduction. The mice given metformin alone appeared healthy, with no signs of illness or distress. Administration of PP 1420 significantly reduced food intake over the first 24 hours (**Figure 22**). This effect persisted over the entire 96 hours of the experiment, as assessed by the cumulative food intake over that time (**Figure 23**). Co-administration of PP 1420 and metformin also caused a sustained reduction in food intake.
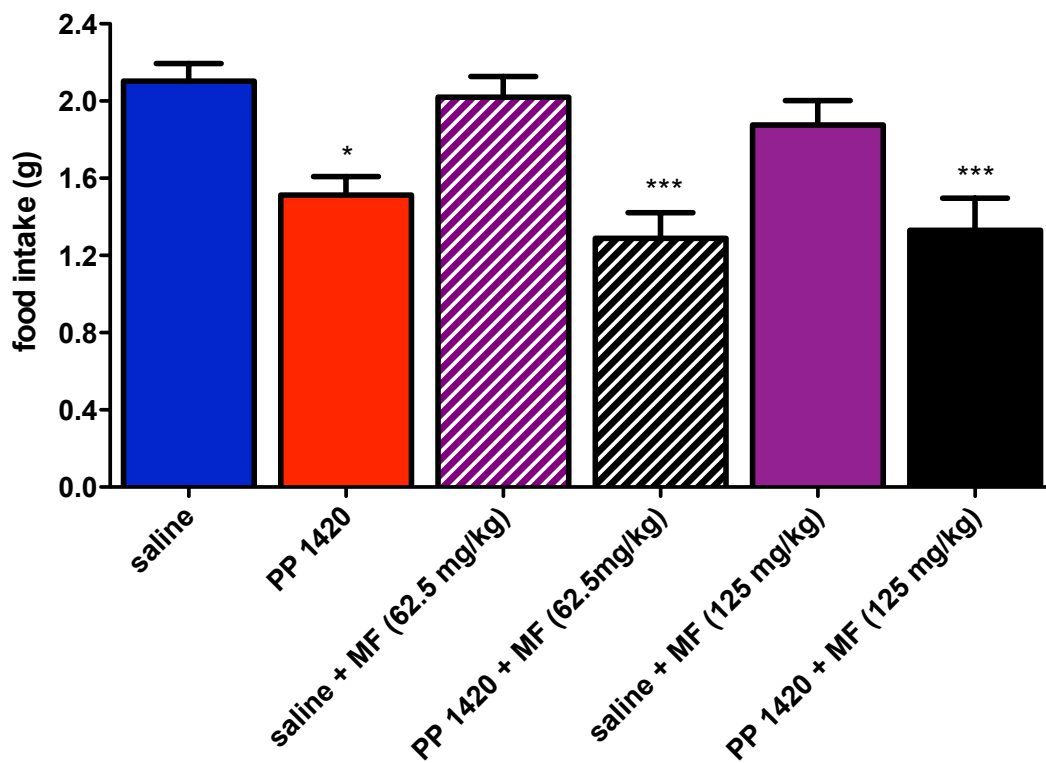
Figure 22: Effect of PP 1420, metformin or PP 1420 + metformin on food intake, 0-24 hours post treatment. MF = metformin. One way analysis of variance (ANOVA) with Tukey's multiple comparison used to assess significance between treatment groups. * = $p<0.05$ vs. saline group, *** = $p<0.001$ vs. saline group.
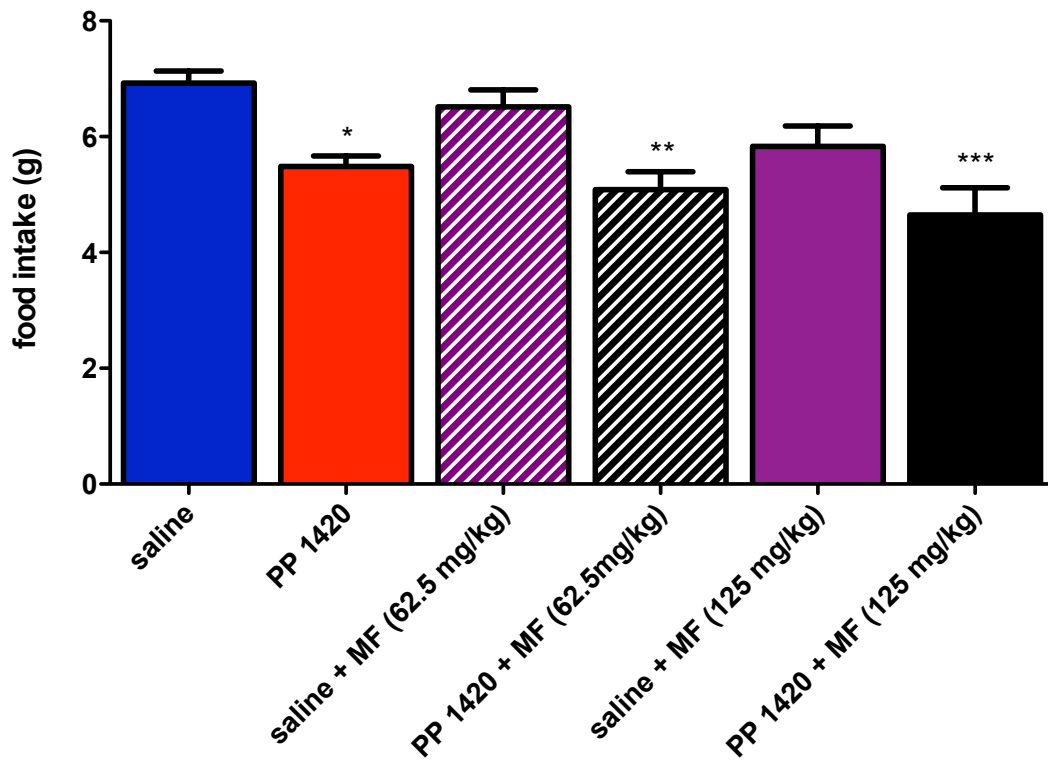
Figure 23: Effect of PP 1420, metformin or PP 1420 + metformin on food intake over 96 hours.
MF = metformin. One way ANOVA with Tukey's multiple comparison used to assess significance between treatment groups. * = p<0.05 vs. saline group, ** = p<0.01 vs. saline group, *** = p<0.001 vs. saline group.

3.7.4.2 Effects of treatments on body weight

Metformin treatment alone did not have significant effects on body weight. The metformin 125 mg/kg group showed a time-dependent reduction in body weight at 0.3% (95% C.I. -1.8 to 2.4%) on day 1, 1.6% (95% C.I. -0.5 to 3.7%) on day 2, 1.8% (95% C.I. -0.3 to 3.9%) on day 3, but this was not statistically significantly different from the saline control group (**Figure 24**). PP 1420 treatment (64 μg/kg) alone, as expected, caused an acute reduction in body weight (corrected for saline control group) at 2.5% (95% C.I. 0.5 to 4.5%) on day 1, 3.0% (95% C.I. 1.0 to 5.1%) on day 2, 2.8% (95% C.I. 0.7 to 4.8%) on day 3. Combination of metformin and PP 1420 resulted in a greater weight loss than administration of either agent alone. When metformin treatment at 62.5 mg/kg was added to PP 1420, there was a reduction in body weights of 3.0% (95% C.I. 0.9 to 5.1%) on day 1, 3.9% (95% C.I. 1.8 to 6.0%) on day 2, 3.6% (95% C.I. 1.5 to 5.7%) on day 3, but this was not statistically significantly different from PP 1420 alone (**Figure 24**).

When PP 1420 and metformin (125 mg/kg) were given together, there was a reduction in body weight of 2.6% (95% C.I. 0.6 to 4.7%) on day 1, 4.0% (95% C.I. 1.9 to 6.1%) on day 2, 4.6% (95% C.I. 2.5 to 6.7%) on day 3. This reduction in body weight was not found to be significantly different from PP 1420 alone but was significantly different from metformin 125 mg/kg alone ($p<0.05$ at day 1, $p<0.01$ at days 2 and 3 for comparison of saline + metformin 125 mg/kg versus PP 1420 + metformin 125 mg/kg).
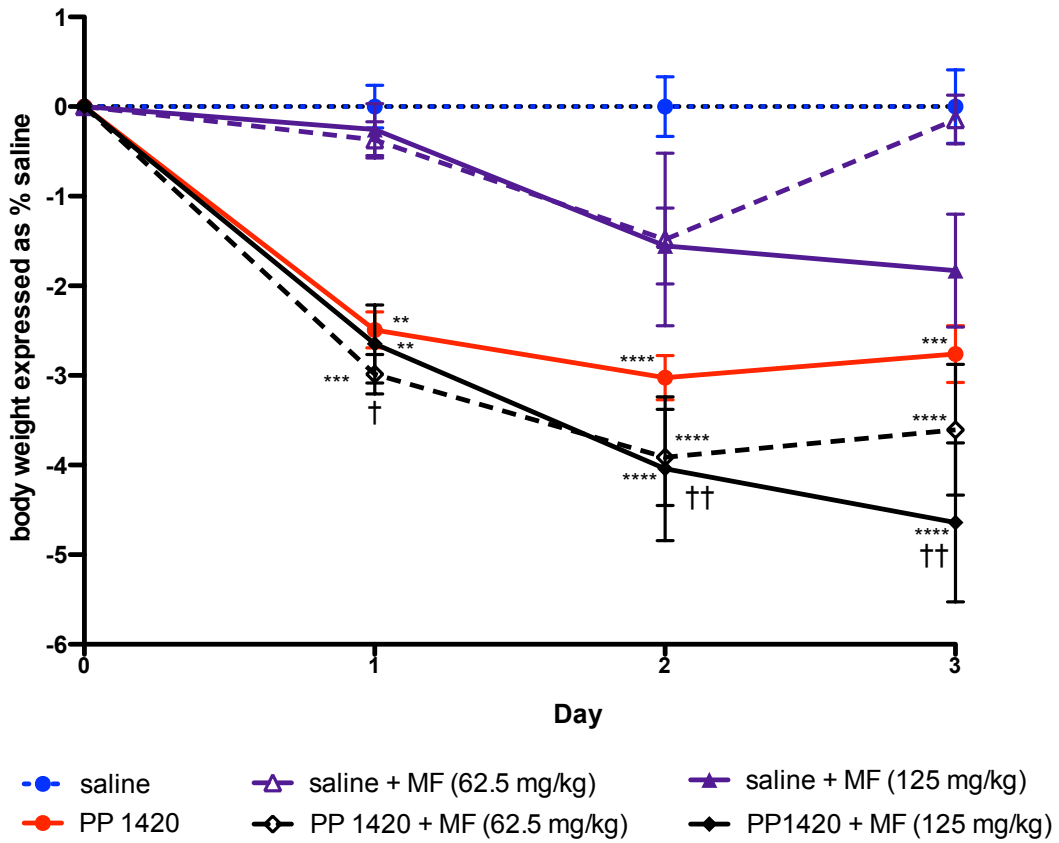
Figure 24: Effect of PP 1420, metformin or PP 1420 + metformin on body weight, corrected for the weight changes in the saline group. MF = metformin. Means ± SEM plotted. Two-way ANOVA analysis: Bonferroni post-hoc test applied. *** = $p<0.001$, **** = $p<0.0001$ for comparison to saline group. † = $p<0.05$, †† = $p<0.01$ for comparison to saline + metformin (125 mg/kg) group.

3.7.4.3 Effects of treatments on fasting glucose

There were no significant effects seen on fasting glucose at the end of the experiment with any treatment group (**Figure 25**).
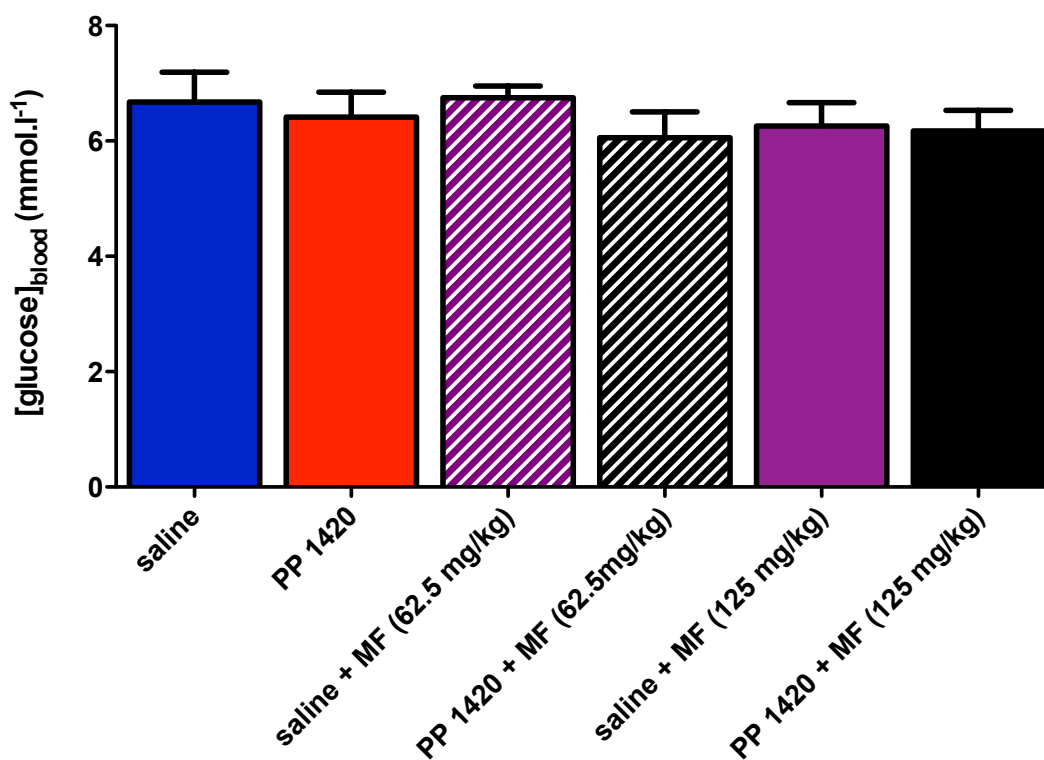
Figure 25: Effect of PP 1420, metformin or PP 1420 + metformin on fasting glucose. Means ± SEM plotted. One way ANOVA analysis: no significant differences between groups detected (p=0.7979).

3.8 Discussion

In this study I have shown that PP 1420 is able to bind to the Y4 receptor in preference to the Y2 receptor, similar to hPP. Similar to PP, a trend to suppression of food intake was observed. This suppression of food intake appears to be sustained during treatment over 69 days, and is accompanied by a trend to reduction in body weight. However, no significant change in glucose tolerance was observed in the PP 1420 treated mice compared to control mice.

I then went on to test the effects of PP 1420 in combination with metformin. In this study, PP 1420 alone significantly reduced food intake and body weight in a dose-dependent manner. At the doses tested, metformin alone did not have any significant effect on food intake and body weight, although there appeared to be a dose-dependent trend towards reduction in both parameters. When given together, metformin and PP 1420 appear to have additive effects on reduction in body weight such that the combination of metformin at 125 mg/kg with PP 1420 caused a greater degree of body weight reduction compared to metformin 125 mg/kg alone. There were no significant acute effects of the treatments, either PP 1420 alone, metformin alone, or both, on fasting glucose.

As mentioned in Section 1.3.6.4, the acute effects of PP treatment on glucose tolerance have been studied in the special context of the increased insulin resistance seen after pancreatectomy, where PP treatment has been shown to restore insulin sensitivity. The studies presented here have aimed to examine the effects of PP 1420 in a context more reflective of its likely application as a therapy for obesity and

diabetes. The data presented here suggest that there is no effect of PP 1420 on insulin sensitivity when this is given to DIO mice over 69 days, as fasting glucose levels and glucose tolerance are not improved. However, the study of Asakawa and colleagues did suggest that insulin resistance, as judged by fasting glucose levels, is improved in fatty liver Shionogi-*ob*/*ob* mice after 14 days' treatment with PP at 3 nmol/mouse every 12 hours (Asakawa et al., 2003). It may be, therefore, that the metabolic impacts of PP and PP 1420 are dependent on the particular animal model used, and this possibility remains to be explored. Another possibility is that a more sensitive technique to study insulin resistance (such as an euglycaemic clamp study) may be necessary to detect the changes induced by PP 1420, and this also remains to be explored.

These studies therefore suggest that the combination of metformin and PP 1420 may be useful in the treatment of obesity, as an additive effect on food intake and body weight was seen. This possibility should therefore be tested in a Phase 2 clinical study, once PP 1420 alone has been shown to be efficacious in reducing weight in human volunteers. However, the utility of the metformin and PP 1420 combination for the treatment of diabetes remains to be proven.

4 A PHASE 1 TRIAL OF PP 1420 IN HEALTHY HUMAN VOLUNTEERS

4.1 Introduction

PP 1420 was shown to be effective in reducing appetite and body weight in pre-clinical models of obesity (Chapter 3), and other pre-clinical studies showed that the drug was safe and well-tolerated. These data supported the continued development of PP 1420 in a first-time-in-human Phase 1 trial. As with other Phase 1 trials, the primary objective of this study was to study PP 1420's safety, tolerability and pharmacokinetics (PK) in healthy human volunteers, in order to establish a suitable dose range and frequency for future clinical trials.

4.2 Hypothesis and Aims

4.2.1 Specific Hypotheses

I hypothesise that:

- PP 1420 is well tolerated in healthy volunteers.

- PP 1420 exhibits extended PK and a longer terminal elimination half-life compared to endogenous hPP.

4.2.2 Specific Aims

The aims of this Phase 1 study were to confirm the safety and tolerability of single ascending subcutaneous doses of PP 1420 in healthy subjects, and to investigate the drug's PK and dose proportionality.

4.3 Methods

4.3.1 Design of study

This was a single centre study performed at the Sir John McMichael Clinical Investigation Unit, Hammersmith Hospital. It was a randomized, placebo-controlled, double-blind study in healthy male subjects.

4.3.2 Subjects

It was planned that 12 subjects would be recruited during each dosing period. Two reserve subjects were also recruited during each dosing period in case of drop-out of subjects. Inclusion criteria were healthy adult males aged between 18 and 50 years with body mass index 18 to 35 kg/m^2 (inclusive) and body weight $\geq$70 kg. Exclusion criteria included a positive pre-study drug/alcohol screen; positive Hepatitis B surface antigen or positive Hepatitis C antibody result within three months of screening; a positive test for human immunodeficiency virus (HIV) antibody; history of migraine; history of or evidence of abnormal eating behaviour, as observed through the Dutch Eating Behaviour (van Strien et al., 1986) and SCOFF questionnaires (Morgan et al., 1999); history of excessive alcohol consumption within six months of the study;

urinary cotinine levels indicative of smoking or history or regular use of tobacco- or nicotine-containing products within 6 months prior to screening; an electrocardiographic corrected QT interval at screening >450 ms; systolic blood pressure outside the range 85–160 mmHg; diastolic blood pressure outside the range 45–100 mmHg, and/or heart rate outside the range 40–110 bpm. The use of any medicine that the investigator considered could interfere with the trial results was not allowed.

All subjects gave their written consent prior to any trial-related procedures. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The study protocol and informed consent information were approved by the Outer West London Research Ethics Committee (reference number 10/H0709/10). Clinical Trial Authorization was obtained from the Medicines and Healthcare products Regulatory Agency, UK (EudraCT number 2009-017522-39). The trial was registered with www.clinicaltrials.gov, number NCT01052493.

4.3.3 PP 1420 administration and sample collection

PP 1420 active pharmaceutical ingredient was synthesized to current International Committee on Harmonization Q7 Good Manufacturing Practice standards by PolyPeptide Laboratories (Hillerød, Denmark), and the Clinical Trial Material (CTM) was manufactured by Nextpharma (Braine l'Alleud, Belgium). The starting dose, exposure escalation strategy and stopping exposures in this study were based on the following specific considerations. A reduction of body weight and food intake for PP1420 given daily for 69 days was seen at 0.282 mg/kg/day when compared to

saline treated controls (Section 3.7.2). For dose conversions from mouse to man, an allometric scaling factor of 12.3 is appropriate (i.e. mouse metabolism and circulation time produces a 12.3 fold faster clearance of peptides of PP's size – FDA Guidance for Industry, p.7: http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf). We considered the fact that human PP receptor (Y4) binding is about two fold lower than that of the mouse (Section 3.7.1). Thus the effective scaling from mouse to man is 6.15 (12.3 ÷ 2). From the above considerations, a 4 mg dose for a 90 kg volunteer was considered appropriate (0.282 mg/kg × 90 kg ÷ 6.15 = 4.12 mg). Based on these findings in both animals and human studies, we anticipated seeing an effect at doses of around 4 mg, with the expectation that 8 mg was likely to represent the maximum tolerated dose.

Coded syringes containing PP 1420 or placebo were prepared by Imperial College Healthcare NHS Trust Pharmacy Department. The doses of PP 1420 were 2, 4 or 8 mg given as subcutaneous injection into the anterior abdominal wall (equivalent to 468, 936, 1872 nmol, respectively, of PP 1420). Placebo was 0.9% saline. Note that mass units (mg) are conventionally used for clinical trials.

Subjects were admitted to the clinical research unit the evening prior to dosing (Day -1). They received their first single dose of study medication the next morning (Day 1) and were discharged from the unit on Day 2 after all 24-hour assessments had been completed, and following satisfactory review by the investigators. Subjects were required to fast from 2200h on Day -1 until 2 hours after study drug administration. Blood samples were obtained for PK at the following times: pre-dose, 0, 15, 30, 45,

60, 90, 120, 150, 180, 210, 240, 360, 720 minutes and 24 hours. Heart rate, blood pressure, temperature, 12-lead ECG, physical examination and blood and urine sampling for standard laboratory assessments were performed during all three dosing periods. Subjects returned for a follow-up visit 7 to 10 days after each dosing period. The duration of each subject's participation in the study from screening to the follow-up visit was approximately 7–12 weeks.

A sentinel dosing strategy, as recommended by the Duff Report (Expert Group on Phase One Trials: Final Report – http://webarchive.nationalarchives.gov.uk/ +/dh.gov.uk/en/publicationsandstatistics/publications/publicationspolicyandguidance/ dh_063117) was used at the start of each dose level, so that any early adverse events could be assessed before all subjects received the PP 1420. There were three dosing periods with each subject randomized to receive one dose of placebo and two ascending doses of PP 1420. On the first day of each dosing period, two subjects were dosed, one receiving active PP 1420 and the other receiving placebo. The remaining subjects were dosed as soon as possible thereafter, depending on scheduling in the clinical unit.

4.3.4 Dose Adjustment and Stopping Criteria

The Investigators and Independent Data Monitoring Committee (IDMC) separately reviewed safety and tolerability data from all subjects, and preliminary PK data from at least four subjects, prior to dose escalation. The Investigators assessed adverse events (AEs) in the blinded state. Standard PK parameters were derived for all available samples and the PK profile was analysed for data up to and including the

24-hour post-dose time point. Whilst it was expected that a linear relationship would exist between dose and drug exposure, if it had become apparent that this relationship was non-linear, the dose escalation would have been modified according to the advice of the study pharmacokineticist. The doses could also have been adjusted on the basis of safety and tolerability data from previous doses. The maximum dose that was given in this study was 8 mg.

Dose escalation in this study would have been stopped if three or more subjects experienced dose limiting, drug-related AEs or if the pattern of AEs observed in a group had been consistent across subjects, poorly tolerable and clinically significant. Dose escalation in this study would also have been stopped if three or more subjects developed significantly high titres of antibodies to human sequence PP or PP 1420 analogue.

4.3.5 Laboratory assessments

Concentrations of PP 1420 were determined in plasma samples using a validated LC-MS/MS analytical methodology by Quotient Bioresearch (Fordham, UK). The following is an extract from Project Report QBR105510/1 that summarises the method.

4.3.5.1 Test Compounds

Compounds were accurately weighed and corrected for purity, water content and salt as necessary.

	Reference Standard 1	Internal Standard 1
Company code/name	PP1420	SIL PP1420
Supplier	PolyPeptide Laboratories	Cambridge Research Biochemicals
Lot / Batch Number	RD570	27700
Storage Conditions	-20°C	-20°C
Retest/Expiry Date	January 2011	N/A
Molecular Formula	$C_{191}H_{300}N_{56}O_{56}$	$C_{173}H_{300}N_{53}O_{56}\,{}^{13}C_{18}\,{}^{15}N_3$
Molecular Weight	4274.7	4294.4
Net peptide content	86%	75%
1 mg compound weighed equals	0.86 mg	0.75 mg

Reference material storage conditions: stored at a nominal temperature -20°C under which conditions they were stable.

4.3.5.2 Biological Matrix

Control blank human plasma containing K_3-EDTA as an anti-coagulant, was obtained from accredited CTLS, London, UK and was stored at a nominal temperature of -20°C when not in use. Blank human plasma was centrifuged for approximately 10 minutes at 2200 x g / 3000 rpm prior to use.

4.3.5.3 Reagents

The following chemicals were used during the course of the Project:

- Acetonitrile (HPLC grade)

- Bovine Serum Albumin (BSA) (reagent grade)

- Formic Acid (analytical grade)

- Methanol (HPLC grade)

Water was obtained from an in house Triple Red Duo water purification system.

Reagent	Function	Preparation	Shelf life/ Storage
methanol:water:formic acid:BSA (20:80:0.1:0.1 v/v/v/w)	Standard solution solvent	1 g of BSA was dissolved in 200 mL of methanol, 800 mL of water and 1 mL formic acid	3 months/ +4ºC
Acetonitrile (AcN):water:0.1% formic acid (75:25:0.1 v/v/v)	Protein precipitation solvent	40 mL of reagent grade water and 120 mL of acetonitrile and 160 μL of formic acid were mixed	1 month
0.2% formic acid in AcN	Mobile phase	2 mL formic acid added to 1000 mL of acetonitrile	1 month
0.2% formic acid (aq)	Mobile phase & reconstitution solvent	2 mL formic acid added to 1000 mL of reagent grade water	3 days
AcN	Strong wash solvent	1000 mL of acetonitrile	1 month
Acetonitrile:water (10:90 v/v)	Weak wash solvent	100 mL acetonitrile: 900 mL UPLC grade water was mixed	1 month

Unless specified, all chemicals and solvents were at least AR and HPLC grade, respectively. Unless specified, all reagents were stored at room temperature (nominally +22°C).

4.3.5.4   Preparation of Solutions

The volumes used in the preparation of solutions were scaled up or down as required.

4.3.5.4.1 Preparation of Analyte Stock Solutions

Initially two sets of stock solutions of PP1420 were prepared from independent weighings. An appropriate amount of PP1420 was dissolved in the required volume of methanol:water:formic acid:BSA (20:80:0.1:0.1 v/v/v/w) to give a 1 mg/mL stock solution (Stock A). A second analyst repeated the procedure (Stock B). The mass

spectrometer responses of each stock solution were compared to check reproducibility of preparation. If the acceptance criterion was not met further stock solutions can be prepared by other additional individuals. Stock solutions of PP1420 were stored at +4ºC for up to 5 months.

4.3.5.4.2 Preparation of Analyte Standard Spiking Solutions (SSS)

Solutions were prepared in methanol:water:formic acid:BSA (20:80:0.1:0.1 v/v/v/w) and nominally stored at +4°C for up to 5 months.

Standard Solution	Target concentration (ng/mL)	Volume of spiking solution	Volume added (mL)	Final Volume (mL)
Int 1	50000	500 µL of Stock A	9.50	10
SSS 8	5000	1000 µL of Int 1	9.00	10
SSS 7	4500	900 µL of Int 1	9.10	10
SSS 6	2000	400 µL of Int 1	9.60	10
SSS 5	500	100 µL of Int 1	9.90	10
SSS 4	200	1000 µL of SSS 6	9.00	10
SSS 3	50	250 µL of SSS 6	9.75	10
SSS 2	20	100 µL of SSS 6	9.90	10
SSS 1	10	50 µL of SSS 6	9.95	10
SSS HIGH	4000	800 µL of Int 1	9.2	10
SSS MED	250	50 µL of Int 1	9.95	10
SSS LOW	30	150 µL of SSS 6	9.85	10
SSS LLOQ1	10	50 µL of SSS 6	9.95	10

4.3.5.4.3 Preparation of Internal Standard Stock Solution

An appropriate amount of SIL PP1420 was weighed out and dissolved in the required

volume of methanol:water:formic acid:BSA (20:80:0.1:0.1 v/v/v/w) to give a 100

μg/mL or 1000 μg/mL stock solution.

4.3.5.4.4 Preparation of Internal Standard Working Solution

Standard Solution	Target Concentration (ng/mL)	Volume of Spiking solution	Volume added (mL)	Final Volume (mL)
Intermediate solution	10000	1 mL of IS stock (100 μg/mL) or 100 μL of IS stock (1000 μg/mL)	9.00 (100 μg/mL) or or 9.90 (1000 μg/mL)	10.0 or 10.0
ISWS	0.500	10 μL of IS intermediate solution	199.9 mL of precipitation solution	200

4.3.5.4.5 Preparation of System Suitability Test (SST) Solution

An extracted Standard 1 was analysed to demonstrate system suitability.

4.3.5.5 Preparation of Calibration Standards

Calibration standards were prepared by adding analyte standard solution to aliquots of

blank human plasma as indicated in the following table. Replicate 0.5 mL aliquots

were transferred to appropriate assay tubes for storage at a nominal temperature of -

20°C for up to 1 month.

Calibration Standard Concentration (ng/mL)	Spiking Solution	Concentration of Spiking Solution (ng/mL)	Volume of Spiking Solution (μL)	Volume of Blank Matrix (μL)	Final Volume (mL)
250	SSS 8	5000	25	475	0.5
225	SSS 7	4500	25	475	0.5

100	SSS 6	2000	25	475	0.5
25	SSS 5	500	25	475	0.5
10	SSS 4	200	25	475	0.5
2.5	SSS 3	50	25	475	0.5
1	SSS 2	20	25	475	0.5
0.5	SSS 1	10	25	475	0.5

4.3.5.6 Preparation of quality control samples

QC samples were prepared in bulk by the addition of QC standard solution to blank human plasma as indicated in the table below. Replicate 1.5 mL aliquots were transferred to appropriate assay tubes for storage at a nominal temperature of -20°C for up to 1 month.

QC Concentration (ng/mL)	Spiking Solution	Concentration of Spiking Solution (ng/mL)	Volume of Spiking Solution (µL)	Volume of Blank Matrix (µL)	Final Volume (mL)
200	SSS HIGH	4000	75	1425	1.5
12.5	SSS MED	250	75	1425	1.5
1.5	SSS LOW	30	75	1425	1.5
0.5	SSS LLOQ2	10	75	1425	1.5

The volumes used in the preparation of QC samples were scaled up or down, as required.

4.3.5.7 Experimental

Typical analytical method parameters are reproduced below, but may have been varied from instrument to instrument in order to achieve an equivalent response, with a record of any changes to chromatographic and mass spectrometer conditions.

4.3.5.7.1 Extraction Procedure

Step	Process
1	Aliquot 200 μL of sample, standard or QC into vial/ tube / plate
2	Add 800 μL protein precipitation solution to double blank
3	Add 800 μL freshly prepared IS working solution to all non double-blank samples as appropriate
4	Cap and vortex mix for 1 minute at 1500 rpm
5	Centrifuge for 10 minutes at approximately 3500 rpm (2400 g)
6	Transfer the supernatant to a Lo-bind plate
7	Dry overnight in the genevac with no heating
8	Reconstitute sample into 100 μL of 0.2% formic acid (aq)
9	Cap plate and centrifuge for 10 minutes at approximately 3500 g
10	Inject onto LC-MS/MS system for analysis

4.3.5.7.2 HPLC Conditions

Autosampler	Acquity BSM
Strong wash	Acetonitrile
Weak wash	10% Acetonitrile in UP water
Typical Injection Volume	20
LC system	Acquity BSM
Flow rate	0.7 mL/min
Analytical Column	100 x 2.1mm i.d. Acquity C18 1.7 μm
Column temperature	Nominally +60°C
Run Time	4 minutes
Mobile phase A	Acetonitrile containing 0.2% (v/v) formic acid
Mobile phase B	Water containing 0.2% (v/v) formic acid
Divert Valve	VICI

4.3.5.7.3 Gradient Profile

Time (mins)	%A	%B	Profile	Divert valve
Initial	2	98	6	Waste
0.10	21	79	6	
0.50	21	79	6	
1.30	22.5	77.5	6	MS
1.40	100	0	6	
2.90	100	0	6	
3.00	2	98	6	
3.10	2	98	6	
3.20	100	0	6	Waste
3.30	100	0	6	
3.40	2	98	6	
4.00	2	98	6	

10.0	90	10	10	

4.3.5.8 Typical MS/MS Conditions

Mass Spectrometer	Applied Biosystems API 5000
Ionisation/Interface	TurboIonSpray™
Source Temperature	+550ºC
GS1	50 psi
GS2	50 psi
Curtain gas setting	30 psi
Collision gas setting	12 PSI
Ionspray Voltage	5,500 V

Analyte	Precursor ion (m/z)	Product ion (m/z)	Dwell time (msec)	Polarity	Typical R.T (mins)
PP1420	714	762	75	Positive	1.2
SIL PP1420	717	766	75	Positive	1.2

Masses for Precursor/Product ions are nominal.

4.3.5.9 Regression Model

The model used peak area ratios with $1/x^2$ weighted linear regression.

4.3.5.10 Additional information

- All solutions were made and stored in plastic containers to minimise protein binding.

- Internal standard working solutions were prepared freshly each day.

4.3.6 Pharmacokinetic analysis

PK calculations were made in collaboration with a qualified clinical trial pharmacokineticist (Dr Charlie Brindley, KinetAssist, Quothquan, Scotland) to statutory standards. The PK endpoints, as listed below, were estimated for each subject using a fully validated version of WinNonlin Pro (Version 5.2.1 – Pharsight Products, Mountain View, CA, USA). The following parameters were derived, where appropriate, from the individual plasma concentration versus time profiles after a single dose: C_{max}, t_{max}, AUC_{0-t} (the area under the concentration versus time curve from time zero to the last sampling time, calculated by the linear trapezoidal rule), λ_z (the apparent terminal rate constant), $t_{1/2}$ (calculated from $\log_e 2 / \lambda_z$), $AUC_{0-\infty}$ (the area under the concentration-time curve estimated from time zero to infinity). Actual sampling times were used for the PK analysis. Plasma concentrations below the limit of quantification of the assay (BLQ) were taken as zero for calculation of concentration summary statistics and all PK parameters.

A non-linear power model was used to assess dose-proportionality (Gough et al., 1995). The proportional relationship between each parameter and dose is written as a power function:

$$y = a \times dose^b \text{ (Equation 1)}$$

where a is a constant, b is the proportionality constant and 'y' is the parameter of interest ($AUC_{0-\infty}$ or C_{max}). The exponent, b, was estimated by performing a linear regression of the logged parameter on log dose. The exponent, b, is the estimated slope of the resulting regression line since taking logs of Equation (1) gives the linear relationship, $\log y = \log a + b \times \log dose$. The relationship is dose-proportional when

b = 1. The exponents and 95% confidence intervals (CIs), b_{lower} (b_l) and b_{upper} (b_u), were estimated. There would have been evidence of non dose-proportionality if this CI excluded one. The estimate of the fold increase in exposure for a doubling in dose (with 95% CI) was also calculated. The increase in exposure expected for a doubling in dose was calculated as 2^b (95% CI: 2^{b_l}, 2^{b_u}).

The assumption of a linear relationship between $\log_e$ transformed parameter and $\log_e$ dose was tested by fitting an analysis of variance (ANOVA) model and portioning the sum of squares for number of treatments (number of treatments -1 degree of freedom [df]) into those for linearity (1df) and departures from linearity or lack of fit (number of treatments -2df). The p-values from this test would indicate significant lack of fit of the power model if <0.05, and would only be reported if there was evidence of lack of fit.

Since each subject received two of the three dose levels, the assessment of dose proportionality required the covariate "subject" to be included as a random effect in the power model. Subject was included as a random effect using the Linear Mixed Effects Wizard in WinNonlin Pro Version 5.2.1.

4.3.7 Safety endpoints

Safety endpoints were AEs, laboratory parameters (haematology, biochemistry, urinalysis), physical examination, vital signs and ECG.

4.3.8 Exploratory endpoints

Exploratory efficacy endpoints were also collected. Subjects were required to eat standard meals and drinks whilst resident in the unit. The food was weighed before and after consumption to calculate food intake. These standardized meals were presented at 1 pm on Day 1, 5 pm on Day 1 and 8 am on Day 2.

Data analysis was carried out in collaboration with the Imperial Clinical Trials Unit (Prof Deborah Ashby and Mr Juan Gonzalez-Maffe). Food intake was described through mean, 95% confidence intervals (95% CIs), difference of means (between PP1420 dose and placebo) and 95% CIs of the difference of means. Data aggregation was done according to dosing level of PP1420, and it was compared with the corresponding values of the same subjects when taking placebo. Energy intake at the first meal after dosing was compared against placebo. In addition, cumulative energy intake at subsequent meals after dosing was compared with the cumulative intake after placebo.

Subjective hunger and nausea was measured using 100 mm VAS (Flint et al., 2000). Descriptive statistics of mean change from baseline, standard error of the mean (SEM), difference of means (between PP1420 dose and placebo) and SEM of the difference of means were calculated. Data aggregation was done according to dosing level of PP1420 and compared with the corresponding values of the same subjects when taking placebo. This descriptive analysis did not show patterns of difference between placebo and PP1420 doses for the VAS considered.

4.4 Results

4.4.1 Study groups

A total of 33 male subjects were screened for the study of which 13 were recruited. These 13 subjects were randomized and exposed to PP 1420. One subject was replaced after the first dosing period for reasons unconnected to the study. This subject's evaluable data was included in the safety and PK analyses. The baseline demographic characteristics of the study group were as follows (mean ± standard deviation): BMI 28.1±3.3 kg/m^2, weight 87.8±9.2 kg, height 1.80±0.1 m, age 34.0±8.9 yr.

4.4.2 PP 1420 pharmacokinetics

The mean concentration-time profiles of PP 1420 are presented in **Figure 26**. The pharmacokinetic variables calculated from the concentration-time profiles are presented in **Table 2**. Following single subcutaneous doses of PP 1420 at 2, 4 and 8 mg to male subjects, the median t_{max} was at approximately 1 h post-dose (range of individual values: 0.32 to 2.00 h). Thereafter, plasma concentrations of PP 1420 declined with geometric mean apparent $t_{1/2}$ ranging from 2.42 to 2.61 h (range of individual values: 1.64 to 3.95 h) across all dose levels.
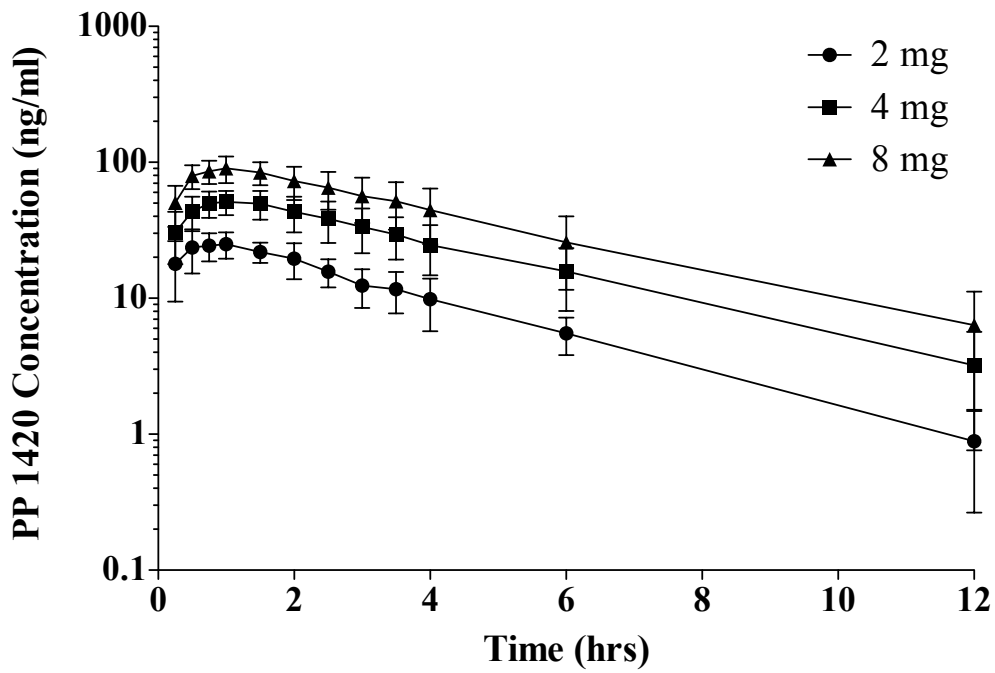
Figure 26: Mean plasma concentrations of PP 1420 following single, subcutaneous, injections of PP 1420 at 2 mg (filled circles), 4 mg (filled squares) and 8 mg (filled triangles). Plotted on a log scale (y axis). Error bars indicate standard deviation.

Dose (mg)	Parameter	C_{max} (ng/mL)	t_{max} (h)	AUC_{0-t} (ng·h/mL)	$AUC_{0-\infty}$ (ng·h/mL)	$t_{1/2}$ (h)
2	Geometric mean	26.3	0.875*	93.6	101	2.42
	Range	15.9–39.1	0.32–2.00	49.7–126	67.6–131	1.64–3.38
	%CV	28.6	NC	31.3	22.7	22.0
4	Geometric mean	55.1	1.00*	229	241	2.49
	Range	37.5–74.2	0.75–1.50	107–316	109-356	1.69–3.60
	%CV	21.5	NC	35.6	37.9	29.0
8	Geometric mean	95.7	1.00*	403	418	2.61
	Range	74.2–126	0.50–1.50	205–589	210–600	2.13–3.95
	%CV	15.1	NC	37.5	38.5	20.8

Table 2: Pharmacokinetic parameters following single SC doses of PP 1420 to healthy male subjects. Geometric mean, range and percentage coefficient of variation (%CV) are presented, with the exception of t_{max} which is presented as median (*). NC = not calculated.

Following single dosing of 2, 4 and 8 mg PP 1420, systemic exposure (C_{max} and $AUC_{0-\infty}$) to PP 1420 increased with increasing doses and the increase was dose proportional. The relationship of $AUC_{0-\infty}$ to increasing doses is shown in **Figure 27**, and for C_{max} is shown in Figure 3. For a doubling in dose, systemic exposure to PP 1420 was predicted to increase, on average, 1.91 to 1.93-fold.

Following single dosing of 2, 4 and 8 mg PP 1420, between-subject variability in systemic exposure ($AUC_{0-\infty}$ and C_{max}) to PP 1420 was low; CVs were 15.1–38.5%.
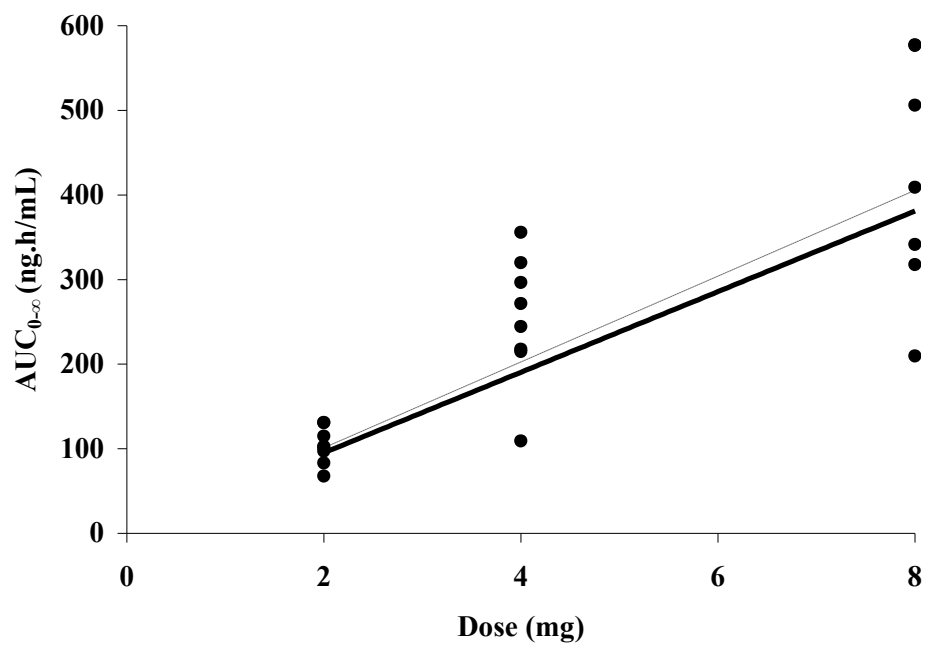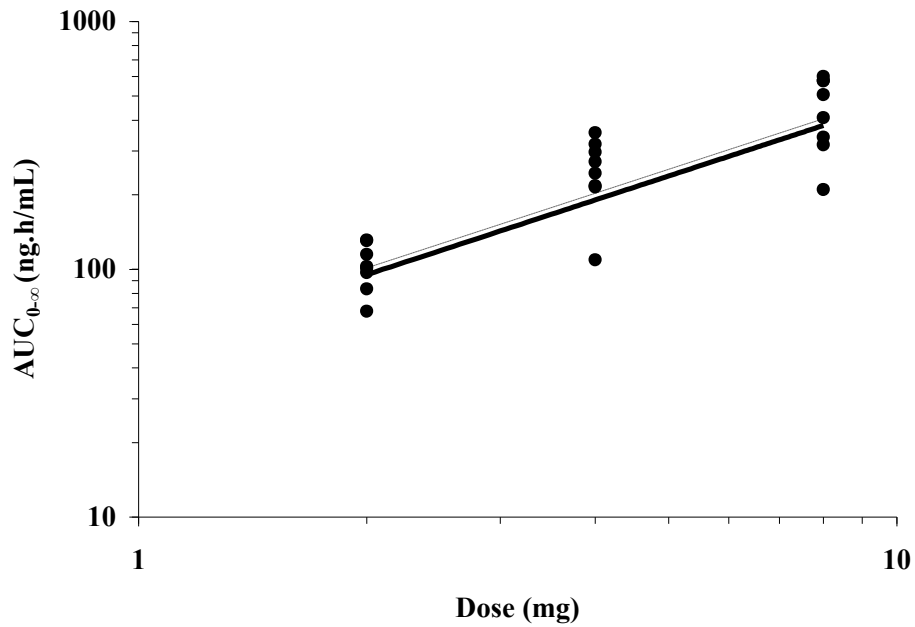
Figure 27: Relationship between $AUC_{0-\infty}$ and dose of PP 1420. The individual data points from each volunteer are plotted as dots. Top figure is plotted as $\log_{10} AUC_{0-\infty}$ vs $\log_{10}$ dose. Bottom figure is plotted as $AUC_{0-\infty}$ vs dose. The dotted line represents the dose-proportional relationship passing through the parameter at the 2 mg dose level. The solid line represents the power function obtained from the linear regression from the log parameter against log dose.
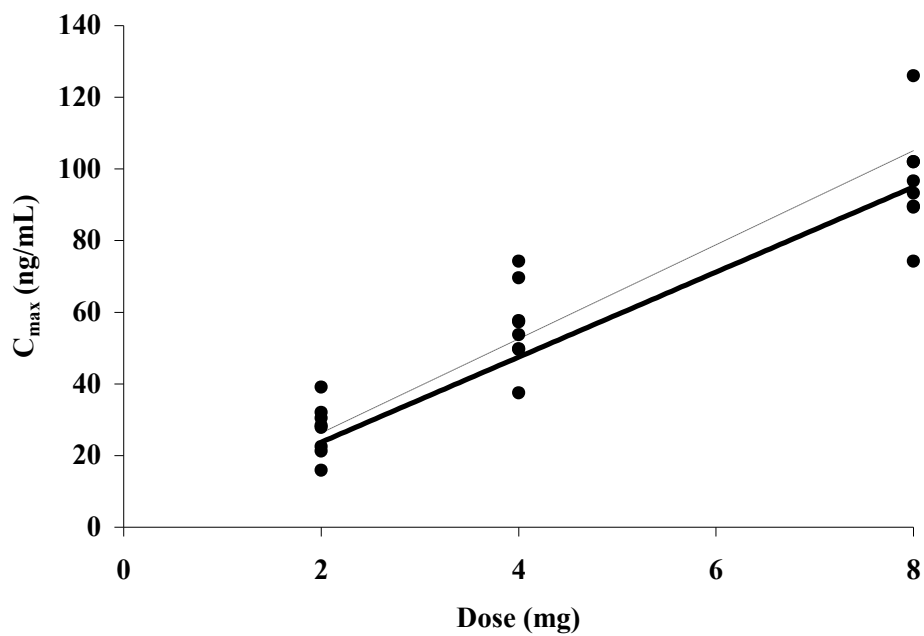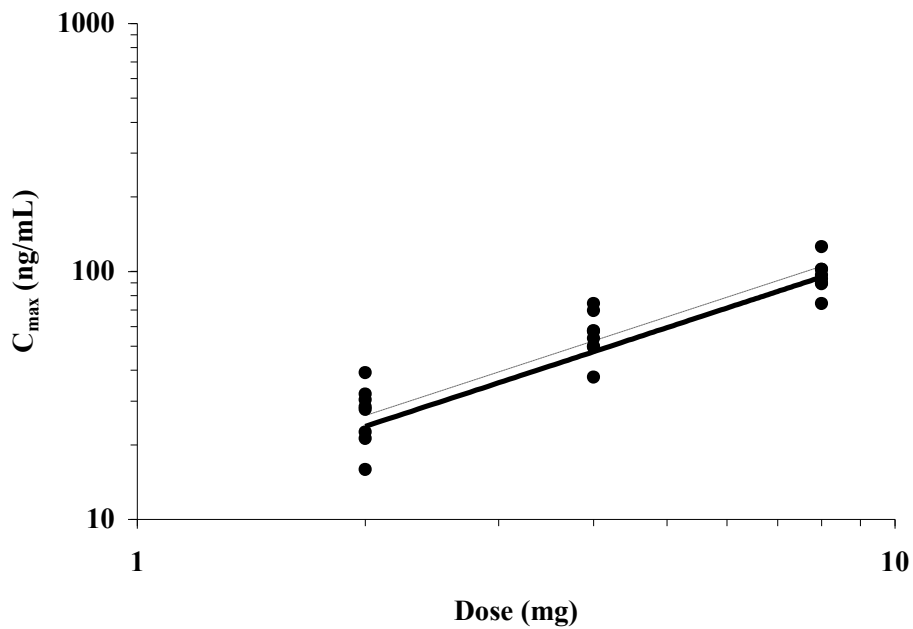
Figure 28: **Relationship between C_{max} and dose of PP 1420.** The individual data points from each volunteer are plotted as dots. Top figure is plotted as $\log_{10} C_{max}$ vs $\log_{10}$ dose. Bottom figure is plotted as C_{max} vs dose. The dotted line represents the dose-proportional relationship passing through the parameter at the 2 mg dose level. The solid line represents the power function obtained from the linear regression from the log parameter against log dose.

4.4.3 Safety and tolerability

PP 1420 appeared to be well tolerated with no evidence of a dose-response relationship for adverse effects (AEs) and no serious AEs that were attributed to PP 1420. Five subjects receiving 4 mg of PP 1420 experienced an AE compared to 3 subjects receiving 8 mg and 2 subjects each receiving 2 mg and placebo. Four subjects did not experience any adverse event during treatment. The most common AE experienced was headache. Inspection of the AEs showed no unexpected pattern in their nature (**Table 3**). There were no effects of any concern in any vital signs, ECG or laboratory parameters.

Event	Placebo	2 mg	4 mg	8 mg
Number of subjects with AE	2	2	5	3
Total number of AEs	4	2	5	6
Headache	2	1	2	1
Injection site reaction/bruising			1	2
Nausea	1	1		
Vomiting	1			1
Diarrhoea			1	
Abdominal pain/bloating				1
Infected finger				1
Cold sore			1	

Table 3: Summary of adverse effects (AEs) in PP 1420 study. Empty cells indicate that there were no AEs.

4.4.4 Exploratory efficacy endpoints

The study design was intended to investigate safety, tolerability and pharmacokinetics; it was not powered to investigate energy intake, but the effect on this parameter was explored. The efficacy of a single administration of PP 1420 on energy intake at each visit generally favoured PP 1420 over placebo at 2 mg and 4 mg visits but shifted to favour placebo on the third visit when 8 mg PP 1420 was administered (**Figure 29**). Apart from one meal at 2 mg (lunch post-dose) the changes in energy intake were not statistically significant. No significant changes in the VAS scores for hunger (**Figure 30**) nor nausea (**Figure 31**) were observed.
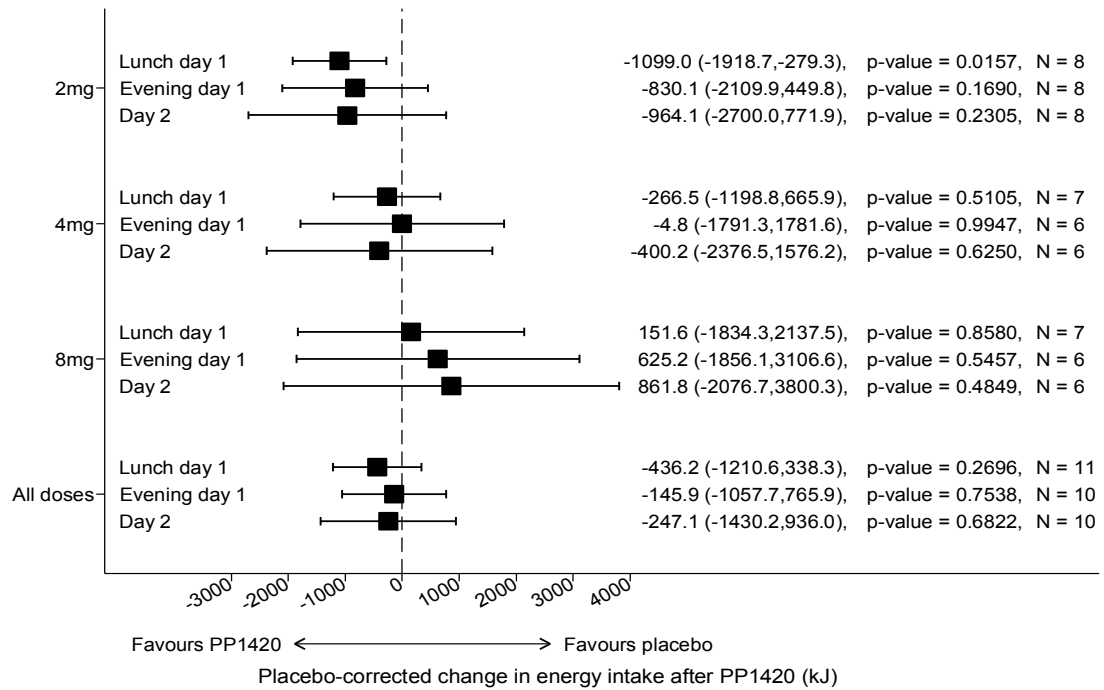
Figure 29: Cumulative mean change and 95% CI of energy intake for the three meals after PP 1420 injection in kJ. Complete-cases analysis. Data is presented for the energy intake at each meal. Estimates of the mean change (corrected for placebo) are plotted as solid squares, with the 95% CI plotted as error bars. One subject, who had missing energy intake data for the evening meal (day 1), is excluded from analyses of energy intake.
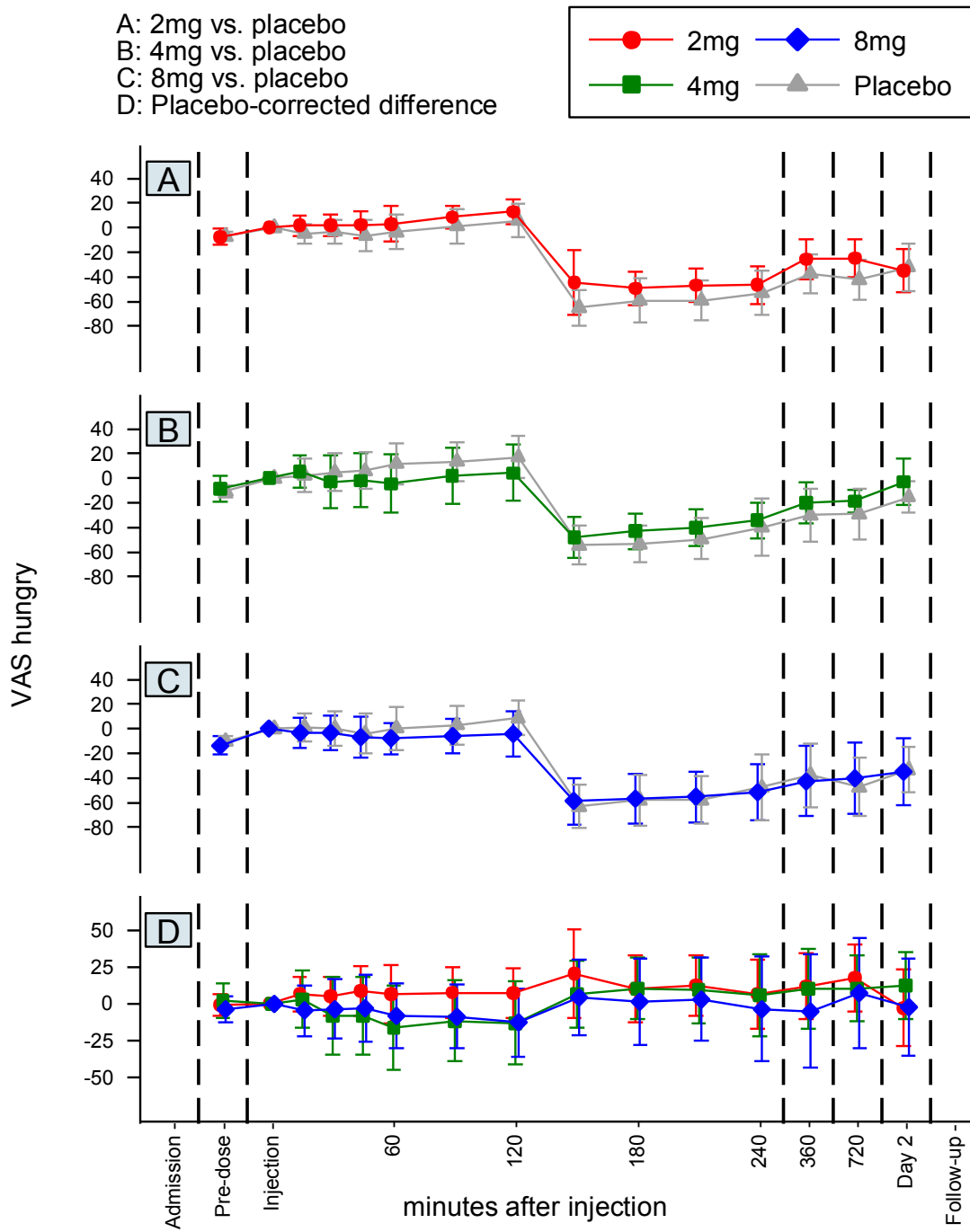
Figure 30: VAS scores for hunger. Difference in VAS from injection timepoint plotted for: (A) 2 mg (red) vs placebo (grey); (B) 4 mg (green) vs placebo (grey); (C) 8 mg (blue) vs placebo (grey). Placebo-corrected differences plotted in (D): 2 mg (red), 4 mg (blue), 8 mg (green).
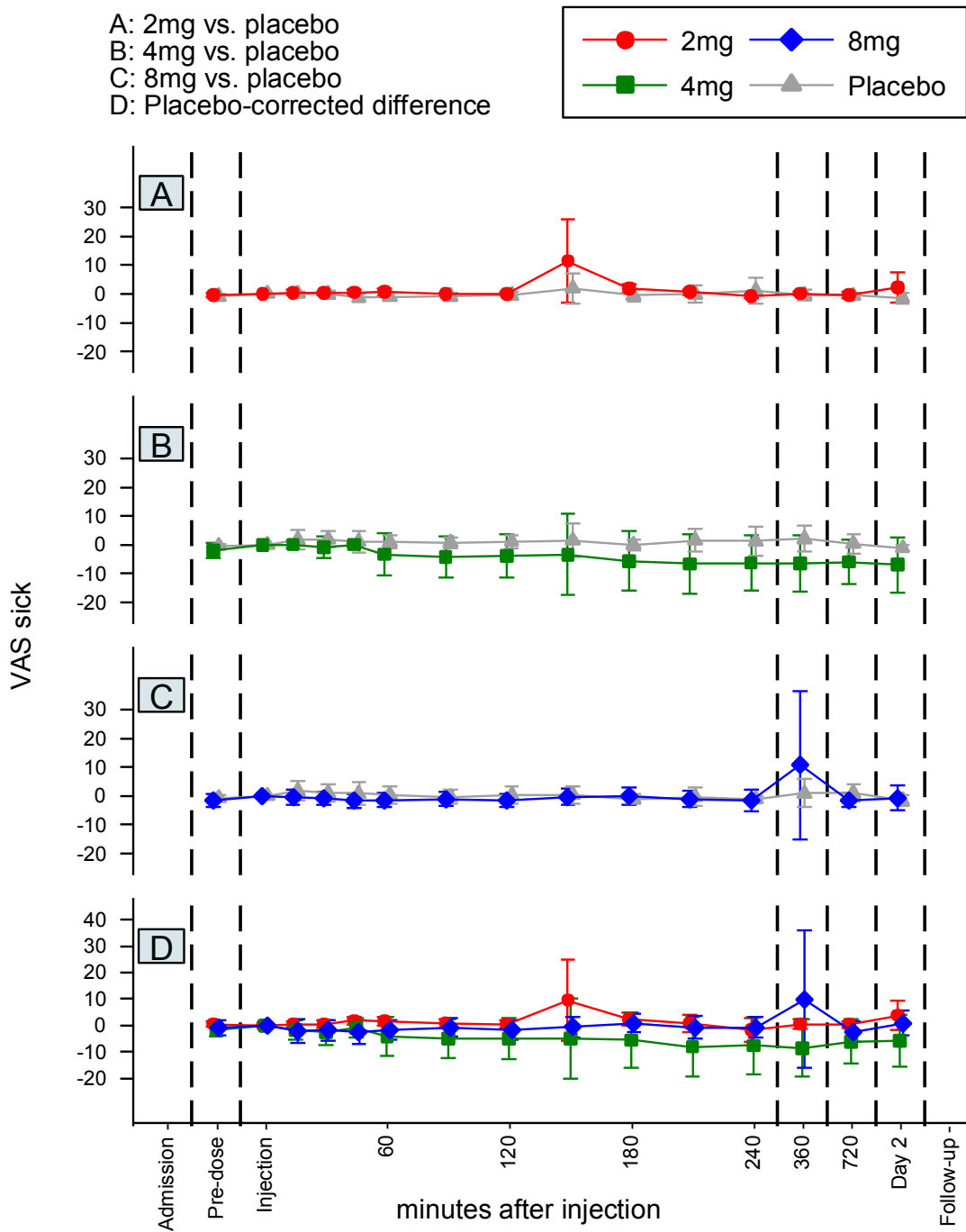
Figure 31: VAS scores for nausea. Difference in VAS from injection timepoint plotted for: (A) 2 mg (red) vs placebo (grey); (B) 4 mg (green) vs placebo (grey); (C) 8 mg (blue) vs placebo (grey). Placebo-corrected differences plotted in (D): 2 mg (red), 4 mg (blue), 8 mg (green).

4.5 Discussion

This study constitutes the first clinical investigation of PP 1420, an Y4 receptor agonist developed as a new treatment for obesity. As expected, the peptidase resistance evident in pre-clinical studies was reflected in a prolonged terminal elimination half-life compared to native hPP.

The current study was not powered to determine the duration of satiety that may be induced by exposure to PP 1420. Nevertheless, the pharmacokinetic profile of PP 1420 may suggest that a twice or three times daily dosing regimen would be required to sustain plasma PP 1420 concentrations over 24 hours if this proved necessary for efficacy. Whilst once daily or less frequent dosing would be desirable, the precedent set by exenatide indicates that twice-daily dosing can be acceptable to patients and is clinically practicable.

A potential advantage of targeting the Y4 receptor is that the satiety induced by a 90 minute IV infusion of hPP may persist for up to 24 hours even after plasma concentrations have returned to baseline (Batterham et al., 2003b). Thus, a relatively brief exposure to a typical post-prandial concentration of hPP can reduce spontaneous food consumption several hours later. Several dosing strategies will thus have to be investigated in future trials to determine PP 1420's pharmacodynamics and hence the optimal dosing regimen.

There were no adverse events in the current study of any clinical concern, nor were there any differences between placebo and PP 1420 in any vital sign measurements, ECG measurements or laboratory parameters measures. Notably, given that nausea was anticipated to be a likely adverse event, PP 1420 did not cause any significant alteration in the VAS scores for nausea.

Overall, the study demonstrated that administration of PP 1420 in healthy human subjects was well tolerated at each dose level, with no tolerability issues arising from either measured clinical endpoints or observed adverse events. However, this trial did not clearly establish a maximum tolerated dose. The results thus support the conduct of further trials of PP 1420 in humans to explore its efficacy, tolerability and pharmacokinetics in multiple doses.

5 THE 'MEDICAL BYPASS': STUDIES ON CARBOHYDRATE METABOLISM WITH A COMBINATION OF GLP-1 AND PEPTIDE YY

5.1 Introduction

As outlined in Section 1.6, bariatric surgery in the morbidly obese, in the form of a Roux-en-Y gastric bypass (RYGB), leads to weight loss of ~30% together with reductions in CVD mortality and deaths from cancer (Sjostrom, 2008; Sjostrom et al., 2009; Sjostrom et al., 2007). It can also induce sustained remission of diabetes and its metabolic consequences, with rapid and marked improvements in insulin sensitivity and secretion, dyslipidaemia and blood pressure. The indications for RYGB surgery have therefore been extended to T2DM associated with only moderate obesity. Trials in T2DM have been relatively small and have lacked adequate control groups. Further, the mechanisms for the favourable changes in glycaemia and body weight are unclear. They do not appear to reflect mere malabsorption of nutrients, but rather a stimulation of insulin secretion and sensitivity together with suppression of appetite mediated in large part by multiple alterations in gut hormone secretion, in particular PYY_{3-36} and GLP-1 (Le Roux et al., 2006a).

Y1 receptor inhibits insulin release from islets (Wang et al., 1994), and sympathetic activity releases both NPY and norepinephrine producing inhibition of insulin secretion (Skoglund et al., 1993). Consistent with this, Y1 receptor knockout mice exhibit a basal hyperinsulinaemia (Kushi et al., 1998). Moreover, PYY_{1-36}, activating

Y1 receptor, has been shown to inhibit stimulated insulin secretion in humans (Greeley et al., 1988). PYY_{3-36} is considerably less active at Y1 receptor but fully active at Y2 receptor. Since the Y2 receptor has been shown to act as a pre-synaptic auto-inhibitor of sympathetic transmission (Malmstrom et al., 2002), Y2 activation might not affect or could even cause disinhibition of insulin release. There is relatively little known about the effects of PYY_{3-36} on glucose metabolism. In animal studies, administration of PYY_{3-36} was associated with increased glucose disposal under hyperinsulinaemic conditions, i.e. an increase in insulin sensitivity (van den Hoek et al., 2004). Sloth et al. found that an acute PYY_{3-36} infusion (at a dose of 0.2 pmol/kg/min) was able to increase the post-prandial insulin response as judged by AUC for insulin concentration (Sloth et al., 2007).

Most of the physiological investigations in this field have concentrated on exploring the effects of infusing single gut hormones at supra-physiological levels, but there have been few studies of combinations of gut hormones to show what happens physiologically. Some combinations of gut hormones e.g. low-dose PYY_{3-36} co-infused with low-dose GLP-1, are known to reduce appetite and food intake in a additive fashion (Neary et al., 2005) but these investigations could not simultaneously study glucose handling due to potential interference with normal appetite due to venepuncture.

Thus we do not know the effects of the normal physiological combination of these gut hormones on glucose homeostasis. No study so far has looked at the acute effects and physiology of combination gut hormone infusions on validated measures of insulin

secretion and resistance. This study of postprandial physiology was therefore designed to establish the effect of low-dose gut hormones in reducing insulin resistance and increasing insulin secretion in humans.

In order to estimate indices of insulin resistance and acute insulin secretion during the low-dose gut hormone infusion, I selected the frequently-sampled IV glucose tolerance test technique. In brief this technique utilizes a provocation in the form of an IV bolus of 20% dextrose (0.3 g/kg) with frequent sampling of glucose and insulin. These results are then modeled using a mathematical model known as the minimal model to describe the glucose-insulin dynamics and to estimate three key indices: the acute insulin response to glucose (AIRg), the index of insulin sensitivity (S_I), and the disposition index (DI = AIRg x S_I). The reasons for choosing this technique were as follows: firstly, this is a well validated technique against the gold standard of clamp studies (Pacini and Mari, 2007); secondly, the technique allows for estimation of both insulin production and insulin resistance in the same procedure, whereas clamp-based techniques would require two studies.

5.2 Hypothesis and Aims

5.2.1 Specific Hypothesis

PYY, when added to GLP-1 in a combination infusion, acutely improves insulin secretion and/or insulin sensitivity.

5.2.2 Specific Aim

The aim was to investigate the acute effect on first-phase insulin secretion and insulin sensitivity of co-administration of "low-dose" PYY_{3-36} and GLP-1 in healthy overweight/obese volunteers.

5.3 Materials & Methods

5.3.1 Peptides

PYY_{3-36} and GLP-1 were purchased from Bachem Ltd. (St Helens, Merseyside). Following initial high fidelity Fmoc solid-phase synthesis, the peptides underwent purification by high resolution HPLC. Sterile 0.9% saline was purchased from Bayer (Haywards Heath, UK). Using an aseptic technique in a laminar flow cabinet, PYY_{3-36} and GLP-1 were separately dissolved in 0.9% saline, aliquoted into vials and freeze dried. Representative PYY_{3-36}, and GLP-1 vials were randomly selected for microbiological examination and for the *Limulus* Amoebocyte Lysate test for pyrogen (Associates of Cape Cod, Liverpool, UK). The vials were sterile on culture after seven days (Department of Microbiology, Hammersmith Hospital, London) and endotoxin levels were within the safe range for human infusion. Further representative vials of both PYY_{3-36} and GLP-1 were randomly selected and sent for amino acid analysis by Alta Bioscience (Birmingham, UK). These results were used to calculate the actual peptide content of the vials. Gelofusine was supplied by B. Braun Medical Ltd (Sheffield, UK).

5.3.2 Subjects

13 healthy overweight volunteers, 11 men and 2 women, of mean age 34.8 ± 2.7 years (range 21–50 years), mean weight 92.8 ± 3.3 kg (range 76.9–115.0), mean height 1.75 ± 0.02 m (range 1.55–1.84 m), mean BMI 30.3 ± 0.9 kg/m^2 (range 26.8 –35.9 kg/m^2), were recruited by advertisement. All volunteers underwent a standardized 75g oral glucose tolerance test to exclude diabetes. Inclusion criteria were: age 18 years and over, male or female, body mass index 23–40 kg/m^2, with stable weight for at least three months. Exclusion criteria were: diabetes mellitus according to WHO criteria, history of alcoholism or substance abuse, history of major haematological, renal, gastrointestinal, hepatic, respiratory, cardiovascular or psychiatric disease or any other illness or use of any medications, including over the counter (OTC) products, which, in the opinion of the investigator, would either interfere with the study or potentially cause harm to the volunteer. Women who were currently pregnant, breastfeeding or unable to maintain adequate contraception for the duration of the study and for one month afterwards were also excluded.

All subjects were screened and determined to be in normal health (or have no significant disease process), by medical history, physical examination, 12 lead electrocardiogram and routine biochemistry and haematology. Women of child bearing age were advized to avoid pregnancy during the study and underwent urine tests to exclude pregnancy prior to each study. The study was approved by the Hammersmith & Queen Charlotte's Ethics Committee (reference no. 09/H0707/77). All subjects gave written informed consent, and the study was planned and performed in accordance with the Declaration of Helsinki and Good Clinical Practice.

5.3.3 Protocol

Each subject attended for 5 study visits. The first visit was to acclimatise the subject to the clinical environment and to experimental procedures. This acclimatization visit was run in identical fashion to subsequent, randomized single-blinded visits, except that the infusion always consisted only of vehicle. Data from the acclimatization visit was not included in the analysis. The subsequent four visits followed a randomized, single-blind, placebo-controlled crossover design comparing four different infusions: (1) Vehicle alone (Gelofusine® – B. Braun Medical Ltd, Sheffield, UK); (2) PYY_{3-36} alone (0.15 pmol/kg/min); (3) $GLP-1_{7-36 \text{ amide}}$ alone (0.2 pmol/kg/min); (4) PYY_{3-36} + $GLP-1_{7-36 \text{ amide}}$ together (0.15 pmol/kg/min and 0.2 pmol/kg/min respectively). The infused doses of the peptide hormones were selected after a preliminary dose-finding phase to achieve plasma concentrations of PYY_{3-36} at 80–120 pmol/L, a level that has previously been shown to increase post-prandial insulin AUC values after an ad libitum meal (Sloth et al., 2007). For $GLP-1_{7-36 \text{ amide}}$, I aimed to achieve 100–140 pmol/L, a level that has previously been shown to increase insulin secretion rate in response to a graded glucose infusion (Kjems et al., 2003). The randomization was carried out by an independent clinician not otherwise involved in the study.

In order to limit adsorption of peptide to the infusion apparatus Gelofusine® was used as the vehicle for all peptide infusions, to dissolve the contents of the randomized vials of peptide and to prime all syringes and infusion lines (Kraegen et al., 1975). Each peptide was drawn up under sterile conditions in a separate 50 ml syringe and, to allow the use of two different infusion rates, delivered by a separate syringe driver

(Graseby 3100, SIMS Graseby Ltd, Watford, UK, or Asena GH Mk III, Alaris Medical Systems Ltd, Basingstoke, UK). Thus on a visit when the volunteer received only one peptide, the second syringe delivered vehicle only, set at the delivery rate calculated for the other hormone.

During the 24-hour period prior to each study visit, participants refrained from strenuous exercise and alcohol consumption. They fasted from 10 p.m. the night before the study, drinking only water. On the morning of each study visit, participants attended a dedicated Clinical Investigation Unit at the Hammersmith Hospital. Female participants had a urine ß-hCG test to exclude pregnancy before the peptide infusion was started. Two cannulae were inserted into the participant's peripheral veins. One cannula was used for sampling, and the other one was used to administer peptide infusion and IV glucose bolus (via a multi-port connector). The infusion containing the peptide hormone(s) was started at 0 minutes.

For evaluation of the acute insulin response to glucose (AIRg) and insulin sensitivity, a frequently-sampled intravenous glucose tolerance test (FSIVGTT) was performed at +60 minutes with an IV glucose bolus of 0.3 g/kg (Pacini and Mari, 2007). Augmentation of FSIVGTT plasma insulin concentrations by tolbutamide or insulin injection was not undertaken since participants were normoglycaemic and insulin release was, in any case, likely to be amplified by the PYY and GLP-1 infusions. The peptide infusion was stopped at +240 minutes. Subjects completed a series of visual analogue scales (VAS) that rated hunger, satiety, prospective food consumption and nausea throughout the study. These consisted of 100 mm lines with text expressing

the most positive and the most negative rating for each variable anchored at either end (Flint et al., 2000). Pulse and blood pressure were regularly monitored.

Blood samples were taken for glucose into fluoride oxalate tubes, and insulin into plain serum tubes (Becton, Dickinson, Portsmouth, UK) at -30, 0, 20, 40, 60, 62, 63, 64, 65, 66, 68, 70, 72, 74, 78, 80, 82, 85, 90, 100, 110, 130, 160, 200, 240 minutes (**Figure 32**). Larger samples were taken at 0, 20, 40, 60, 80, 100, 160, and 240 minutes for plasma gut hormone analysis in lithium heparin coated tubes (International Scientific Supplies Ltd, Bradford, UK) containing 2000 kallikrein inhibitor units (0.2 ml) aprotinin (Trasylol, Bayer Schering Pharma, Berlin, Germany). The insulin samples were allowed to clot for ten minutes at room temperature, after which they were centrifuged and separated and stored at -20°C until analysis. All other samples underwent immediate centrifugation at 4°C, after which plasma was promptly separated and stored at -20°C until analysis.
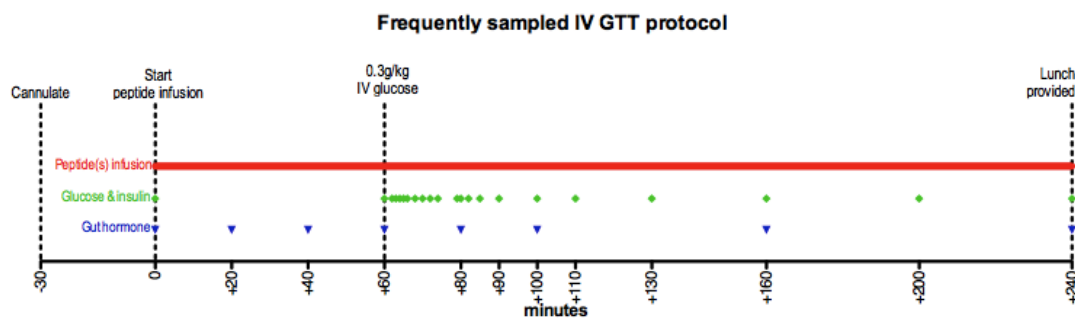
Figure 32: Infusion and sampling protocol for frequently sampled IV GTT study.

5.3.4 Plasma Hormone and Other Assays

All samples were assayed in duplicate and within a single assay to eliminate inter-assay variation. Serum insulin was assayed using the Siemens Immulite 2000 immunoassay (Erlangen, Germany), which is a solid-phase, two-site chemiluminescent immunoassay with an analytical range of 2 to 300 mIU/L and an intra-assay coefficient of variation of 3.3–5.5%. Plasma glucose was assayed using an Abbott Architect automated analyzer (Maidenhead, UK), utilizing a hexokinase-glucose-6-phosphate dehydrogenase method. The analytical range was 0.278–44.4 mmol/L, with an intra-assay coefficient of variation of 0.65–1.98% and an inter-assay coefficient of variation of 0.84–0.93%. Plasma total PYY and amidated GLP-1 were measured using established in-house RIAs (Adrian et al., 1985b; Ghatei et al., 1983) – see Section 2.3.4 for the method. The PYY assay's functional detection limit was 16.8 pmol/L (95% confidence limit 14.4–19.3) with an intra-assay coefficient of variation of 7.4%. The GLP-1 assay's functional detection limit was 13.4 pmol/L (95% confidence limit 12.5–14.2) with an intra-assay coefficient of variation of 3.1%.

5.3.5 Statistical Analysis

Data is expressed as mean ± standard error of the mean (S.E.M.) except where noted. Statistical analysis was carried out using Prism 5.0f (GraphPad Software, San Diego, CA). The acute plasma insulin concentration response to glucose (AIRg: 0–10 minutes), a sensitive index of beta cell function (Kahn et al., 2008), was calculated as the area under the FSIVGTT insulin concentration profile (area-under-the-curve: AUC) from 0 to 10 minutes following glucose administration, calculated using the trapezoid rule (Matthews et al., 1990). Insulin sensitivity (S_I), a measure of the ability

of insulin to enhance glucose disposal, was determined from FSIVGTT glucose and insulin concentrations using the minimal model of glucose disappearance (Bergman et al., 1979) implemented as previously described (Godsland et al., 2006). The FSIVGTT-derived measures, AIRg and S_I, provide the so-called disposition index (DI), calculated as $S_I \times$ AIRg (Kahn et al., 1993). This widely-used dimensionless measure of beta cell function, quantifies beta cell adaptation to variation in insulin sensitivity, according to the hyperbolic relationship between insulin resistance and insulin secretion.

5.4 Results

PYY exposures were similar between the two arms that included PYY_{3-36} in the infusion (**Figure 33A**), as were GLP-1 exposures comparing the arms that included $GLP-1_{7-36amide}$ in the infusion (**Figure 33B**). There were no significant variations in pulse and blood pressure across infusions and analysis of VAS scores revealed no nausea in response to the gut hormone infusions (data not shown).

A

B
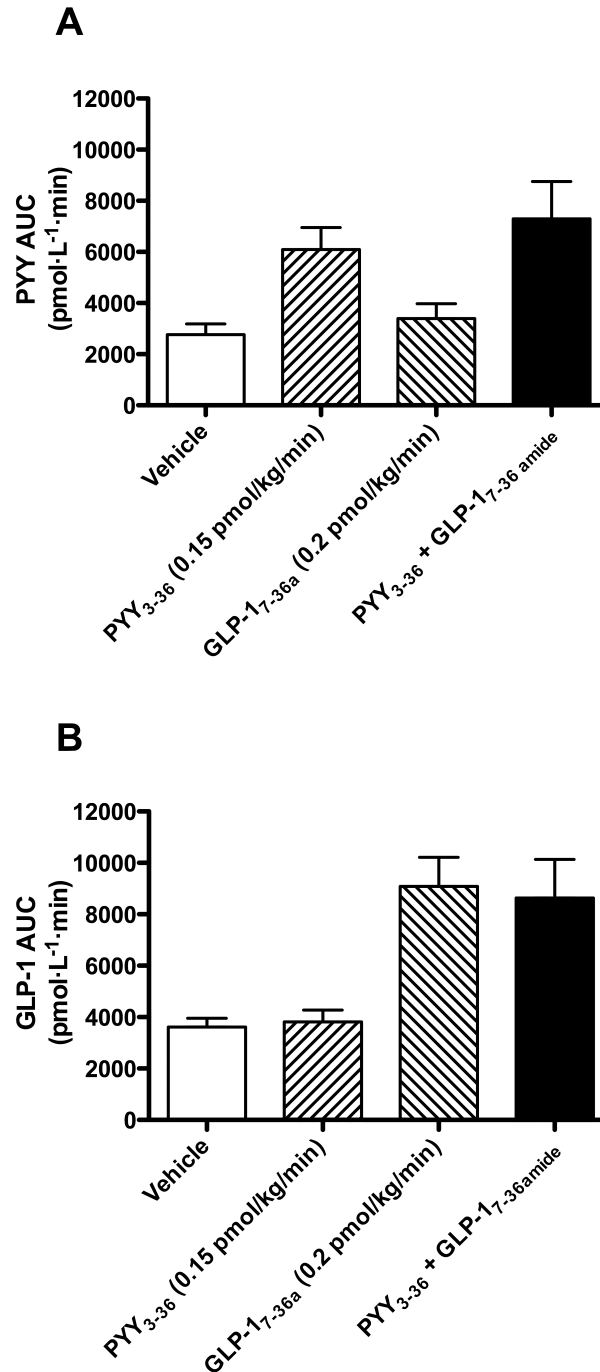
Figure 33: (A) PYY and (B) GLP-1 exposure during the FSIVGTT. Integrated area under the concentration curve (AUC) for 0 to 100 minutes, from the start of the infusion to the end of the intensive minimal modeling period, is plotted on the Y-axis. The X-axis indicates infusion given. Mean ± S.E.M. plotted. Baseline plasma PYY levels (at 0 mins) were vehicle: 47.7 ± 8.7 pmol/L; PYY_{3-36}: 45.8 ± 8.1 pmol/L; GLP-$1_{7-36\ amide}$: 34.1 ± 5.3 pmol/L; PYY_{3-36} + GLP-$1_{7-36\ amide}$: 52.2 ± 10.9 pmol/L). End-infusion (+240min: steady state) levels were vehicle: 26.7 ± 15.8 pmol/L; PYY_{3-36}: 113.5 ± 13.7 pmol/L; GLP-$1_{7-36\ amide}$: 21.3 ± 13.9 pmol/L; PYY_{3-36} + GLP-$1_{7-36\ amide}$: 97.8   ± 37.2 pmol/L. To estimate the exposure of volunteers to PYY_{3-36} from 0 to 100 mins, the respective AUC for each infusion arm was calculated as follows: vehicle: 2766 ± 423.7 pmol·L-1·min, PYY_{3-36}: 6091 ± 861.2 pmol·L^{-1}·min, GLP-$1_{7-36\ amide}$: 3395 ± 575.9 pmol·L-1·min, PYY_{3-36} + GLP-$1_{7-36\ amide}$ 7297 ± 1460 pmol·L^{-1}·min. Baseline plasma GLP-1 levels (at 0 mins) across different infusion arms were vehicle:

43.7 ± 6.2 pmol/L; PYY_{3-36}: 43.8 ± 7.7 pmol/L; GLP-$1_{7-36 \text{ amide}}$: 55.6 ± 9.7 pmol/L; PYY_{3-36} + GLP-$1_{7-36 \text{ amide}}$: 52.6 ± 15.2 pmol/L). End-infusion (+240min: steady state) levels were vehicle: 44.0 ± 8.5 pmol/L; PYY_{3-36}: 33.4 ± 2.6 pmol/L; GLP-$1_{7-36 \text{ amide}}$: 142.2 ± 22.3 pmol/L; PYY_{3-36} + GLP-$1_{7-36 \text{ amide}}$: 140.4 ± 22.0 pmol/L. To estimate the exposure of volunteers to GLP-$1_{7-36 \text{ amide}}$ from 0 to 100 mins, the respective AUC for each infusion arm was calculated as follows: vehicle: 3614 ± 344.2 pmol·L-1·min, PYY_{3-36}: 3813 ± 458.7 pmol·L-1·min, GLP-$1_{7-36 \text{ amide}}$: 9084 ± 1134 pmol·L-1·min, PYY_{3-36} + GLP-$1_{7-36 \text{ amide}}$ 8639 ± 1495 pmol·L-1·min.

Fasting glucose levels were very similar between all infusion arms (**Figure 34**). With the administration of the IV glucose bolus, glucose levels peaked at 15.5–16.2 mmol/L (64 mins) and fell back to baseline by 110 min. In no case did any volunteer experience a biochemical or symptomatic hypoglycaemia as a result of the endogenous insulin release in response to the large IV glucose bolus.

The insulin response to the IV glucose bolus is shown in **Figure 35**. Infusion of GLP-$1_{7\text{-}36\ amide}$, either alone or in combination with $PYY_{3\text{-}36}$, augmented the insulin secretory response following the IV glucose bolus compared with either vehicle or PYY alone.
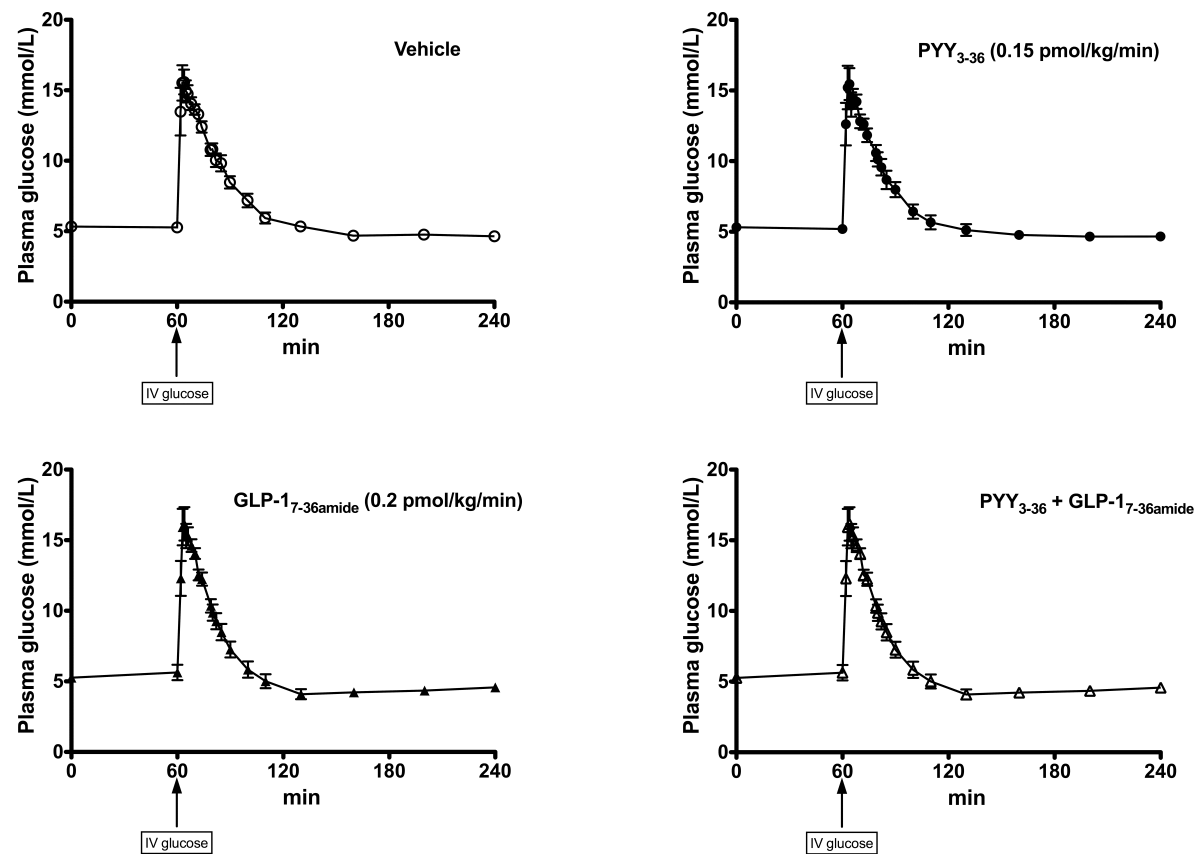
Figure 34: Plasma glucose levels during the FSIVGTT. Y-axis shows plasma glucose levels (mmol/L). X-axis shows time (min). IV glucose bolus (0.3 g/kg) given at 60 minutes. Mean ± S.E.M. plotted. Open circles, dashed line: placebo infusion arm; Closed circles, solid line: PYY_{3-36} infusion (0.15 pmol/kg/min); Closed triangles, solid line: $GLP-1_{7-36 \text{ amide}}$ infusion (0.2 pmol/kg/min); Open triangles, solid line: combined PYY_{3-36} + $GLP-1_{7-36 \text{ amide}}$ infusion. Fasting glucose values for vehicle: 5.3 ± 0.1 mmol/L; PYY_{3-36}: 5.3 ± 0.2 mmol/L; $GLP-1_{7-36amide}$: 5.3 ± 0.1 mmol/L; combined PYY_{3-36} + $GLP-1_{7-36amide}$: 5.4 ± 0.1 mmol/L.
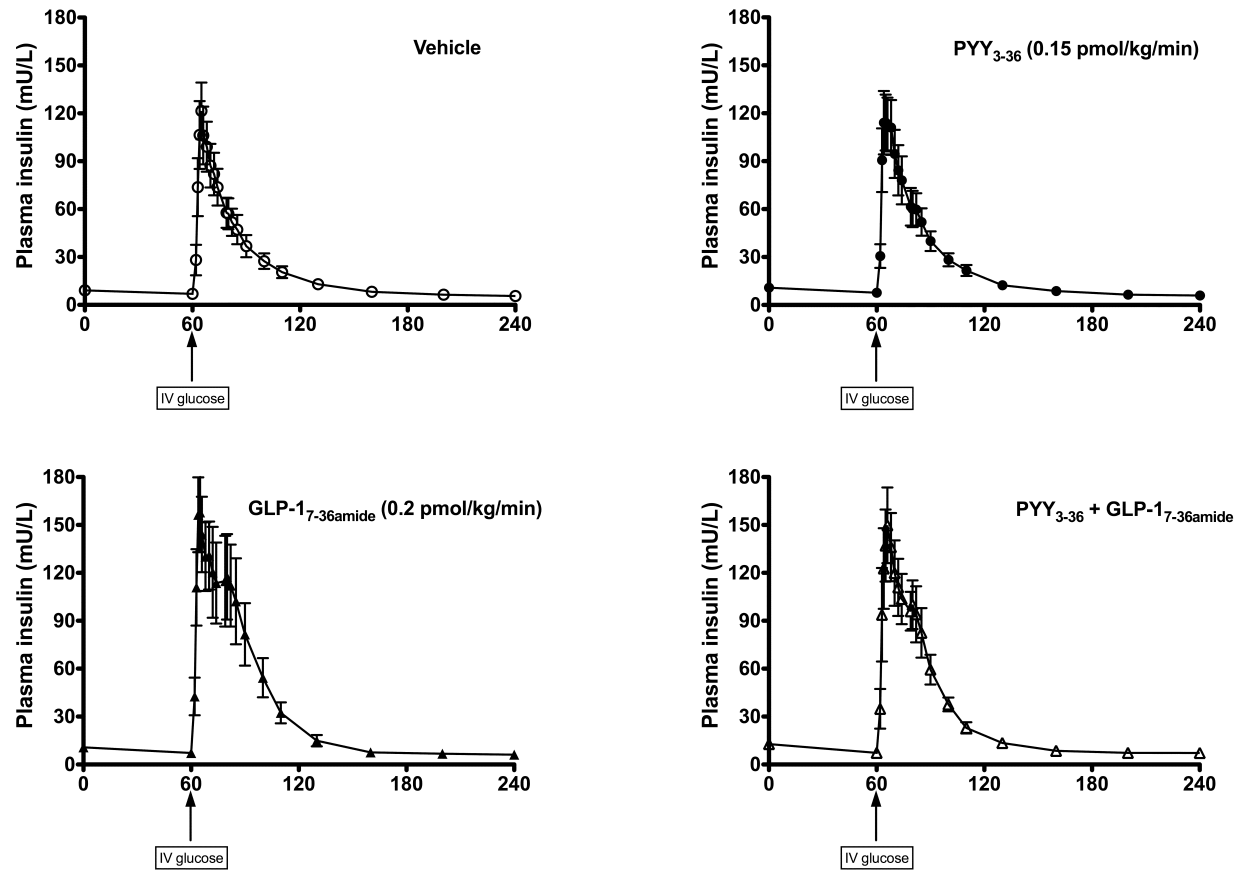
Figure 35: Plasma insulin levels during the FSIVGTT. Y-axis shows insulin levels (mU/L). X-axis shows time (min). IV glucose bolus (0.3 g/kg) given at 60 minutes. Mean ± S.E.M. plotted. Open circles, dashed line: placebo infusion arm; Closed circles, solid line: PYY$_{3-36}$ infusion (0.15 pmol/kg/min); Closed triangles, solid line: GLP-1$_{7-36 amide}$ infusion (0.2 pmol/kg/min); Open triangles, solid line: combined PYY$_{3-36}$ + GLP-1$_{7-36 amide}$ infusion.

In line with the observations on insulin secretion, the AIRg during each infusion showed a significant difference in means (p=0.004 – **Figure 36A**). No significant difference was detected on post-hoc testing between vehicle and PYY$_{3-36}$ (mean difference in AIRg 93.77 mU·L^{-1}·min, 95% C.I. for difference -159 to 346.5). A significant difference was detected between vehicle and GLP-1$_{7-36 \text{ amide}}$ (p <0.01: mean difference in AIRg 377.5 mU·L^{-1}·min, 95% C.I. for difference 120.4 to 630.2). The PYY$_{3-36}$ + GLP-1$_{7-36 \text{ amide}}$ combination also increased AIRg compared to vehicle, similar to GLP-1$_{7-36 \text{ amide}}$, although the difference in AIRg did not quite reach statistical significance (mean difference 251.8 mU·L^{-1}·min, 95% C.I. for difference -0.98 to 504.2). No significant differences in the insulin sensitivity index (S$_I$) were discerned between infusion arms (p=0.99 – **Figure 36B**). There was a borderline significant difference in mean DI between infusion arms (p=0.07 – **Figure 36C**).
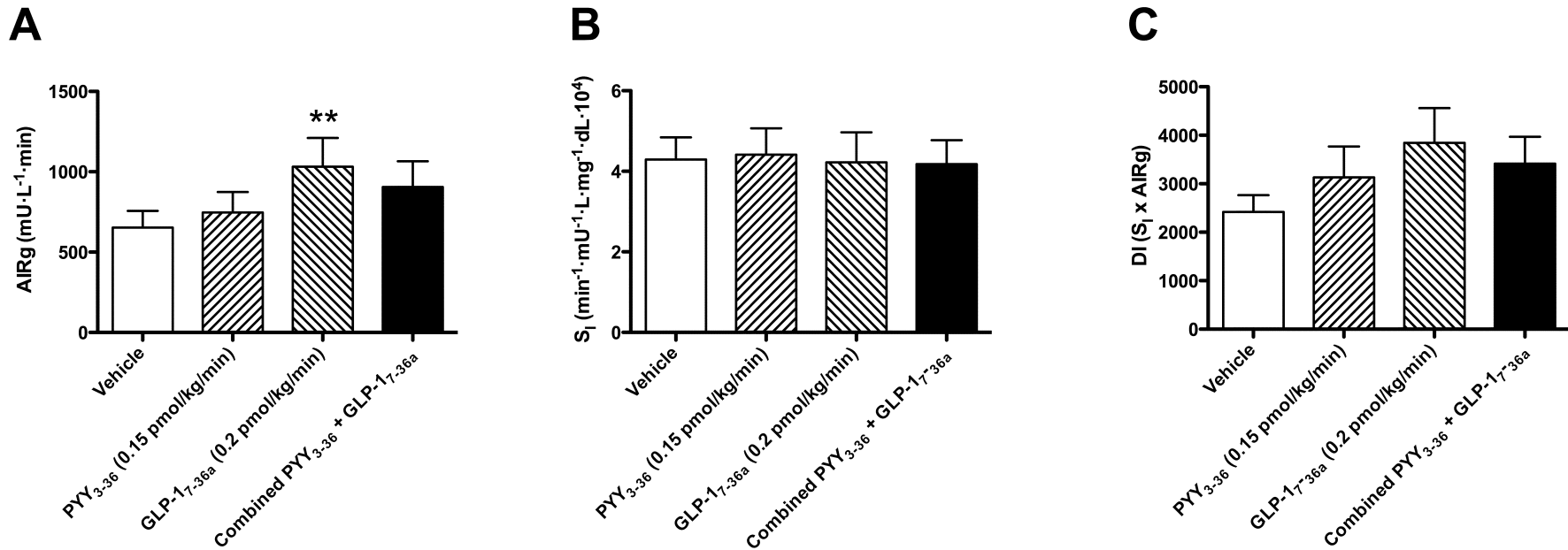
Figure 36: (A) First-phase insulin (AIRg) response to IV glucose. Means plotted ± S.E.M., one-way repeated measures ANOVA p=0.004. AIRg means for vehicle 653.4 ± 103.6 $mU·L^{-1}·min$; GLP-1$_{7-36amide}$ infusion 1031 ± 178.3 $mU·L^{-1}·min$; PYY$_{3-36}$ 747.2 ± 127.7 $mU·L^{-1}·min$; combined PYY$_{3-36}$ + GLP-1$_{7-36amide}$ 905.2 ± 161 $mU·L^{-1}·min$. ** = p<0.01 for comparison of GLP-1 to vehicle for AIRg by Dunnett's multiple correction test. **(B) Insulin sensitivity index (S$_I$).** Means plotted ± S.E.M., one-way repeated measures ANOVA p=0.004. S$_I$ for vehicle: 4.29 ± 0.55 $min^{-1}·mU^{-1}·L·mg^{-1}·dL·10^4$; PYY$_{3-36}$: 4.41 ± 0.65 $min^{-1}·mU^{-1}·L·mg^{-1}·dL·10^4$; GLP-1$_{7-36amide}$: 4.22 ± 0.74 $min^{-1}·mU^{-1}·L·mg^{-1}·dL·10^4$; combined PYY$_{3-36}$ + GLP-1$_{7-36amide}$: 4.18 ± 0.60 $min^{-1}·mU^{-1}·L·mg^{-1}·dL·10^4$. **(C) Disposition index (DI).** Means plotted ± S.E.M., one-way repeated measures ANOVA p=0.07. DI for vehicle: 2417 ± 349.5; PYY$_{3-36}$: 3131 ± 638.1; GLP-1$_{7-36\ amide}$ 3844 ± 716.8; combined PYY$_{3-36}$+ GLP-1$_{7-36\ amide}$: 3414 ± 553.6.

5.5 Discussion

In this study, I measured the acute changes in first-phase insulin secretion, insulin sensitivity and β-cell function in response to an infusion of PYY_{3-36} and $GLP-1_{7-36\ amide}$ in healthy, overweight, non-diabetic humans using an FSIVGTT. As expected from its known action as an incretin hormone (Section 1.5.5.1), $GLP-1_{7-36\ amide}$ infusion significantly increased first-phase insulin secretion in response to the IV glucose, compared with vehicle. The observed elevation in AIRg with combination PYY_{3-36} + $GLP-1_{7-36\ amide}$ infusion just failed to reach significance. However, the observation that PYY_{3-36} infusion alone resulted in a slight, non-significant rise in AIRg compared with vehicle suggests that it is unlikely that PYY_{3-36} is actively counteracting the insulinotropic effect of $GLP-1_{7-36\ amide}$ but rather that there appears to be no additive or synergistic effect between PYY_{3-36} and $GLP-1_{7-36\ amide}$ in combination on insulin secretion. Furthermore, neither hormone had any acute effect on measures of insulin sensitivity in this cohort, hence the changes in disposition indices mirrored the changes in AIRg across all infusion arms.

As noted in Chapter 1 and above, PYY_{3-36} has been shown to increase insulin sensitivity in animal studies (van den Hoek et al., 2004) and has been associated with an increase in insulin secretion in response to an ad libitum meal when given to humans (Sloth et al., 2007). In contrast, we have shown that acute, low dose administration of PYY_{3-36} to overweight humans has no effect on insulin sensitivity and no significant effect on β-cell secretory function. The physiological relevance of the results from previous animal studies is questionable as supraphysiological doses

of PYY_{3-36} were usually used. In the human study of Sloth et al., the insulin response was examined after an ad libitum lunch, and, surprisingly the PYY_{3-36} group did eat slightly more than the placebo group. This may be because the PYY infusion day always followed the placebo day; when subjects acclimatize to the study environment they typically eat more in the second day, introducing a bias in measurements of food intake (an 'order effect'). Thus the increased insulin response with PYY_{3-36} may be merely a response to an increased energy intake at the meal (Sloth et al., 2007). This study incorporated the following elements to minimize bias: firstly, a double blinded design; secondly, an acclimatization visit was included; thirdly, the infusions were given in a random order; fourthly, a standardized method was used to examine insulin secretion in response to a fixed IV glucose stimulus. The observations from the present study are therefore likely to be more robust than in the previous studies mentioned above.

A limitation of this study is that it only examined the effects of PYY_{3-36} and GLP-1 in an acute setting. I speculate that longer-term treatment with PYY_{3-36} may ultimately improve insulin sensitivity through a reduction in food intake and therefore weight loss. Future studies may need to concentrate on measuring the effects of chronic administration of these gut hormones on insulin sensitivity and secretion.

6 GENERAL DISCUSSION AND CONCLUSIONS

The only treatment that has been shown to be most effective and beneficial for patients with obesity and Type 2 diabetes mellitus has been bariatric surgery (Buchwald et al., 2009; Pournaras et al., 2012; Sjostrom et al., 2007). However, in many of these patients, surgery is not a feasible option as it carries a significant peri-operative mortality rate. Co-morbidities such as hypertension, ischaemic heart disease and diabetes may add to this danger (Buchwald et al., 2004). Moreover, surgery is a relatively expensive solution and, because of the requirement for specialist surgeons and facilities, this cannot be effectively delivered to the 25-30% of the population that are obese without a radical re-orientation of health systems. Therefore an efficacious and safe medical therapy is urgently required as an alternative solution for obesity and diabetes.

Unfortunately, the record of medical anti-obesity treatments has been dismal. All previously licensed medications have been withdrawn from marketing due to issues with side effects, some very serious. The only exception to this, the intestinal lipase inhibitor orlistat, is only modestly effective (2.6% mean body weight reduction over placebo) and associated with significant side effects such as fatty/oily stool and faecal urgency (Rucker et al., 2007).

Even new and emerging agents for weight loss are relatively inefficacious. Contrave® (bupropion/naltrexone – Orexigen Therapeutics) is associated with a mean weight loss of only 3.2% on average over placebo in a 52-week randomized, controlled trial

in overweight/obese type 2 diabetes patients (Hollander et al., 2013). Similarly the 5HT$_{2C}$ receptor antagonist lorcaserin (Arena Pharmaceuticals) causes a mean weight loss of 3.65% over placebo in a 52-week randomized, controlled trial in overweight/obese patients (Smith et al., 2010). Qysmia® (phentermine/topiramate controlled release – Vivus, Inc.) is more effective, causing a mean weight loss of 6.8% (7.5/46 mg dose) and 7.5% (15/92 mg dose) over placebo in a 28-week randomized, controlled trial (Aronne et al., 2013). Each of these drugs is associated with significant side effects: nausea, constipation and vomiting in the case of Contrave (Hollander et al., 2013), headache, dizziness and nausea with lorcaserin (Smith et al., 2010), and paraesthesiae, dry mouth and headache with Qsymia (Aronne et al., 2013), limiting their applicability to patients.

New targets for drug development in obesity are therefore required. Gut hormones represent an extremely promising avenue for anti-obesity drug development, for the following reasons.

Firstly, gut hormones already play a physiological role in controlling appetite. This is unlike some other treatments, which exploit non-physiological effects as a means to suppressing appetite and causing weight loss, such as Qsymia® (Garvey et al., 2012) and Contrave® (Greenway et al., 2010).

Secondly, there is already strong evidence, discussed in Section 1.6, that elevations in gut hormones are responsible for the suppression of food intake and improvements in metabolism seen after bariatric surgery, particularly the bypass procedures (Clements

et al., 2004; Korner et al., 2007; Le Roux et al., 2006a; Pournaras and Le Roux, 2009; Rubino et al., 2004).

Thirdly, oxyntomodulin and GLP-1 analogues have already been shown to induce weight loss during chronic treatment (Astrup et al., 2012; Astrup et al., 2009; Wynne et al., 2005).

Fourth, the GLP-1 analogues appear to be safe for chronic treatment. Despite some concerns regarding pancreatitis and pancreatic cancer associated with GLP-1 analogue treatment (Butler et al., 2013), so far there does not appear to be an excess risk of these complications in diabetic patients treated with GLP-1 analogues compared to other treatments (Funch et al., 2013).

Fifth, as mentioned above, GLP-1 appears to have salutary effects on the cardiovascular system.

However, much needs to be established before gut hormones can be developed into viable therapies for obesity, as follows.

- What the exact effects of gut hormones are on appetite and on carbohydrate metabolism.
- What the efficacious doses are, without side effects.
- A formulation which is safe and tolerated.

- Whether such therapies can be conveniently delivered to patients, e.g. in the form of self-administered subcutaneous injections through a fine gauge needle, preferably once a day or less frequently.

- Whether combination therapies bring additional benefits compared to the individual hormones, for example, by increasing efficacy, combining desired individual effects, and reducing side effects.

The work presented in this thesis has focused on several aspects of the physiology of the gut hormones PP, PYY_{3-36} and GLP-1, and explored how these might be used as potential treatments for Type 2 diabetes and obesity, both when administered alone and in combination. The work in Chapter 2 confirmed that SC injections of hPP suppress appetite in human volunteers, in a similar way to IV infusion of hPP (Batterham et al., 2003b; Jesudason et al., 2007). However, there appeared to be no clear dose-response relationship between the dose and the appetite-suppressive effect. Nevertheless, the results presented in Chapter 2 lend support to the notion that it is feasible to deliver PP via SC injections as an anti-obesity drug.

Following on from this, I went on to characterize the effects of the PP analogue PP 1420 (Chapter 3). This analogue was engineered to possess an extended terminal elimination half-life by virtue of sequence changes in comparison to hPP that make it more peptidase resistant, but yet to retain PP's physiological characteristics. This was in order to allow PP 1420 to be administered less frequently than PP, and to improve its practicability as a treatment. I showed that PP 1420 was able to bind to the human Y4 PP receptor with a similar affinity to hPP. When PP 1420 was given to DIO mice

for an extended period of time, I observed a trend towards reduction in food intake and body weight, but no improvements were seen in glucose tolerance. I then proceeded to show that there was an additive effect from PP 1420 when this was given together with metformin, in terms of food intake suppression and body weight reduction in DIO mice, although no improvement was seen in glucose levels in this short study. This result suggests that the combination PP 1420 plus metformin may have potential as a treatment for obesity with enhanced efficacy compared to the individual components, but these findings will need to be confirmed in Phase II/III trials in diabetic obese patients.

PP 1420 was then tested in a Phase 1 trial for the first time in humans, and it was found that this was well tolerated and safe (Chapter 4). It was confirmed that the sequence alterations in PP 1420 conferred an extended terminal elimination half-life of approximately 2.5 h, compared to seven minutes for PP (Adrian et al., 1978b). As Phase 1 trials are by nature designed to examine tolerability and safety in a small number of volunteers (to minimize drug exposure), the design of the Phase 1 trial precluded definitive examination of PP 1420's efficacy in reducing food intake and body weight, although there were some preliminary signs that PP 1420 might be able to reduce food intake.

Finally, I went on to examine the effect of a combination of PYY_{3-36} and GLP-1 on insulin secretion and insulin sensitivity during a frequently sampled IV glucose tolerance test (Chapter 5). I showed that although GLP-1 had the expected effect in increasing acute insulin secretion, PYY_{3-36} did not appear to contribute to this effect

when combined with GLP-1. Neither PYY$_{3-36}$ nor GLP-1 appear to have any significant acute effect on insulin sensitivity. However, it is possible that chronic treatment with PYY and GLP-1 may improve insulin sensitivity by inducing weight loss, and this hypothesis remains to be tested in definitive studies.

In this thesis, therefore, I have drawn together studies that encompass the spectrum of translational medicine, starting from physiological studies (Chapters 2, 5), going on to pre-clinical development (Chapter 3), and then to early phase development in humans (Chapter 4). My work illustrates some of the key issues in translation of basic scientific findings to clinical treatment, as follows.

The proper design of physiological studies to provide a solid basis for translational development (Chapters 2, 5).

The design and pre-clinical development of new drugs to characterise their safety and tolerability (Chapter 3).

Meeting regulatory requirements for the development of drugs through the pre-clinical and early clinical stages (Chapters 3, 4).

Ensuring that studies in which human volunteers are involved are first and foremost safe (Chapter 4).

Rigorous statistical analysis to ensure that the correct conclusions are drawn from the necessarily limited data generated during studies of small numbers of volunteers (Chapter 4).

What are the realistic prospects of gut hormone analogues being developed into treatments for obesity? GLP-1 has been an important paradigm for the field, as the development of the GLP-1 analogues has shown that these represent feasible, practical and efficacious treatments for diabetes (Todd and Bloom, 2007). Unfortunately, as previously mentioned, GLP-1 analogues only have limited efficacy in the treatment of obesity, and the doses given are limited by their side effect profiles (Astrup et al., 2009). Alternatives are required that are more efficacious in reducing weight without causing side effects. Two strategies might deliver such efficacy, as follows.

Development of alternative gut hormones with more favourable side effect profiles, enabling dose titration to achieve efficacy targets. In this case, PP and its analogue PP 1420 was developed as such an alternative (Chapters 2, 3, 4). As may be seen from the data presented in this thesis, encouraging progress has been made in the development of PP 1420, and future studies will build on this progress.

Development of gut hormone combinations that would deliver favourable efficacy, reduced side effect profiles and favourable combinations desired individual effects, for example the appetite-suppressive effects of PYY$_{3-36}$ combined with the insulinotropic effects of GLP-1. Given that the regulation of

appetite and metabolism involves the finely regulated secretion of multiple gut hormones, it is likely that future therapies will involve the administration of such combinations of gut hormones to obtain optimal effects on obesity and Type 2 diabetes. It is therefore vital that an understanding of the physiological effects of such combinations is obtained. In Chapter 5, I presented a study that was designed to illuminate the physiological changes when gut hormones are combined. More such studies will be required to delineate these physiological changes so that combination treatments may be rationally designed in future.

Most gut hormones levels rise and fall on a timescale of a few hours, i.e. chronic elevation is not a usual physiological situation. Gastric bypass surgery causes many metabolic changes including suppression of food intake, alterations in food preference, alterations in hedonic responses to eating, amelioration of diabetes and insulin resistance. Although many of these changes are likely to be due to the chronic elevation in gut hormones observed after bariatric surgery, the specific roles of gut hormones in mediating these changes are unknown. To build on the studies I have presented in this thesis, I am currently conducting the following studies.

Phase 1B and 1C studies on PP 1420 (funded by the Wellcome Trust). The aim of these studies is: (1) to test PP 1420 in an extended dose range of up to 64 mg per single dose; (2) to establish the dose of PP 1420 that can significantly reduce food intake in healthy volunteers; (3) to establish the safety, tolerability and the efficacy of multiple daily doses of PP 1420; and (4) to ascertain the food intake reduction and effect on body weight in overweight healthy volunteers when given these multiple

daily doses of PP. Completion of this study would give definitive proof of PP 1420's efficacy in humans.

A mechanistic study comparing the effects of RYGB surgery with those of a chronic infusion (28 days) of a combination of GLP-1, oxyntomodulin and PYY$_{3-36}$ (funded by the MRC under the Experimental Medicine Challenge Grant scheme). This study seeks to examine the effects of the abovementioned treatments in obese patients on food intake, body weight, carbohydrate tolerance, and activation of brain areas by food cues using functional MRI. Completion of this study would answer some key questions about the roles of chronic gut hormone elevations in mediating the metabolic effects of RYGB.

These studies, which will study the chronic effects of gut hormone combinations on physiology in a comprehensive and validated manner, will meet the challenge of understanding the true physiological role and therapeutic value of gut hormones.

7 APPENDICES

7.1 Abbreviations for amino acids

Three-letter abbreviation	One-letter abbreviation	Amino acid name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid (Aspartate)
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid (Glutamate)
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

8 REFERENCES

Abbott, C.R., Monteiro, M., Small, C.J., Sajedi, A., Smith, K.L., Parkinson, J.R., Ghatei, M.A., and Bloom, S.R. (2005a). The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. Brain Res *1044*, 127-131.

Abbott, C.R., Small, C.J., Kennedy, A.R., Neary, N.M., Sajedi, A., Ghatei, M.A., and Bloom, S.R. (2005b). 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*, 139-144.

Abdallah, B., Hassan, A., Benoist, C., Goula, D., Behr, J.P., and Demeneix, B.A. (1996). A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethylenimine. Hum Gene Ther *7*, 1947-1954.

Acosta, A., Hurtado, M.D., Gorbatyuk, O., La Sala, M., Duncan, D., Aslanidi, G., Campbell-Thompson, M., Zhang, L., Herzog, H., Voutetakis, A.*, et al.* (2011). Salivary PYY: a putative bypass to satiety. PloS One *6*, e26137.

Adrian, T.E., Ballantyne, G.H., Longo, W.E., Bilchik, A.J., Graham, S., Basson, M.D., Tierney, R.P., and Modlin, I.M. (1993). Deoxycholate is an important releaser of peptide YY and enteroglucagon from the human colon. Gut *34*, 1219-1224.

Adrian, T.E., Besterman, H.S., Cooke, T.J., Bloom, S.R., Barnes, A.J., and Russell, R.C. (1977). Mechanism of pancreatic polypeptide release in man. Lancet *1*, 161-163.

Adrian, T.E., Bloom, S.R., Bryant, M.G., Polak, J.M., and Heitz, P. (1976a). Proceedings: Radioimmunoassay of a new gut hormone-human pancreatic polypeptide. Gut *17*, 393-394.

Adrian, T.E., Bloom, S.R., Bryant, M.G., Polak, J.M., Heitz, P.H., and Barnes, A.J. (1976b). Distribution and release of human pancreatic polypeptide. Gut *17*, 940-944.

Adrian, T.E., Bloom, S.R., Hermansen, K., and Iversen, J. (1978a). Pancreatic polypeptide, glucagon and insulin secretion from the isolated perfused canine pancreas. Diabetologia *14*, 413-417.

Adrian, T.E., Ferri, G.L., Bacarese-Hamilton, A.J., Fuessl, H.S., Polak, J.M., and Bloom, S.R. (1985a). Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology *89*, 1070-1077.

Adrian, T.E., Greenberg, G.R., Barnes, A.J., Christofides, N.D., Alberti, K.G., and Bloom, S.R. (1980). Effects of pancreatic polypeptide on motilin and circulating metabolites in man. Eur J Clin Invest *10*, 235-240.

Adrian, T.E., Greenberg, G.R., Besterman, H.S., and Bloom, S.R. (1978b). Pharmacokinetics of pancreatic polypeptide in man. Gut *19*, 907-909.

Adrian, T.E., Greenberg, G.R., Fitzpatrick, M.L., and Bloom, S.R. (1981). Lack of effect of pancreatic polypeptide in the rate of gastric emptying and gut hormone release during breakfast. Digestion *21*, 214-218.

Adrian, T.E., Savage, A.P., Sagor, G.R., Allen, J.M., Bacarese-Hamilton, A.J., Tatemoto, K., Polak, J.M., and Bloom, S.R. (1985b). Effect of peptide YY on gastric, pancreatic, and biliary function in humans. Gastroenterology *89*, 494-499.

Ahlkvist, L., Vikman, J., Pacini, G., and Ahren, B. (2012). Synergism by individual macronutrients explains the marked early GLP-1 and islet hormone responses to mixed meal challenge in mice. Regul Pept *178*, 29-35.

Ahren, B., Pacini, G., Foley, J.E., and Schweizer, A. (2005). Improved meal-related beta-cell function and insulin sensitivity by the dipeptidyl peptidase-IV inhibitor vildagliptin in metformin-treated patients with type 2 diabetes over 1 year. Diabetes care *28*, 1936-1940.

Ahren, B., Pettersson, M., Uvnas-Moberg, K., Gutniak, M., and Efendic, S. (1991). Effects of cholecystokinin (CCK)-8, CCK-33, and gastric inhibitory polypeptide (GIP) on basal and meal-stimulated pancreatic hormone secretion in man. Diabetes Res Clin Pract *13*, 153-161.

Arakawa, M., Mita, T., Azuma, K., Ebato, C., Goto, H., Nomiyama, T., Fujitani, Y., Hirose, T., Kawamori, R., and Watada, H. (2010). Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4. Diabetes *59*, 1030-1037.

Aronne, L.J., Wadden, T.A., Peterson, C., Winslow, D., Odeh, S., and Gadde, K.M. (2013). Evaluation of phentermine and topiramate versus phentermine/topiramate extended-release in obese adults. Obesity (Silver Spring).

Asakawa, A., Inui, A., Ueno, N., Fujimiya, M., Fujino, M.A., and Kasuga, M. (1999). Mouse pancreatic polypeptide modulates food intake, while not influencing anxiety in mice. Peptides *20*, 1445-1448.

Asakawa, A., Inui, A., Yuzuriha, H., Ueno, N., Katsuura, G., Fujimiya, M., Fujino, M.A., Niijima, A., Meguid, M.M., and Kasuga, M. (2003). Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. Gastroenterology *124*, 1325-1336.

Astrup, A., Carraro, R., Finer, N., Harper, A., Kunesova, M., Lean, M.E., Niskanen, L., Rasmussen, M.F., Rissanen, A., Rossner, S.*, et al.* (2012). Safety, tolerability and sustained weight loss over 2 years with the once-daily human GLP-1 analog, liraglutide. Int J Obes (Lond) *36*, 843-854.

Astrup, A., Rossner, S., Van Gaal, L., Rissanen, A., Niskanen, L., Al Hakim, M., Madsen, J., Rasmussen, M.F., and Lean, M.E. (2009). Effects of liraglutide in the treatment of obesity: a randomised, double-blind, placebo-controlled study. Lancet *374*, 1606-1616.

Baetens, D., Malaisse-Lagae, F., Perrelet, A., and Orci, L. (1979). Endocrine pancreas: three-dimensional reconstruction shows two types of islets of langerhans. Science *206*, 1323-1325.

Baggio, L., Kieffer, T.J., and Drucker, D.J. (2000). Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, regulates fasting glycemia and nonenteral glucose clearance in mice. Endocrinology *141*, 3703-3709.

Ballantyne, G.H. (2006). Peptide YY(1-36) and peptide YY(3-36): Part II. Changes after gastrointestinal surgery and bariatric surgery. Obes Surg *16*, 795-803.

Ballantyne, G.H., Longo, W.E., Savoca, P.E., Adrian, T.E., Vukasin, A.P., Bilchik, A.J., Sussman, J., and Modlin, I.M. (1989). Deoxycholate-stimulated release of peptide YY from the isolated perfused rabbit left colon. Am J Physiol *257*, G715-724.

Bard, J.A., Walker, M.W., Branchek, T.A., and Weinshank, R.L. (1995). Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. J Biol Chem *270*, 26762-26765.

Barragan, J.M., Rodriguez, R.E., and Blazquez, E. (1994). Changes in arterial blood pressure and heart rate induced by glucagon-like peptide-1-(7-36) amide in rats. Am J Physiol *266*, E459-466.

Batterham, R.L., Cohen, M.A., Ellis, S.M., Le Roux, C.W., Withers, D.J., Frost, G.S., Ghatei, M.A., and Bloom, S.R. (2003a). Inhibition of food intake in obese subjects by peptide YY3-36. N Engl J Med *349*, 941-948.

Batterham, R.L., Cowley, M.A., Small, C.J., Herzog, H., Cohen, M.A., Dakin, C.L., Wren, A.M., Brynes, A.E., Low, M.J., Ghatei, M.A.*, et al.* (2002). Gut hormone PYY(3-36) physiologically inhibits food intake. Nature *418*, 650-654.

Batterham, R.L., ffytche, D.H., Rosenthal, J.M., Zelaya, F.O., Barker, G.J., Withers, D.J., and Williams, S.C. (2007). PYY modulation of cortical and hypothalamic brain areas predicts feeding behaviour in humans. Nature *450*, 106-109.

Batterham, R.L., Heffron, H., Kapoor, S., Chivers, J.E., Chandarana, K., Herzog, H., Le Roux, C.W., Thomas, E.L., Bell, J.D., and Withers, D.J. (2006). Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab *4*, 223-233.

Batterham, R.L., Le Roux, C.W., Cohen, M.A., Park, A.J., Ellis, S.M., Patterson, M., Frost, G.S., Ghatei, M.A., and Bloom, S.R. (2003b). Pancreatic polypeptide reduces appetite and food intake in humans. J Clin Endocrinol Metab *88*, 3989-3992.

Baxter, J., Minnion, J.S., Cuenco-Shillito, J., Ghatei, M.A., and Bloom, S. (2009). "Characterisation of the breakdown of Pancreatic Polypeptide (PP) in vitro and in vivo by the enzymes dipeptidyl peptidase-IV (DPPIV) and neprilysin (24.11)". Presented in the 7th James Black Conference.

Baxter, J., Minnion, J.S., Cuenco-Shillito, J., Tan, T., Murphy, K., Ghatei, M.A., and Bloom, S. (2010). Pancreatic polypeptide: a novel substrate for the endopeptidase neprilysin. Endocr Abs *21*, P133.

Bell, G.I., Sanchez-Pescador, R., Laybourn, P.J., and Najarian, R.C. (1983a). Exon duplication and divergence in the human preproglucagon gene. Nature *304*, 368-371.

Bell, G.I., Santerre, R.F., and Mullenbach, G.T. (1983b). Hamster preproglucagon contains the sequence of glucagon and two related peptides. Nature *302*, 716-718.

Berglund, M.M., Hipskind, P.A., and Gehlert, D.R. (2003a). Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. Exp Biol Med *228*, 217-244.

Berglund, M.M., Schober, D.A., Esterman, M.A., and Gehlert, D.R. (2003b). Neuropeptide Y Y4 receptor homodimers dissociate upon agonist stimulation. J Pharmacol Exp Ther *307*, 1120-1126.

Bergman, R.N., Ider, Y.Z., Bowden, C.R., and Cobelli, C. (1979). Quantitative estimation of insulin sensitivity. Am J Physiol *236*, E667-677.

Bharucha, A.E., Charkoudian, N., Andrews, C.N., Camilleri, M., Sletten, D., Zinsmeister, A.R., and Low, P.A. (2008). Effects of glucagon-like peptide-1, yohimbine, and nitrergic modulation on sympathetic and parasympathetic activity in humans. Am J Physiol Regul Integr Compr Physiol *295*, R874-880.

Bjerre Knudsen, L., Madsen, L.W., Andersen, S., Almholt, K., de Boer, A.S., Drucker, D.J., Gotfredsen, C., Egerod, F.L., Hegelund, A.C., Jacobsen, H*., et al.* (2010). Glucagon-like Peptide-1 receptor agonists activate rodent thyroid C-cells causing calcitonin release and C-cell proliferation. Endocrinology *151*, 1473-1486.

Blevins, J.E., Chelikani, P.K., Haver, A.C., and Reidelberger, R.D. (2008). PYY(3-36) induces Fos in the arcuate nucleus and in both catecholaminergic and non-catecholaminergic neurons in the nucleus tractus solitarius of rats. Peptides *29*, 112-119.

Bloom, S. (2007). Hormonal regulation of appetite. Obes Rev *8 Suppl 1*, 63-65.

Bloom, S.R., Kuhajda, F.P., Laher, I., Pi-Sunyer, X., Ronnett, G.V., Tan, T.M., and Weigle, D.S. (2008). The obesity epidemic: pharmacological challenges. Mol Interv *8*, 82-98.

Boel, E., Schwartz, T.W., Norris, K.E., and Fiil, N.P. (1984). A cDNA encoding a small common precursor for human pancreatic polypeptide and pancreatic icosapeptide. EMBO J *3*, 909-912.

Borg, C.M., le Roux, C.W., Ghatei, M.A., Bloom, S.R., Patel, A.G., and Aylwin, S.J. (2006). Progressive rise in gut hormone levels after Roux-en-Y gastric bypass suggests gut adaptation and explains altered satiety. Br J Surg *93*, 210-215.

Borowsky, B., Walker, M.W., Bard, J., Weinshank, R.L., Laz, T.M., Vaysse, P., Branchek, T.A., and Gerald, C. (1998). Molecular biology and pharmacology of multiple NPY Y5 receptor species homologs. Regul Pept *75-76*, 45-53.

Bose, A.K., Mocanu, M.M., Carr, R.D., Brand, C.L., and Yellon, D.M. (2005). Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury. Diabetes *54*, 146-151.

Bradbury, A.F., Finnie, M.D., and Smyth, D.G. (1982). Mechanism of C-terminal amide formation by pituitary enzymes. Nature *298*, 686-688.

Bradbury, A.F., and Smyth, D.G. (1987). Enzyme-catalysed peptide amidation. Isolation of a stable intermediate formed by reaction of the amidating enzyme with an imino acid. Eur J Biochem *169*, 579-584.

Broberger, C., Johansen, J., Johansson, C., Schalling, M., and Hokfelt, T. (1998). The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proc Natl Acad Sci USA *95*, 15043-15048.

Brunicardi, F.C., Chaiken, R.L., Ryan, A.S., Seymour, N.E., Hoffmann, J.A., Lebovitz, H.E., Chance, R.E., Gingerich, R.L., Andersen, D.K., and Elahi, D. (1996). Pancreatic polypeptide administration

improves abnormal glucose metabolism in patients with chronic pancreatitis. J Clin Endocrinol Metab *81*, 3566-3572.

Bryant, M.G., Bloom, S.R., Polak, J.M., Hobbs, S., Domschke, W., Domschke, S., Mitznegg, P., Ruppin, H., and Demling, L. (1983). Measurement of gut hormonal peptides in biopsies from human stomach and proximal small intestine. Gut *24*, 114-119.

Buchwald, H., Avidor, Y., Braunwald, E., Jensen, M.D., Pories, W., Fahrbach, K., and Schoelles, K. (2004). Bariatric surgery: a systematic review and meta-analysis. JAMA *292*, 1724-1737.

Buchwald, H., Estok, R., Fahrbach, K., Banel, D., Jensen, M.D., Pories, W.J., Bantle, J.P., and Sledge, I. (2009). Weight and type 2 diabetes after bariatric surgery: systematic review and meta-analysis. Am J Med *122*, 248-256 e245.

Bullock, B.P., Heller, R.S., and Habener, J.F. (1996). Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. Endocrinology *137*, 2968-2978.

Burdyga, G., de Lartigue, G., Raybould, H.E., Morris, R., Dimaline, R., Varro, A., Thompson, D.G., and Dockray, G.J. (2008). Cholecystokinin regulates expression of Y2 receptors in vagal afferent neurons serving the stomach. J Neurosci *28*, 11583-11592.

Buse, J.B., Drucker, D.J., Taylor, K.L., Kim, T., Walsh, B., Hu, H., Wilhelm, K., Trautmann, M., Shen, L.Z., Porter, L.E.*, et al.* (2010). DURATION-1: exenatide once weekly produces sustained glycemic control and weight loss over 52 weeks. Diabetes Care *33*, 1255-1261.

Buse, J.B., Rosenstock, J., Sesti, G., Schmidt, W.E., Montanya, E., Brett, J.H., Zychma, M., and Blonde, L. (2009). Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6). Lancet *374*, 39-47.

Butler, A.E., Campbell-Thompson, M., Gurlo, T., Dawson, D.W., Atkinson, M., and Butler, P.C. (2013). Marked expansion of exocrine and endocrine pancreas with incretin therapy in humans with increased exocrine pancreas dysplasia and the potential for glucagon-producing neuroendocrine tumors. Diabetes *62*, 2595-2604.

Campbell, R.M., and Scanes, C.G. (1992). Evolution of the growth hormone-releasing factor (GRF) family of peptides. Growth Regul *2*, 175-191.

Carr, R.D., Larsen, M.O., Winzell, M.S., Jelic, K., Lindgren, O., Deacon, C.F., and Ahren, B. (2008). Incretin and islet hormonal responses to fat and protein ingestion in healthy men. Am J Physiol Endocrinol Metab *295*, E779-784.

Challis, B.G., Coll, A.P., Yeo, G.S., Pinnock, S.B., Dickson, S.L., Thresher, R.R., Dixon, J., Zahn, D., Rochford, J.J., White, A.*, et al.* (2004). Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3-36). Proc Natl Acad Sci USA *101*, 4695-4700.

Chang, S.H., Stoll, C.R., Song, J., Varela, J.E., Eagon, C.J., and Colditz, G.A. (2013). The Effectiveness and Risks of Bariatric Surgery: An Updated Systematic Review and Meta-analysis, 2003-2012. JAMA Surg. doi:10.1001/jamasurg.2013.3654

Chelikani, P.K., Shah, I.H., Taqi, E., Sigalet, D.L., and Koopmans, H.H. (2010). Comparison of the effects of Roux-en-Y gastric bypass and ileal transposition surgeries on food intake, body weight, and circulating peptide YY concentrations in rats. Obes Surg *20*, 1281-1288.

Chen, C.H., and Rogers, R.C. (1995). Central inhibitory action of peptide YY on gastric motility in rats. Am J Physiol *269*, R787-792.

Christensen, R., Kristensen, P.K., Bartels, E.M., Bliddal, H., and Astrup, A. (2007). Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. Lancet *370*, 1706-1713.

Clark, J.T., Kalra, P.S., Crowley, W.R., and Kalra, S.P. (1984). Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology *115*, 427-429.

Clements, R.H., Gonzalez, Q.H., Long, C.I., Wittert, G., and Laws, H.L. (2004). Hormonal changes after Roux-en Y gastric bypass for morbid obesity and the control of type-II diabetes mellitus. Am Surg *70*, 1-4; discussion 4-5.

Cohen, M.A., Ellis, S.M., Le Roux, C.W., Batterham, R.L., Park, A., Patterson, M., Frost, G.S., Ghatei, M.A., and Bloom, S.R. (2003). Oxyntomodulin suppresses appetite and reduces food intake in humans. J Clin Endocrinol Metab *88*, 4696-4701.

Conlon, J.M. (2002). The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). Peptides *23*, 269-278.

Conlon, J.M., Platz, J.E., Chartrel, N., Vaudry, H., and Nielsen, P.F. (1998). Amino acid sequence diversity of pancreatic polypeptide among the amphibia. Gen Comp Endocrinol *112*, 146-152.

Considine, R.V., Sinha, M.K., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannesian, J.P., Marco, C.C., McKee, L.J., Bauer, T.L.*, et al.* (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med *334*, 292-295.

Crajoinas, R.O., Oricchio, F.T., Pessoa, T.D., Pacheco, B.P., Lessa, L.M., Malnic, G., and Girardi, A.C. (2011). Mechanisms mediating the diuretic and natriuretic actions of the incretin hormone glucagon-like peptide-1. Am J Physiol Renal Physiol *301*, F355-363.

Cummings, D.E. (2009). Endocrine mechanisms mediating remission of diabetes after gastric bypass surgery. Int J Obes (Lond) *33 Suppl 1*, S33-40.

de Heer, J., Rasmussen, C., Coy, D.H., and Holst, J.J. (2008). Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, inhibits glucagon secretion via somatostatin (receptor subtype 2) in the perfused rat pancreas. Diabetologia *51*, 2263-2270.

De Silva, A., Salem, V., Long, C.J., Makwana, A., Newbould, R.D., Rabiner, E.A., Ghatei, M.A., Bloom, S.R., Matthews, P.M., Beaver, J.D.*, et al.* (2011). The gut hormones PYY 3-36 and GLP-1 7-36 amide reduce food intake and modulate brain activity in appetite centers in humans. Cell metabolism *14*, 700-706.

Degen, L., Drewe, J., Piccoli, F., Grani, K., Oesch, S., Bunea, R., D'Amato, M., and Beglinger, C. (2007). Effect of CCK-1 receptor blockade on ghrelin and PYY secretion in men. Am J Physiol Regul Integr Comp Physiol *292*, R1391-1399.

DeMar, A.R., Taylor, I.L., and Fink, A.S. (1991). Pancreatic polypeptide and peptide YY inhibit the denervated canine pancreas. Pancreas *6*, 419-426.

Diakogiannaki, E., Pais, R., Tolhurst, G., Parker, H.E., Horscroft, J., Rauscher, B., Zietek, T., Daniel, H., Gribble, F.M., and Reimann, F. (2013). Oligopeptides stimulate glucagon-like peptide-1 secretion in mice through proton-coupled uptake and the calcium-sensing receptor. Diabetologia *56*, 2688-2696.

Dockray, G.J. (2009). Cholecystokinin and gut-brain signalling. Regul Pept *155*, 6-10.

Drucker, D.J., Buse, J.B., Taylor, K., Kendall, D.M., Trautmann, M., Zhuang, D., and Porter, L. (2008). Exenatide once weekly versus twice daily for the treatment of type 2 diabetes: a randomised, open-label, non-inferiority study. Lancet *372*, 1240-1250.

Drucker, D.J., Philippe, J., Mojsov, S., Chick, W.L., and Habener, J.F. (1987). Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci USA *84*, 3434-3438.

Dumont, Y., Moyse, E., Fournier, A., and Quirion, R. (2007). Distribution of peripherally injected peptide YY ([125I] PYY (3-36)) and pancreatic polypeptide ([125I] hPP) in the CNS: enrichment in the area postrema. J Mol Neurosci *33*, 294-304.

Eberlein, G.A., Eysselein, V.E., Schaeffer, M., Layer, P., Grandt, D., Goebell, H., Niebel, W., Davis, M., Lee, T.D., Shively, J.E.*, et al.* (1989). A new molecular form of PYY: structural characterization of human PYY(3-36) and PYY(1-36). Peptides *10*, 797-803.

Edfalk, S., Steneberg, P., and Edlund, H. (2008). Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. Diabetes *57*, 2280-2287.

Edwards, C.M., Abbott, C.R., Sunter, D., Kim, M., Dakin, C.L., Murphy, K.G., Abusnana, S., Taheri, S., Rossi, M., and Bloom, S.R. (2000). Cocaine- and amphetamine-regulated transcript, glucagon-like peptide-1 and corticotrophin releasing factor inhibit feeding via agouti-related protein independent pathways in the rat. Brain Res *866*, 128-134.

Ekblad, E., and Sundler, F. (2002). Distribution of pancreatic polypeptide and peptide YY. Peptides *23*, 251-261.

Elahi, D., Egan, J.M., Shannon, R.P., Meneilly, G.S., Khatri, A., Habener, J.F., and Andersen, D.K. (2008). GLP-1 (9-36) amide, cleavage product of GLP-1 (7-36) amide, is a glucoregulatory peptide. Obesity (Silver Spring) *16*, 1501-1509.

Elias, C.F., Lee, C., Kelly, J., Aschkenasi, C., Ahima, R.S., Couceyro, P.R., Kuhar, M.J., Saper, C.B., and Elmquist, J.K. (1998). Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron *21*, 1375-1385.

European Medicines Agency (2013). Investigation into GLP-1-based diabetes therapies concluded. http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2013/07/WC500146619.pdf

Feinle-Bisset, C., Patterson, M., Ghatei, M.A., Bloom, S.R., and Horowitz, M. (2005). Fat digestion is required for suppression of ghrelin and stimulation of peptide YY and pancreatic polypeptide secretion by intraduodenal lipid. Am J Physiol Endocrinol Metab *289*, E948-953.

Feltrin, K.L., Patterson, M., Ghatei, M.A., Bloom, S.R., Meyer, J.H., Horowitz, M., and Feinle-Bisset, C. (2006). Effect of fatty acid chain length on suppression of ghrelin and stimulation of PYY, GLP-2 and PP secretion in healthy men. Peptides *27*, 1638-1643.

Ferrier, L., Segain, J.P., Bonnet, C., Cherbut, C., Lehur, P.A., Jarry, A., Galmiche, J.P., and Blottiere, H.M. (2002). Functional mapping of NPY/PYY receptors in rat and human gastro-intestinal tract. Peptides *23*, 1765-1771.

Flegal, K.M. (2005). Epidemiologic aspects of overweight and obesity in the United States. Physiol Behav *86*, 599-602.

Flint, A., Raben, A., Astrup, A., and Holst, J.J. (1998). Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest *101*, 515-520.

Flint, A., Raben, A., Blundell, J.E., and Astrup, A. (2000). Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. Int J Obes Relat Metab Disord *24*, 38-48.

Flynn, M.C., Plata-Salaman, C.R., and Ffrench-Mullen, J.M. (1999). Neuropeptide Y-related compounds and feeding. Physiol Behav *65*, 901-905.

Fu-Cheng, X., Anini, Y., Chariot, J., Castex, N., Galmiche, J.P., and Roze, C. (1997). Mechanisms of peptide YY release induced by an intraduodenal meal in rats: neural regulation by proximal gut. Pflugers Archiv *433*, 571-579.

Fuhlendorff, J., Johansen, N.L., Melberg, S.G., Thøgersen, H., and Schwartz, T.W. (1990). The antiparallel pancreatic polypeptide fold in the binding of neuropeptide Y to Y1 and Y2 receptors. J Biol Chem *265*, 11706-11712.

Funch, D., Gydesen, H., Tornoe, K., Major-Pedersen, A., and Chan, K.A. (2013). A prospective, claims-based assessment of the risk of pancreatitis and pancreatic cancer with liraglutide compared to other antidiabetic drugs. Diabetes Obes Metab. doi: 10.1111/dom.12230

Gallwitz, B., Vaag, A., Falahati, A., and Madsbad, S. (2010). Adding liraglutide to oral antidiabetic drug therapy: onset of treatment effects over time. Int J Clin Pract *64*, 267-276.

Garvey, W.T., Ryan, D.H., Look, M., Gadde, K.M., Allison, D.B., Peterson, C.A., Schwiers, M., Day, W.W., and Bowden, C.H. (2012). Two-year sustained weight loss and metabolic benefits with controlled-release phentermine/topiramate in obese and overweight adults (SEQUEL): a randomized, placebo-controlled, phase 3 extension study. Am J Clin Nutr *95*, 297-308.

Gaspari, T., Welungoda, I., Widdop, R.E., Simpson, R.W., and Dear, A.E. (2013). The GLP-1 receptor agonist liraglutide inhibits progression of vascular disease via effects on atherogenesis, plaque stability and endothelial function in an ApoE(-/-) mouse model. Diabetes Vasc Dis Res *10*, 353-360.

Gehlert, D.R., Schober, D.A., Beavers, L., Gadski, R., Hoffman, J.A., Smiley, D.L., Chance, R.E., Lundell, I., and Larhammar, D. (1996). Characterization of the peptide binding requirements for the cloned human pancreatic polypeptide-preferring receptor. Mol Pharmacol *50*, 112-118.

Gerspach, A.C., Steinert, R.E., Schonenberger, L., Graber-Maier, A., and Beglinger, C. (2011). The role of the gut sweet taste receptor in regulating GLP-1, PYY, and CCK release in humans. Am J Physiol Endocrinol Metab *301*, E317-325.

Ghatei, M.A., Uttenthal, L.O., Bryant, M.G., Christofides, N.D., Moody, A.J., and Bloom, S.R. (1983). Molecular forms of glucagon-like immunoreactivity in porcine intestine and pancreas. Endocrinology *112*, 917-923.

Ghiglione, M., Uttenthal, L.O., George, S.K., and Bloom, S.R. (1984). How glucagon-like is glucagon-like peptide-1? Diabetologia *27*, 599-600.

Glaser, B., Vinik, A.I., Sive, A.A., and Floyd, J.C., Jr. (1980). Plasma human pancreatic polypeptide responses to administered secretin: effects of surgical vagotomy, cholinergic blockade, and chronic pancreatitis. J Clin Endocrinol Metab *50*, 1094-1099.

Glover, I.D., Barlow, D.J., Pitts, J.E., Wood, S.P., Tickle, I.J., Blundell, T.L., Tatemoto, K., Kimmel, J.R., Wollmer, A., Strassburger, W*., et al.* (1984). Conformational studies on the pancreatic polypeptide hormone family. Eur J Biochem *142*, 379-385.

Godsland, I.F., Agbaje, O.F., and Hovorka, R. (2006). Evaluation of nonlinear regression approaches to estimation of insulin sensitivity by the minimal model with reference to Bayesian hierarchical analysis. Am J Physiol Endocrinol Metab *291*, E167-174.

Gonzalez, N., Moody, T.W., Igarashi, H., Ito, T., and Jensen, R.T. (2008). Bombesin-related peptides and their receptors: recent advances in their role in physiology and disease states. Curr Opin Endocr Obes *15*, 58-64.

Gough, K., Hutchison, M., Keene, O., Byrom, B., Ellis, S., Lacey, L., and McKellar, J. (1995). Assessment of dose-proportionality: report from the statisticians in the pharmaceutical industry/pharmacokinetics UK joint working party. Drug Inform J *29*, 1039-1048.

Goumain, M., Voisin, T., Lorinet, A.M., and Laburthe, M. (1998). Identification and distribution of mRNA encoding the Y1, Y2, Y4, and Y5 receptors for peptides of the PP-fold family in the rat intestine and colon. Biochem Biophys Res Commun *247*, 52-56.

Grandt, D., Dahms, P., Schimiczek, M., Eysselein, V.E., Reeve, J.R., Jr., and Mentlein, R. (1993). [Proteolytic processing by dipeptidyl aminopeptidase IV generates receptor selectivity for peptide YY (PYY)]. Med Klin (Munich) *88*, 143-145.

Greeley, G.H., Jr., Hashimoto, T., Izukura, M., Gomez, G., Jeng, J., Hill, F.L., Lluis, F., and Thompson, J.C. (1989a). A comparison of intraduodenally and intracolonically administered nutrients on the release of peptide-YY in the dog. Endocrinology *125*, 1761-1765.

Greeley, G.H., Jr., Jeng, Y.J., Gomez, G., Hashimoto, T., Hill, F.L., Kern, K., Kurosky, T., Chuo, H.F., and Thompson, J.C. (1989b). Evidence for regulation of peptide-YY release by the proximal gut. Endocrinology *124*, 1438-1443.

Greeley, G.H., Jr., Lluis, F., Gomez, G., Ishizuka, J., Holland, B., and Thompson, J.C. (1988). Peptide YY antagonizes beta-adrenergic-stimulated release of insulin in dogs. Am J Physiol *254*, E513-517.

Greenberg, G.R., McCloy, R.F., Adrian, T.E., Chadwick, V.S., Baron, J.H., and Bloom, S.R. (1978). Inhibition of pancreas and gallbladder by pancreatic polypeptide. Lancet *2*, 1280-1282.

Greenough, A., Cole, G., Lewis, J., Lockton, A., and Blundell, J. (1998). Untangling the effects of hunger, anxiety, and nausea on energy intake during intravenous cholecystokinin octapeptide (CCK-8) infusion. Physiol Behav *65*, 303-310.

Greenway, F.L., Fujioka, K., Plodkowski, R.A., Mudaliar, S., Guttadauria, M., Erickson, J., Kim, D.D., Dunayevich, E., and Group, C.-I.S. (2010). Effect of naltrexone plus bupropion on weight loss in overweight and obese adults (COR-I): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet *376*, 595-605.

Gregor, P., Feng, Y., DeCarr, L.B., Cornfield, L.J., and McCaleb, M.L. (1996). Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. J Biol Chem *271*, 27776-27781.

Gribble, F.M., Williams, L., Simpson, A.K., and Reimann, F. (2003). A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line. Diabetes *52*, 1147-1154.

Guo, Y.S., Fujimura, M., Lluis, F., Tsong, Y., Greeley, G.H., Jr., and Thompson, J.C. (1987a). Inhibitory action of peptide YY on gastric acid secretion. Am J Physiol *253*, G298-302.

Guo, Y.S., Singh, P., Gomez, G., Greeley, G.H., Jr., and Thompson, J.C. (1987b). Effect of peptide YY on cephalic, gastric, and intestinal phases of gastric acid secretion and on the release of gastrointestinal hormones. Gastroenterology *92*, 1202-1208.

Gutzwiller, J.P., Drewe, J., Goke, B., Schmidt, H., Rohrer, B., Lareida, J., and Beglinger, C. (1999). Glucagon-like peptide-1 promotes satiety and reduces food intake in patients with diabetes mellitus type 2. Am J Physiol *276*, R1541-1544.

Gutzwiller, J.P., Tschopp, S., Bock, A., Zehnder, C.E., Huber, A.R., Kreyenbuehl, M., Gutmann, H., Drewe, J., Henzen, C., Goeke, B.*, et al.* (2004). Glucagon-like peptide 1 induces natriuresis in healthy subjects and in insulin-resistant obese men. J Clin Endocrinol Metab *89*, 3055-3061.

Halatchev, I.G., Ellacott, K.L., Fan, W., and Cone, R.D. (2004). Peptide YY3-36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology *145*, 2585-2590.

Halbirk, M., Norrelund, H., Moller, N., Holst, J.J., Schmitz, O., Nielsen, R., Nielsen-Kudsk, J.E., Nielsen, S.S., Nielsen, T.T., Eiskjaer, H.*, et al.* (2010). Cardiovascular and metabolic effects of 48-h glucagon-like peptide-1 infusion in compensated chronic patients with heart failure. Am J Physiol Heart Circ Physiol *298*, H1096-1102.

Hankir, M.K., Parkinson, J.R., Minnion, J.S., Addison, M.L., Bloom, S.R., and Bell, J.D. (2011). Peptide YY 3-36 and pancreatic polypeptide differentially regulate hypothalamic neuronal activity in mice in vivo as measured by manganese-enhanced magnetic resonance imaging. Neuroendocrinology *23*, 371-380.

Hedo, J.A., Villanueva, M.L., and Marco, J. (1978). Stimulation of pancreatic polypeptide and glucagon secretion by 2-deoxy-D-glucose in man: evidence for cholinergic mediation. J Clin Endocrinol Metab *47*, 366-371.

Heredia, M.d.P., Delgado, C., Pereira, L., Perrier, R., Richard, S., Vassort, G., Benitah, J.P., and Gomez, A.M. (2005). Neuropeptide Y rapidly enhances [Ca2+]i transients and Ca2+ sparks in adult rat ventricular myocytes through Y1 receptor and PLC activation. J Mol Cell Cardiol *38*, 205-212.

Herman, G.A., Bergman, A., Stevens, C., Kotey, P., Yi, B., Zhao, P., Dietrich, B., Golor, G., Schrodter, A., Keymeulen, B.*, et al.* (2006). Effect of single oral doses of sitagliptin, a dipeptidyl peptidase-4 inhibitor, on incretin and plasma glucose levels after an oral glucose tolerance test in patients with type 2 diabetes. J Clin Endocrinol Metab *91*, 4612-4619.

Herrmann, C., Goke, R., Richter, G., Fehmann, H.C., Arnold, R., and Goke, B. (1995). Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. Digestion *56*, 117-126.

Hirata, K., Kume, S., Araki, S., Sakaguchi, M., Chin-Kanasaki, M., Isshiki, K., Sugimoto, T., Nishiyama, A., Koya, D., Haneda, M.*, et al.* (2009). Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model. Biochem Biophys Res Commun *380*, 44-49.

Hollander, P., Gupta, A.K., Plodkowski, R., Greenway, F., Bays, H., Burns, C., Klassen, P., Fujioka, K., and Group, C.O.-D.S. (2013). Effects of naltrexone sustained- release/bupropion sustained-release combination therapy on body weight and glycemic parameters in overweight and obese patients with type 2 diabetes. Diabetes Care *36*, 4022-4029.

Holst, J.J., Schwartz, T.W., Lovgreen, N.A., Pedersen, O., and Beck-Nielsen, H. (1983). Diurnal profile of pancreatic polypeptide, pancreatic glucagon, gut glucagon and insulin in human morbid obesity. International journal of *7*, 529-538.

Holz, G.G. (2004). Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell. Diabetes *53*, 5-13.

Hort, Y., Baker, E., Sutherland, G.R., Shine, J., and Herzog, H. (1995). Gene duplication of the human peptide YY gene (PYY) generated the pancreatic polypeptide gene (PPY) on chromosome 17q21.1. Genomics *26*, 77-83.

Hsieh, J., Longuet, C., Baker, C.L., Qin, B., Federico, L.M., Drucker, D.J., and Adeli, K. (2010). The glucagon-like peptide 1 receptor is essential for postprandial lipoprotein synthesis and secretion in hamsters and mice. Diabetologia *53*, 552-561.

Inui, A., Okita, M., Nakajima, M., Inoue, T., Sakatani, N., Oya, M., Morioka, H., Okimura, Y., Chihara, K., and Baba, S. (1991). Neuropeptide regulation of feeding in dogs. Am J Physiol *261*, R588-594.

Izukura, M., Hashimoto, T., Gomez, G., Uchida, T., Greeley, G.H., Jr., and Thompson, J.C. (1991). Intracolonic infusion of bile salt stimulates release of peptide YY and inhibits cholecystokinin-stimulated pancreatic exocrine secretion in conscious dogs. Pancreas *6*, 427-432.

Jesudason, D.R., Monteiro, M.P., McGowan, B.M., Neary, N.M., Park, A.J., Philippou, E., Small, C.J., Frost, G.S., Ghatei, M.A., and Bloom, S.R. (2007). Low-dose pancreatic polypeptide inhibits food intake in man. The British journal of nutrition *97*, 426-429.

Johns, C.E., Newton, J.L., Westley, B.R., and May, F.E. (2006). Human pancreatic polypeptide has a marked diurnal rhythm that is affected by ageing and is associated with the gastric TFF2 circadian rhythm. Peptides *27*, 1341-1348.

Jorde, R., and Burhol, P.G. (1984). Fasting and postprandial plasma pancreatic polypeptide (PP) levels in obesity. Int J Obes (Lond) *8*, 393-397.

Jung, G., Louie, D.S., and Owyang, C. (1987). Pancreatic polypeptide inhibits pancreatic enzyme secretion via a cholinergic pathway. Am J Physiol *253*, G706-710.

Kahn, S.E., Carr, D.B., Faulenbach, M.V., and Utzschneider, K.M. (2008). An examination of beta-cell function measures and their potential use for estimating beta-cell mass. Diabetes, obesity & metabolism *10 Suppl 4*, 63-76.

Kahn, S.E., Haffner, S.M., Heise, M.A., Herman, W.H., Holman, R.R., Jones, N.P., Kravitz, B.G., Lachin, J.M., O'Neill, M.C., Zinman, B.*, et al.* (2006). Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N Engl J Med *355*, 2427-2443.

Kahn, S.E., Prigeon, R.L., McCulloch, D.K., Boyko, E.J., Bergman, R.N., Schwartz, M.W., Neifing, J.L., Ward, W.K., Beard, J.C., Palmer, J.P.*, et al.* (1993). Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function. Diabetes *42*, 1663-1672.

Karaki, S., Mitsui, R., Hayashi, H., Kato, I., Sugiya, H., Iwanaga, T., Furness, J.B., and Kuwahara, A. (2006). Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res *324*, 353-360.

Kavianipour, M., Ehlers, M.R., Malmberg, K., Ronquist, G., Ryden, L., Wikstrom, G., and Gutniak, M. (2003). Glucagon-like peptide-1 (7-36) amide prevents the accumulation of pyruvate and lactate in the ischemic and non-ischemic porcine myocardium. Peptides *24*, 569-578.

Keire, D.A., Kobayashi, M., Solomon, T.E., and Reeve, J.R., Jr. (2000a). Solution structure of monomeric peptide YY supports the functional significance of the PP-fold. Biochemistry *39*, 9935-9942.

Keire, D.A., Mannon, P., Kobayashi, M., Walsh, J.H., Solomon, T.E., and Reeve, J.R., Jr. (2000b). Primary structures of PYY, [Pro(34)]PYY, and PYY-(3-36) confer different conformations and receptor selectivity. Am J Physiol Gastrointest Liver Physiol *279*, G126-131.

Kieffer, T.J., and Habener, J.F. (1999). The glucagon-like peptides. Endocr Rev *20*, 876-913.

Kimmel, J.R., Hayden, L.J., and Pollock, H.G. (1975). Isolation and characterization of a new pancreatic polypeptide hormone. J Biol Chem *250*, 9369-9376.

Kimmel, J.R., Pollock, H.G., and Hazelwood, R.L. (1968). Isolation and characterization of chicken insulin. Endocrinology *83*, 1323-1330.

Kirkpatrick, A., Heo, J., Abrol, R., and Goddard, W.A., 3rd (2012). Predicted structure of agonist-bound glucagon-like peptide 1 receptor, a class B G protein-coupled receptor. Proc Natl Acad Sci USA *109*, 19988-19993.

Kjems, L.L., Holst, J.J., Volund, A., and Madsbad, S. (2003). The influence of GLP-1 on glucose-stimulated insulin secretion: effects on beta-cell sensitivity in type 2 and nondiabetic subjects. Diabetes *52*, 380-386.

Kleinman, R., Gingerich, R., Ohning, G., Wong, H., Olthoff, K., Walsh, J., and Brunicardi, F.C. (1995). The influence of somatostatin on glucagon and pancreatic polypeptide secretion in the isolated perfused human pancreas. Int J Pancreatol *18*, 51-57.

Koda, S., Date, Y., Murakami, N., Shimbara, T., Hanada, T., Toshinai, K., Niijima, A., Furuya, M., Inomata, N., Osuye, K.*, et al.* (2005). The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. Endocrinology *146*, 2369-2375.

Kohler, H., Nustede, R., Barthel, M., Muller, C., and Schafmayer, A. (1991). Total denervation of the pancreas does not alter the pancreatic polypeptide-induced inhibition of pancreatic exocrine secretion in dogs. Res Exp Med (Berl) *191*, 359-369.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature *402*, 656-660.

Kojima, S., Ueno, N., Asakawa, A., Sagiyama, K., Naruo, T., Mizuno, S., and Inui, A. (2007). A role for pancreatic polypeptide in feeding and body weight regulation. Peptides *28*, 459-463.

Konturek, S.J., Bielanski, W., Kwiecien, N., Konturek, J., and Czarnobilski, K. (1987). Immunoneutralization of circulating pancreatic polypeptide and pancreatic secretion. Pancreas *2*, 536-544.

Korner, J., Bessler, M., Inabnet, W., Taveras, C., and Holst, J.J. (2007). Exaggerated glucagon-like peptide-1 and blunted glucose-dependent insulinotropic peptide secretion are associated with Roux-en-Y gastric bypass but not adjustable gastric banding. Surg Obes Relat Dis *3*, 597-601.

Koska, J., DelParigi, A., de Courten, B., Weyer, C., and Tataranni, P.A. (2004). Pancreatic polypeptide is involved in the regulation of body weight in pima Indian male subjects. Diabetes *53*, 3091-3096.

Kraegen, E.W., Lazarus, L., Meler, H., Campbell, L., and Chia, Y.O. (1975). Carrier solutions for low-level intravenous insulin infusion. BMJ *3*, 464-466.

Kreymann, B., Williams, G., Ghatei, M.A., and Bloom, S.R. (1987). Glucagon-like peptide-1 7-36: a physiological incretin in man. Lancet *2*, 1300-1304.

Kuo, S.C., Gananadha, S., Scarlett, C.J., Gill, A., and Smith, R.C. (2008). Sporadic pancreatic polypeptide secreting tumors (PPomas) of the pancreas. World J Surg *32*, 1815-1822.

Kushi, A., Sasai, H., Koizumi, H., Takeda, N., Yokoyama, M., and Nakamura, M. (1998). Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. Proc Natl Acad Sci USA *95*, 15659-15664.

Lamers, C.B., Diemel, C.M., and Jansen, J.B. (1984). Comparative study of plasma pancreatic polypeptide responses to food, secretin, and bombesin in normal subjects and in patients with chronic pancreatitis. Dig Dis Sci *29*, 102-108.

Larhammar, D., Soderberg, C., and Lundell, I. (1998). Evolution of the neuropeptide Y family and its receptors. Ann NY Acad Sci *839*, 35-40.

Larsen, P.J., and Kristensen, P. (1997). The neuropeptide Y (Y4) receptor is highly expressed in neurones of the rat dorsal vagal complex. Brain Res Mol Brain Res *48*, 1-6.

Larsen, P.J., and Kristensen, P. (2000). Central Y4 receptor distribution. Radioactive ribonucleotide probe in situ hybridization with in vitro receptor autoradiography. Methods Mol Biol *153*, 185-198.

Larsen, P.J., Tang-Christensen, M., Holst, J.J., and Orskov, C. (1997a). Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience *77*, 257-270.

Larsen, P.J., Tang-Christensen, M., and Jessop, D.S. (1997b). Central administration of glucagon-like peptide-1 activates hypothalamic neuroendocrine neurons in the rat. Endocrinology *138*, 4445-4455.

Larsson, L.I., Schwartz, T., Lundqvist, G., Chance, R.E., Sundler, F., Rehfeld, J.F., Grimelius, L., Fahrenkrug, J., Schaffalitzky de Muckadell, O., and Moon, N. (1976). Occurrence of human pancreatic polypeptide in pancreatic endocrine tumors. Possible implication in the watery diarrhea syndrome. Am J Pathol *85*, 675-684.

Larsson, L.I., Sundler, F., and Hakanson, R. (1975). Immunohistochemical localization of human pancreatic polypeptide (HPP) to a population of islet cells. Cell Tissue Res *156*, 167-171.

Lassmann, V., Vague, P., Vialettes, B., and Simon, M.C. (1980). Low plasma levels of pancreatic polypeptide in obesity. Diabetes *29*, 428-430.

Lauffer, L.M., Iakoubov, R., and Brubaker, P.L. (2009). GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell. Diabetes *58*, 1058-1066.

Le Roux, C.W., Aylwin, S.J., Batterham, R.L., Borg, C.M., Coyle, F., Prasad, V., Shurey, S., Ghatei, M.A., Patel, A.G., and Bloom, S.R. (2006a). Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. Ann Surg *243*, 108-114.

Le Roux, C.W., Batterham, R.L., Aylwin, S.J., Patterson, M., Borg, C.M., Wynne, K.J., Kent, A., Vincent, R.P., Gardiner, J., Ghatei, M.A.*, et al.* (2006b). Attenuated peptide YY release in obese subjects is associated with reduced satiety. Endocrinology *147*, 3-8.

le Roux, C.W., Borg, C.M., Murphy, K.G., Vincent, R.P., Ghatei, M.A., and Bloom, S.R. (2008). Supraphysiological doses of intravenous PYY3-36 cause nausea, but no additional reduction in food intake. Ann Clin Biochem *45*, 93-95.

Leiter, A.B., Montminy, M.R., Jamieson, E., and Goodman, R.H. (1985). Exons of the human pancreatic polypeptide gene define functional domains of the precursor. J Biol Chem *260*, 13013-13017.

Lieverse, R.J., Masclee, A.A., Jansen, J.B., and Lamers, C.B. (1994). Plasma cholecystokinin and pancreatic polypeptide secretion in response to bombesin, meal ingestion and modified sham feeding in lean and obese persons. Int J Obes Relat Metab Disord *18*, 123-127.

Lin, S., Shi, Y.C., Yulyaningsih, E., Aljanova, A., Zhang, L., Macia, L., Nguyen, A.D., Lin, E.J., During, M.J., Herzog, H.*, et al.* (2009). Critical role of arcuate Y4 receptors and the melanocortin system in pancreatic polypeptide-induced reduction in food intake in mice. PloS One *4*, e8488.

Lin, T.M., and Chance, R.E. (1974). Candidate hormones of the gut. VI. Bovine pancreatic polypeptide (BPP) and avian pancreatic polypeptide (APP). Gastroenterology *67*, 737-738.

Lin, T.M., Evans, D.C., Chance, R.E., and Spray, G.F. (1977). Bovine pancreatic peptide: action on gastric and pancreatic secretion in dogs. Am J Physiol *232*, E311-315.

Lockie, S.H., Heppner, K.M., Chaudhary, N., Chabenne, J.R., Morgan, D.A., Veyrat-Durebex, C., Ananthakrishnan, G., Rohner-Jeanrenaud, F., Drucker, D.J., DiMarchi, R.*, et al.* (2012). Direct control of brown adipose tissue thermogenesis by central nervous system glucagon-like peptide-1 receptor signaling. Diabetes *61*, 2753-2762.

Lonborg, J., Vejlstrup, N., Kelbaek, H., Botker, H.E., Kim, W.Y., Mathiasen, A.B., Jorgensen, E., Helqvist, S., Saunamaki, K., Clemmensen, P.*, et al.* (2012). Exenatide reduces reperfusion injury in patients with ST-segment elevation myocardial infarction. Eur Heart J *33*, 1491-1499.

Longo, W.E., Ballantyne, G.H., Savoca, P.E., Adrian, T.E., Bilchik, A.J., and Modlin, I.M. (1991). Short-chain fatty acid release of peptide YY in the isolated rabbit distal colon. Scand J Gastroenterol *26*, 442-448.

Louie, D.S., Williams, J.A., and Owyang, C. (1985). Action of pancreatic polypeptide on rat pancreatic secretion: in vivo and in vitro. Am J Physiol *249*, G489-495.

Lu, M., Wheeler, M.B., Leng, X.H., and Boyd, A.E., 3rd (1993). The role of the free cytosolic calcium level in beta-cell signal transduction by gastric inhibitory polypeptide and glucagon-like peptide I(7-37). Endocrinology *132*, 94-100.

Lund, P.K. (2005). The discovery of glucagon-like peptide 1. Regul Pept *128*, 93-96.

Lund, P.K., Goodman, R.H., and Habener, J.F. (1981). Pancreatic pre-proglucagons are encoded by two separate mRNAs. J Biol Chem *256*, 6515-6518.

Lundell, I., Blomqvist, A.G., Berglund, M.M., Schober, D.A., Johnson, D., Statnick, M.A., Gadski, R.A., Gehlert, D.R., and Larhammar, D. (1995). Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. J Biol Chem *270*, 29123-29128.

Lundell, I., Statnick, M.A., Johnson, D., Schober, D.A., Starback, P., Gehlert, D.R., and Larhammar, D. (1996). The cloned rat pancreatic polypeptide receptor exhibits profound differences to the orthologous receptor. Proc Natl Acad Sci USA *93*, 5111-5115.

Ma, X., Bruning, J., and Ashcroft, F.M. (2007). Glucagon-like peptide 1 stimulates hypothalamic proopiomelanocortin neurons. J Neurosci *27*, 7125-7129.

Malaisse-Lagae, F., Carpentier, J.L., Patel, Y.C., Malaisse, W.J., and Orci, L. (1977). Pancreatic polypeptide: a possible role in the regulation of food intake in the mouse. Hypothesis. Experientia *33*, 915-917.

Malik, S., McGlone, F., Bedrossian, D., and Dagher, A. (2008). Ghrelin modulates brain activity in areas that control appetitive behavior. Cell Metab *7*, 400-409.

Malmstrom, R.E. (1997). Neuropeptide Y Y1 receptor mechanisms in sympathetic vascular control. Acta Physiol Scand Suppl *636*, 1-55.

Malmstrom, R.E., Lundberg, J.O., and Weitzberg, E. (2002). Autoinhibitory function of the sympathetic prejunctional neuropeptide Y Y(2) receptor evidenced by BIIE0246. Eur J Pharmacol *439*, 113-119.

Marco, J., Hedo, J.A., and Villanueva, M.L. (1977). Inhibitory effect of somatostatin on human pancreatic polypeptide secretion. Life Sci *21*, 789-792.

Martinez, A., Montuenga, L.M., Springall, D.R., Treston, A., Cuttitta, F., and Polak, J.M. (1993). Immunocytochemical localization of peptidylglycine alpha-amidating monooxygenase enzymes (PAM) in human endocrine pancreas. J Histochem Cytochem *41*, 375-380.

Matsuda, H., Brumovsky, P.R., Kopp, J., Pedrazzini, T., and Hokfelt, T. (2002). Distribution of neuropeptide Y Y1 receptors in rodent peripheral tissues. J Compr Neurol *449*, 390-404.

Matsumoto, M., Nomura, T., Momose, K., Ikeda, Y., Kondou, Y., Akiho, H., Togami, J., Kimura, Y., Okada, M., and Yamaguchi, T. (1996). Inactivation of a novel neuropeptide Y/peptide YY receptor gene in primate species. J Biol Chem *271*, 27217-27220.

Matthews, J.N., Altman, D.G., Campbell, M.J., and Royston, P. (1990). Analysis of serial measurements in medical research. BMJ *300*, 230-235.

Meier, J.J., Gethmann, A., Gotze, O., Gallwitz, B., Holst, J.J., Schmidt, W.E., and Nauck, M.A. (2006). Glucagon-like peptide 1 abolishes the postprandial rise in triglyceride concentrations and lowers levels of non-esterified fatty acids in humans. Diabetologia *49*, 452-458.

Mentlein, R., Gallwitz, B., and Schmidt, W.E. (1993). Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem *214*, 829-835.

Michel, M.C. (1991). Receptors for neuropeptide Y: multiple subtypes and multiple second messengers. Trends Pharmacol Sci *12*, 389-394.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998). XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol Rev *50*, 143-150.

Migita, K., Loewy, A.D., Ramabhadran, T.V., Krause, J.E., and Waters, S.M. (2001). Immunohistochemical localization of the neuropeptide Y Y1 receptor in rat central nervous system. Brain Res *889*, 23-37.

Mingrone, G., Panunzi, S., De Gaetano, A., Guidone, C., Iaconelli, A., Leccesi, L., Nanni, G., Pomp, A., Castagneto, M., Ghirlanda, G.*, et al.* (2012). Bariatric surgery versus conventional medical therapy for type 2 diabetes. N Engl J Med *366*, 1577-1585.

Misra, S., Murthy, K.S., Zhou, H., and Grider, J.R. (2004). Coexpression of Y1, Y2, and Y4 receptors in smooth muscle coupled to distinct signaling pathways. J Pharmacol Exp Ther *311*, 1154-1162.

Mojsov, S., Heinrich, G., Wilson, I.B., Ravazzola, M., Orci, L., and Habener, J.F. (1986). Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. J Biol Chem *261*, 11880-11889.

Morgan, J.F., Reid, F., and Lacey, J.H. (1999). The SCOFF questionnaire: assessment of a new screening tool for eating disorders. BMJ *319*, 1467-1468.

Munzberg, H., and Myers, M.G., Jr. (2005). Molecular and anatomical determinants of central leptin resistance. Nature Neurosci *8*, 566-570.

Murphy, K.G., and Bloom, S.R. (2006). Gut hormones and the regulation of energy homeostasis. Nature *444*, 854-859.

Myers, M.G., Cowley, M.A., and Munzberg, H. (2008). Mechanisms of leptin action and leptin resistance. Annu Rev Physiol *70*, 537-556.

Nagashima, M., Watanabe, T., Terasaki, M., Tomoyasu, M., Nohtomi, K., Kim-Kaneyama, J., Miyazaki, A., and Hirano, T. (2011). Native incretins prevent the development of atherosclerotic lesions in apolipoprotein E knockout mice. Diabetologia *54*, 2649-2659.

Nakagawa, A., Satake, H., Nakabayashi, H., Nishizawa, M., Furuya, K., Nakano, S., Kigoshi, T., Nakayama, K., and Uchida, K. (2004). Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells. Autonomic Neurosci *110*, 36-43.

Nakamura, M., Yokoyama, M., Watanabe, H., and Matsumoto, T. (1997). Molecular cloning, organization and localization of the gene for the mouse neuropeptide Y-Y5 receptor. Biochim Biophys Acta *1328*, 83-89.

Nauck, M.A., Heimesaat, M.M., Orskov, C., Holst, J.J., Ebert, R., and Creutzfeldt, W. (1993a). Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. J Clin Invest *91*, 301-307.

Nauck, M.A., Kleine, N., Orskov, C., Holst, J.J., Willms, B., and Creutzfeldt, W. (1993b). Normalization of fasting hyperglycaemia by exogenous glucagon-like peptide 1 (7-36 amide) in type 2 (non-insulin-dependent) diabetic patients. Diabetologia *36*, 741-744.

Neary, N.M., Small, C.J., Druce, M.R., Park, A.J., Ellis, S.M., Semjonous, N.M., Dakin, C.L., Filipsson, K., Wang, F., Kent, A.S.*, et al.* (2005). Peptide YY3-36 and glucagon-like peptide-17-36 inhibit food intake additively. Endocrinology *146*, 5120-5127.

Nguyen, N.Q., Fraser, R.J., Chapman, M., Bryant, L.K., Wishart, J., Holloway, R.H., and Horowitz, M. (2006). Fasting and nutrient-stimulated plasma peptide-YY levels are elevated in critical illness and associated with feed intolerance: an observational, controlled study. Crit Care *10*, R175.

Nguyen, T.D., Wolfe, M.S., Heintz, G.G., Whitcomb, D.C., and Taylor, I.L. (1992). High affinity binding proteins for pancreatic polypeptide on rat liver membranes. J Biol Chem *267*, 9416-9421.

Nikolaidis, L.A., Mankad, S., Sokos, G.G., Miske, G., Shah, A., Elahi, D., and Shannon, R.P. (2004). Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion. Circulation *109*, 962-965.

Nogueiras, R., Perez-Tilve, D., Veyrat-Durebex, C., Morgan, D.A., Varela, L., Haynes, W.G., Patterson, J.T., Disse, E., Pfluger, P.T., Lopez, M.*, et al.* (2009). Direct control of peripheral lipid deposition by CNS GLP-1 receptor signaling is mediated by the sympathetic nervous system and blunted in diet-induced obesity. J Neurosci *29*, 5916-5925.

Nohr, M.K., Pedersen, M.H., Gille, A., Egerod, K.L., Engelstoft, M.S., Husted, A.S., Sichlau, R.M., Grunddal, K.V., Seier Poulsen, S., Han, S.*, et al.* (2013). GPR41/FFAR3 and GPR43/FFAR2 as Cosensors for Short-Chain Fatty Acids in Enteroendocrine Cells vs FFAR3 in Enteric Neurons and FFAR2 in Enteric Leukocytes. Endocrinology *154*, 3552-3564.

Nygaard, R., Nielbo, S., Schwartz, T.W., and Poulsen, F.M. (2006). The PP-fold solution structure of human polypeptide YY and human PYY3-36 as determined by NMR. Biochemistry *45*, 8350-8357.

Nystrom, T., Gutniak, M.K., Zhang, Q., Zhang, F., Holst, J.J., Ahren, B., and Sjoholm, A. (2004). Effects of glucagon-like peptide-1 on endothelial function in type 2 diabetes patients with stable coronary artery disease. Am J Physiol Endocrinol Metab *287*, E1209-1215.

O'Hare, M.M., Huda, I., Sloan, J.M., Kennedy, T.L., and Buchanan, K.D. (1985). Characterization of immunoreactive forms of pancreatic polypeptide in islet cell tumors using antisera with different regional specificities. Cancer *55*, 1895-1898.

Orci, L., Malaisse-Lagae, F., Baetens, D., and Perrelet, A. (1978). Pancreatic-polypeptide-rich regions in human pancreas. Lancet *2*, 1200-1201.

Owji, A.A., Gardiner, J.V., Upton, P.D., Mahmoodi, M., Ghatei, M.A., Bloom, S.R., and Smith, D.M. (1996). Characterisation and molecular identification of adrenomedullin binding sites in the rat spinal cord: a comparison with calcitonin gene-related peptide receptors. J Neurochem *67*, 2172-2179.

Pacini, G., and Mari, A. (2007). "Assessment of Insulin Sensitivity from Steady-State and Dynamic Tests". In "Clinical Diabetes Research: Methods and Techniques", M. Roden, ed. (Chichester, England: John Wiley & Sons), pp. 27-41.

Parker, H.E., Wallis, K., le Roux, C.W., Wong, K.Y., Reimann, F., and Gribble, F.M. (2012). Molecular mechanisms underlying bile acid-stimulated glucagon-like peptide-1 secretion. Br J Pharmacol *165*, 414-423.

Parks, D.L., Gingerich, R.L., Jaffe, B.M., and Akande, B. (1979). Role of pancreatic polypeptide in canine gastric acid secretion. Am J Physiol *236*, E488-494.

Patriti, A., Aisa, M.C., Annetti, C., Sidoni, A., Galli, F., Ferri, I., Gulla, N., and Donini, A. (2007). How the hindgut can cure type 2 diabetes. Ileal transposition improves glucose metabolism and beta-cell function in Goto-kakizaki rats through an enhanced Proglucagon gene expression and L-cell number. Surgery *142*, 74-85.

Plamboeck, A., Holst, J.J., Carr, R.D., and Deacon, C.F. (2005). Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both mediators of the degradation of glucagon-like peptide 1 in the anaesthetised pig. Diabetologia *48*, 1882-1890.

Polak, J.M., Bloom, S.R., Adrian, T.E., Heitz, P., Bryant, M.G., and Pearse, A.G. (1976). Pancreatic polypeptide in insulinomas, gastrinomas, vipomas, and glucagonomas. Lancet *1*, 328-330.

Portela-Gomes, G.M., Grimelius, L., and Stridsberg, M. (2008). Prohormone convertases 1/3, 2, furin and protein 7B2 (Secretogranin V) in endocrine cells of the human pancreas. Regul Pept *146*, 117-124.

Pournaras, D.J., Aasheim, E.T., Sovik, T.T., Andrews, R., Mahon, D., Welbourn, R., Olbers, T., and le Roux, C.W. (2012). Effect of the definition of type II diabetes remission in the evaluation of bariatric surgery for metabolic disorders. Br J Surg *99*, 100-103.

Pournaras, D.J., and Le Roux, C.W. (2009). The effect of bariatric surgery on gut hormones that alter appetite. Diabetes Metab *35*, 508-512.

Pournaras, D.J., Osborne, A., Hawkins, S.C., Mahon, D., Ghatei, M.A., Bloom, S.R., Welbourn, R., and le Roux, C.W. (2010). The gut hormone response following Roux-en-Y gastric bypass: cross-sectional and prospective study. Obesity Surg *20*, 56-60.

Prillaman, H.M., Cox, S.B., Freedlender, A.E., Cornett, G.E., Jones, H.A., Flanagan, T.L., Chance, R.E., Hoffmann, J.A., Andersen, D.K., Elahi, D.*, et al.* (1992). The effect of pancreatic polypeptide on glucose disposal after surgical alterations of the pancreas. Ann Surg *216*, 574-582.

Qader, S.S., Hakanson, R., Rehfeld, J.F., Lundquist, I., and Salehi, A. (2008). Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: a study on isolated islets from mouse and rat pancreas. Regul Pept *146*, 230-237.

Rachman, J., Gribble, F.M., Barrow, B.A., Levy, J.C., Buchanan, K.D., and Turner, R.C. (1996). Normalization of insulin responses to glucose by overnight infusion of glucagon-like peptide 1 (7-36) amide in patients with NIDDM. Diabetes *45*, 1524-1530.

Raz, I., Hanefeld, M., Xu, L., Caria, C., Williams-Herman, D., Khatami, H., and Group, S.S. (2006). Efficacy and safety of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy in patients with type 2 diabetes mellitus. Diabetologia *49*, 2564-2571.

Ritzel, R., Orskov, C., Holst, J.J., and Nauck, M.A. (1995). Pharmacokinetic, insulinotropic, and glucagonostatic properties of GLP-1 [7-36 amide] after subcutaneous injection in healthy volunteers. Dose-response-relationships. Diabetologia *38*, 720-725.

Roberts, R.E., Zhao, M., Whitelaw, B.C., Ramage, J., Diaz-Cano, S., le Roux, C.W., Quaglia, A., Huang, G.C., and Aylwin, S.J. (2012). GLP-1 and glucagon secretion from a pancreatic neuroendocrine tumor causing diabetes and hyperinsulinemic hypoglycemia. J Clin Endocrinol Metab *97*, 3039-3045.

Rocca, A.S., and Brubaker, P.L. (1999). Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. Endocrinology *140*, 1687-1694.

Rosenstock, J., Balas, B., Charbonnel, B., Bolli, G.B., Boldrin, M., Ratner, R., Balena, R., and Group, T.e.S. (2013a). The fate of taspoglutide, a weekly GLP-1 receptor agonist, versus twice-daily exenatide for type 2 diabetes: the T-emerge 2 trial. Diabetes Care *36*, 498-504.

Rosenstock, J., Raccah, D., Koranyi, L., Maffei, L., Boka, G., Miossec, P., and Gerich, J.E. (2013b). Efficacy and Safety of Lixisenatide Once Daily Versus Exenatide Twice Daily in Type 2 Diabetes Inadequately Controlled on Metformin: A 24-Week, Randomized, Open-Label, Active-Controlled Study (GetGoal-X). Diabetes Care *36*, 2945-2951.

Rouille, Y., Kantengwa, S., Irminger, J.C., and Halban, P.A. (1997). Role of the prohormone convertase PC3 in the processing of proglucagon to glucagon-like peptide 1. J Biol Chem *272*, 32810-32816.

Rouille, Y., Westermark, G., Martin, S.K., and Steiner, D.F. (1994). Proglucagon is processed to glucagon by prohormone convertase PC2 in alpha TC1-6 cells. Proc Natl Acad Sci USA *91*, 3242-3246.

Rowland, N.E., Crews, E.C., and Gentry, R.M. (1997). Comparison of Fos induced in rat brain by GLP-1 and amylin. Regul Pept *71*, 171-174.

Rubino, F., Gagner, M., Gentileschi, P., Kini, S., Fukuyama, S., Feng, J., and Diamond, E. (2004). The early effect of the Roux-en-Y gastric bypass on hormones involved in body weight regulation and glucose metabolism. Ann Surg *240*, 236-242.

Rucker, D., Padwal, R., Li, S.K., Curioni, C., and Lau, D.C. (2007). Long term pharmacotherapy for obesity and overweight: updated meta-analysis. BMJ *335*, 1194-1199.

Salehi, M., Aulinger, B., Prigeon, R.L., and D'Alessio, D.A. (2010). Effect of endogenous GLP-1 on insulin secretion in type 2 diabetes. Diabetes *59*, 1330-1337.

Schmidt, P.T., Naslund, E., Gryback, P., Jacobsson, H., Holst, J.J., Hilsted, L., and Hellstrom, P.M. (2005). A role for pancreatic polypeptide in the regulation of gastric emptying and short-term metabolic control. J Clin Endocrinol Metab *90*, 5241-5246.

Schwartz, T.W. (1979). Pancreatic-polypeptide (PP) and endocrine tumours of the pancreas. Scand J Gastroenterol Suppl *53*, 93-100.

Schwartz, T.W., Hansen, H.F., Hakanson, R., Sundler, F., and Tager, H.S. (1984). Human pancreatic icosapeptide: isolation, sequence, and immunocytochemical localization of the COOH-terminal fragment of the pancreatic polypeptide precursor. Proc Natl Acad Sci USA *81*, 708-712.

Schwartz, T.W., Holst, J.J., Fahrenkrug, J., Jensen, S.L., Nielsen, O.V., Rehfeld, J.F., de Muckadell, O.B., and Stadil, F. (1978). Vagal, cholinergic regulation of pancreatic polypeptide secretion. J Clin Invest *61*, 781-789.

Schwartz, T.W., and Tager, H.S. (1981). Isolation and biogenesis of a new peptide from pancreatic islets. Nature *294*, 589-591.

Scirica, B.M., Bhatt, D.L., Braunwald, E., Steg, P.G., Davidson, J., Hirshberg, B., Ohman, P., Frederich, R., Wiviott, S.D., Hoffman, E.B.*, et al.* (2013). Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. N Engl J Med *369*, 1317-1326.

Seghieri, M., Rebelos, E., Gastaldelli, A., Astiarraga, B.D., Casolaro, A., Barsotti, E., Pocai, A., Nauck, M., Muscelli, E., and Ferrannini, E. (2013). Direct effect of GLP-1 infusion on endogenous glucose production in humans. Diabetologia *56*, 156-161.

Seymour, N.E., Spector, S.A., Andersen, D.K., Elm, M.S., and Whitcomb, D.C. (1998). Overexpression of hepatic pancreatic polypeptide receptors in chronic pancreatitis. J Surg Res *76*, 47-52.

Seymour, N.E., Volpert, A.R., and Andersen, D.K. (1996). Regulation of hepatic insulin receptors by pancreatic polypeptide in fasting and feeding. J Surg Res *65*, 1-4.

Sharma, A.M., Caterson, I.D., Coutinho, W., Finer, N., Van Gaal, L., Maggioni, A.P., Torp-Pedersen, C., Bacher, H.P., Shepherd, G.M., and James, W.P. (2009). Blood pressure changes associated with sibutramine and weight management - an analysis from the 6-week lead-in period of the sibutramine cardiovascular outcomes trial (SCOUT). Diab Obes Metab *11*, 239-250.

Shiratori, K., Lee, K.Y., Chang, T.M., Jo, Y.H., Coy, D.H., and Chey, W.Y. (1988). Role of pancreatic polypeptide in the regulation of pancreatic exocrine secretion in dogs. Am J Physiol *255*, G535-541.

Sive, A.A., Vinik, A.I., and van Tonder, S.V. (1979). Pancreatic polypeptide (PP) responses to oral and intravenous glucose in man. Am J Gastroenterol *71*, 183-185.

Sjostrom, L. (2008). Bariatric surgery and reduction in morbidity and mortality: experiences from the SOS study. Int J Obes (Lond) *32 Suppl 7*, S93-97.

Sjostrom, L., Gummesson, A., Sjostrom, C.D., Narbro, K., Peltonen, M., Wedel, H., Bengtsson, C., Bouchard, C., Carlsson, B., Dahlgren, S.*, et al.* (2009). Effects of bariatric surgery on cancer incidence in obese patients in Sweden (Swedish Obese Subjects Study): a prospective, controlled intervention trial. Lancet Oncol *10*, 653-662.

Sjostrom, L., Narbro, K., Sjostrom, C.D., Karason, K., Larsson, B., Wedel, H., Lystig, T., Sullivan, M., Bouchard, C., Carlsson, B.*, et al.* (2007). Effects of bariatric surgery on mortality in Swedish obese subjects. N Engl J Med *357*, 741-752.

Skoglund, G., Gross, R., Ahren, B., and Loubatieres-Mariani, M.M. (1993). Different mechanisms are involved in neuropeptide Y-induced pancreatic vasoconstriction and inhibition of insulin secretion. Eur J Pharmacol *236*, 69-74.

Skov, J., Dejgaard, A., Frokiaer, J., Holst, J.J., Jonassen, T., Rittig, S., and Christiansen, J.S. (2013). Glucagon-like peptide-1 (GLP-1): effect on kidney hemodynamics and renin-angiotensin-aldosterone system in healthy men. J Clin Endocrinol Metab *98*, E664-671.

Sloth, B., Holst, J.J., Flint, A., Gregersen, N.T., and Astrup, A. (2007). Effects of PYY1-36 and PYY3-36 on appetite, energy intake, energy expenditure, glucose and fat metabolism in obese and lean subjects. Am J Physiol Endocrinol Metab *292*, E1062-1068.

Smith, S.R., Weissman, N.J., Anderson, C.M., Sanchez, M., Chuang, E., Stubbe, S., Bays, H., and Shanahan, W.R. (2010). Multicenter, placebo-controlled trial of lorcaserin for weight management. N Engl J Med *363*, 245-256.

Sokos, G.G., Nikolaidis, L.A., Mankad, S., Elahi, D., and Shannon, R.P. (2006). Glucagon-like peptide-1 infusion improves left ventricular ejection fraction and functional status in patients with chronic heart failure. J Card Failure *12*, 694-699.

Sonoda, N., Imamura, T., Yoshizaki, T., Babendure, J.L., Lu, J.C., and Olefsky, J.M. (2008). Beta-Arrestin-1 mediates glucagon-like peptide-1 signaling to insulin secretion in cultured pancreatic beta cells. Proc Natl Acad Sci USA *105*, 6614-6619.

Stanley, S., Wynne, K., and Bloom, S. (2004). Gastrointestinal satiety signals III. Glucagon-like peptide 1, oxyntomodulin, peptide YY, and pancreatic polypeptide. Am J Physiol Gastrointest Liver Physiol *286*, G693-697.

Stoffers, D.A., Kieffer, T.J., Hussain, M.A., Drucker, D.J., Bonner-Weir, S., Habener, J.F., and Egan, J.M. (2000). Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes *49*, 741-748.

Strodel, W.E., Vinik, A.I., Lloyd, R.V., Glaser, B., Eckhauser, F.E., Fiddian-Green, R.G., Turcotte, J.G., and Thompson, N.W. (1984). Pancreatic polypeptide-producing tumors. Silent lesions of the pancreas? Arch Surg *119*, 508-514.

Svegliati-Baroni, G., Saccomanno, S., Rychlicki, C., Agostinelli, L., De Minicis, S., Candelaresi, C., Faraci, G., Pacetti, D., Vivarelli, M., Nicolini, D.*, et al.* (2011). Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis. Liver Int *31*, 1285-1297.

Tang-Christensen, M., Vrang, N., and Larsen, P.J. (2001). Glucagon-like peptide containing pathways in the regulation of feeding behaviour. Int J Obes Relat Metab Disord *25 Suppl 5*, S42-47.

Tatemoto, K., and Mutt, V. (1980). Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. Nature *285*, 417-418.

Tazoe, H., Otomo, Y., Kaji, I., Tanaka, R., Karaki, S.I., and Kuwahara, A. (2008). Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. J Physiol Pharmacol *59 Suppl 2*, 251-262.

Thaler, J.P., and Cummings, D.E. (2009). Minireview: Hormonal and metabolic mechanisms of diabetes remission after gastrointestinal surgery. Endocrinology *150*, 2518-2525.

Tharakan, G., Tan, T., and Bloom, S. (2011). Emerging therapies in the treatment of 'diabesity': beyond GLP-1. Trends Pharmacol Sci *32*, 8-15.

The Comptroller and Auditor General (2001). Tackling Obesity in England. In "Report by the Auditor and Comptroller General (HC 220 Session 2000-2001)" (London: National Audit Office).

Thorens, B., Porret, A., Buhler, L., Deng, S.P., Morel, P., and Widmann, C. (1993). Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin-(9-39) an antagonist of the receptor. Diabetes *42*, 1678-1682.

Todd, J.F., and Bloom, S.R. (2007). Incretins and other peptides in the treatment of diabetes. Diabetic Medicine *24*, 223-232.

Todd, J.F., Stanley, S.A., Roufosse, C.A., Bishop, A.E., Khoo, B., Bloom, S.R., and Meeran, K. (2003). A tumour that secretes glucagon-like peptide-1 and somatostatin in a patient with reactive hypoglycaemia and diabetes. Lancet *361*, 228-230.

Tomas, E., Stanojevic, V., and Habener, J.F. (2010). GLP-1 (9-36) amide metabolite suppression of glucose production in isolated mouse hepatocytes. Horm Metab Res *42*, 657-662.

Tong, J., Utzschneider, K.M., Carr, D.B., Zraika, S., Udayasankar, J., Gerchman, F., Knopp, R.H., and Kahn, S.E. (2007). Plasma pancreatic polypeptide levels are associated with differences in body fat distribution in human subjects. Diabetologia *50*, 439–442.

Track, N.S., McLeod, R.S., and Mee, A.V. (1980). Human pancreatic polypeptide: studies of fasting and postprandial plasma concentrations. Can J Physiol Pharmacol *58*, 1484-1489.

Turton, M.D., O'Shea, D., Gunn, I., Beak, S.A., Edwards, C.M., Meeran, K., Choi, S.J., Taylor, G.M., Heath, M.M., Lambert, P.D.*, et al.* (1996). A role for glucagon-like peptide-1 in the central regulation of feeding. Nature *379*, 69-72.

Ueno, N., Inui, A., Iwamoto, M., Kaga, T., Asakawa, A., Okita, M., Fujimiya, M., Nakajima, Y., Ohmoto, Y., Ohnaka, M.*, et al.* (1999). Decreased food intake and body weight in pancreatic polypeptide-overexpressing mice. Gastroenterology *117*, 1427-1432.

Underwood, C.R., Garibay, P., Knudsen, L.B., Hastrup, S., Peters, G.H., Rudolph, R., and Reedtz-Runge, S. (2010). Crystal structure of glucagon-like peptide-1 in complex with the extracellular domain of the glucagon-like peptide-1 receptor. J Biol Chem *285*, 723-730.

Ussher, J.R., and Drucker, D.J. (2012). Cardiovascular biology of the incretin system. Endocr Rev *33*, 187-215.

Valentini, L., Schuetz, T., Omar, A., Glaser, S., Kasim, E., Nowotny, P., Kroencke, T., and Ockenga, J. (2011). Abnormal plasma peptide YY(3-36) levels in patients with liver cirrhosis. Nutrition *27*, 880-884.

van den Hoek, A.M., Heijboer, A.C., Corssmit, E.P., Voshol, P.J., Romijn, J.A., Havekes, L.M., and Pijl, H. (2004). PYY3-36 reinforces insulin action on glucose disposal in mice fed a high-fat diet. Diabetes *53*, 1949-1952.

van den Hoek, A.M., Heijboer, A.C., Voshol, P.J., Havekes, L.M., Romijn, J.A., Corssmit, E.P., and Pijl, H. (2007). Chronic PYY3-36 treatment promotes fat oxidation and ameliorates insulin resistance in C57BL6 mice. Am J Physiol Endocrinol Metab *292*, E238-245.

van Strien, T., Frijters, J.E.R., Bergers, G.P.A., and Defares, P.B. (1986). The Dutch Eating Behavior Questionnaire (DEBQ) for assessment of restrained, emotional, and external eating behavior. Int J Eat Disord *5*, 295-315.

Vidal, J., Nicolau, J., Romero, F., Casamitjana, R., Momblan, D., Conget, I., Morinigo, R., and Lacy, A.M. (2009). Long-term effects of Roux-en-Y gastric bypass surgery on plasma glucagon-like peptide-1 and islet function in morbidly obese subjects. J Clin Endocrinol Metab *94*, 884-891.

Villanueva, M.L., Hedo, J.A., and Marco, J. (1977). Heterogeneity of pancreatic polypeptide immunoreactivity in human plasma. FEBS Lett *80*, 99-102.

Villanueva-Penacarrillo, M.L., Delgado, E., Trapote, M.A., Alcantara, A., Clemente, F., Luque, M.A., Perea, A., and Valverde, I. (1995). Glucagon-like peptide-1 binding to rat hepatic membranes. J Endocrinol *146*, 183-189.

Vilsboll, T., Krarup, T., Deacon, C.F., Madsbad, S., and Holst, J.J. (2001). Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. Diabetes *50*, 609-613.

Voisin, T., Goumain, M., Lorinet, A.M., Maoret, J.J., and Laburthe, M. (2000). Functional and molecular properties of the human recombinant Y4 receptor: resistance to agonist-promoted desensitization. J Pharmacol Exp Ther *292*, 638-646.

von Zastrow, M., Tritton, T.R., and Castle, J.D. (1986). Exocrine secretion granules contain peptide amidation activity. Proc Natl Acad Sci USA *83*, 3297-3301.

Wang, Z.L., Bennet, W.M., Wang, R.M., Ghatei, M.A., and Bloom, S.R. (1994). Evidence of a paracrine role of neuropeptide-Y in the regulation of insulin release from pancreatic islets of normal and dexamethasone-treated rats. Endocrinology *135*, 200-206.

Wei, Y., and Mojsov, S. (1995). Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences. FEBS Lett *358*, 219-224.

Wenten, M., Gaebler, J.A., Hussein, M., Pelletier, E.M., Smith, D.B., Girase, P., Noel, R.A., Braun, D.K., and Bloomgren, G.L. (2012). Relative risk of acute pancreatitis in initiators of exenatide twice daily compared with other anti-diabetic medication: a follow-up study. Diabetic Medicine *29*, 1412-1418.

Wharton, J., Gordon, L., Byrne, J., Herzog, H., Selbie, L.A., Moore, K., Sullivan, M.H., Elder, M.G., Moscoso, G., Taylor, K.M.*, et al.* (1993). Expression of the human neuropeptide tyrosine Y1 receptor. Proc Natl Acad Sci USA *90*, 687-691.

Whitcomb, D.C., Puccio, A.M., Vigna, S.R., Taylor, I.L., and Hoffman, G.E. (1997). Distribution of pancreatic polypeptide receptors in the rat brain. Brain Res *760*, 137-149.

Whitwell, J.L., Sampson, E.L., Loy, C.T., Warren, J.E., Rossor, M.N., Fox, N.C., and Warren, J.D. (2007). VBM signatures of abnormal eating behaviours in frontotemporal lobar degeneration. NeuroImage *35*, 207-213.

Willms, B., Werner, J., Holst, J.J., Orskov, C., Creutzfeldt, W., and Nauck, M.A. (1996). Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients. J Clin Endocrinol Metab *81*, 327-332.

Wisen, O., Bjorvell, H., Cantor, P., Johansson, C., and Theodorsson, E. (1992). Plasma concentrations of regulatory peptides in obesity following modified sham feeding (MSF) and a liquid test meal. Regul Pept *39*, 43-54.

Wood, S.P., Pitts, J.E., Blundell, T.L., Tickle, I.J., and Jenkins, J.A. (1977). Purification, crystallisation and preliminary X-ray studies on avian pancreatic polypeptide. Eur J Biochem *78*, 119-126.

Wood, W.G., Wachter, C., and Scriba, P.C. (1981). Experiences using chloramine-T and 1, 3, 4, 6-tetrachloro-3 alpha, 6 alpha-diphenylglycoluril (Iodogen) for radioiodination of materials for radioimmunoassay. J Clin Chem Clin Biochem *19*, 1051-1056.

Wren, A.M., Small, C.J., Ward, H.L., Murphy, K.G., Dakin, C.L., Taheri, S., Kennedy, A.R., Roberts, G.H., Morgan, D.G., Ghatei, M.A.*, et al.* (2000). The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology *141*, 4325-4328.

Wulff, B.S., Johansen, T.E., Dalboge, H., O'Hare, M.M., and Schwartz, T.W. (1993). Processing of two homologous precursors, pro-neuropeptide Y and pro-pancreatic polypeptide, in transfected cell lines expressing different precursor convertases. J Biol Chem *268*, 13327-13335.

Wynne, K., Park, A.J., Small, C.J., Patterson, M., Ellis, S.M., Murphy, K.G., Wren, A.M., Frost, G.S., Meeran, K., Ghatei, M.A.*, et al.* (2005). Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. Diabetes *54*, 2390-2395.

Xu, J., McNearney, T.A., and Chen, J.D. (2011). Impaired postprandial releases/syntheses of ghrelin and PYY(3-36) and blunted responses to exogenous ghrelin and PYY(3-36) in a rodent model of diet-induced obesity. J Gastroenterol Hepatol *26*, 700-705.

Yang, H. (2002). Central and peripheral regulation of gastric acid secretion by peptide YY. Peptides *23*, 349-358.

Zander, M., Madsbad, S., Madsen, J.L., and Holst, J.J. (2002). Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. Lancet *359*, 824-830.

Zhang, T., Uchida, T., Gomez, G., Lluis, F., Thompson, J.C., and Greeley, G.H., Jr. (1993). Neural regulation of peptide YY secretion. Regul Pept *48*, 321-328.

Zhou, J., Hegsted, M., McCutcheon, K.L., Keenan, M.J., Xi, X., Raggio, A.M., and Martin, R.J. (2006). Peptide YY and proglucagon mRNA expression patterns and regulation in the gut. Obesity (Silver Spring) *14*, 683-689.

Zhou, J., Martin, R.J., Tulley, R.T., Raggio, A.M., McCutcheon, K.L., Shen, L., Danna, S.C., Tripathy, S., Hegsted, M., and Keenan, M.J. (2008). Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. Am J Physiol Endocrinol Metab *295*, E1160-1166.

Zulueta, M.A., Vincent, E., Correas, I., Villanueva, M.L., Feliu, J.E., and Marco, J. (1982). Characterization of the pancreatic polypeptide response to hypoglycemia in man. Diabete Metab *8*, 47-51.