

The role of gut hormones in energy homeostasis

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ABSTRACT

Obesity is an increasing worldwide problem and yet current pharmaceutical treatments only produce modest weight loss which is rarely sustained. Glucagon and glucagon-like peptide-1 (GLP-1) are members of the secretin family of peptide hormones. Both hormones are products of the preproglucagon gene, and are produced in the pancreas and gut respectively. GLP-1 is an incretin hormone that enhances glucose-mediated insulin release and inhibits glucagon secretion. In contrast, glucagon counter-regulates insulin and stimulates gluconeogenesis in response to low circulating levels of glucose. Despite their opposing roles in glucose homeostasis, both hormones reduce food intake in rodents and humans. In addition, glucagon increases energy expenditure.

In this thesis, I have investigated for the first time the effects of co-administration of glucagon and GLP-1 in energy balance. Co-administration of glucagon and GLP-1 in mice significantly reduced food intake compared to controls and appeared to do so in an additive manner. Glucagon and GLP-1 alone and in combination activated similar areas within the brainstem and amygdala. Prolonged co-administration of novel, protease-resistant analogues of glucagon and GLP-1 reduced body weight in diet-induced obese (DIO) mice, despite similar food intake compared to saline controls. Furthermore, glucagon and GLP-1 analogue co-administration improved glucose homeostasis compared to saline controls in DIO mice.

Intravenous infusion of glucagon alone and in combination with GLP-1 increased energy expenditure in overweight humans whilst GLP-1 alone had no effect. This represents a first in man study of the effects of co-administration of glucagon and GLP-1 on energy homeostasis. Sub-anorectic doses of glucagon and GLP-1 in combination reduced food intake whilst alone they had no effect. Co-administration of both hormones ameliorated the rise in plasma glucose seen following glucagon infusion alone, demonstrating an additional benefit of GLP-1 and glucagon in combination.

These findings suggest that the combination of GLP-1 and glucagon represents an exciting therapeutic target for development of a novel anti-obesity agent.

DECLARATION OF CONTRIBUTORS

The majority of the work described in this thesis was undertaken by the author. All collaborations and assistance are detailed below:

Chapter 2: Rodent feeding studies, glucose measurements and c-fos immunohistochemistry were performed in collaboration with Dr Jennifer Parker.

Chapter 3: Acute rodent feeding studies, behavioural studies and c-fos immunohistochemistry following the administration of the glucagon/GLP-1 analogue, G(X) were performed in collaboration with Dr Jennifer Parker. Chronic rodent feeding studies following administration of G(Y) and G(Z), glucose tolerance tests and tissue harvesting were undertaken in collaboration with Dr Rachel Troke, Dr James Minnion, Dr Vicky Salem and Joy Shillito with assistance from members of the G series group. mRNA extraction was performed in collaboration with Dr Rachel Troke, Dr Vicky Salem and Joy Shillito. rtPCR was performed in collaboration with Dr Rachel Troke. Plasma hormone assays following chronic administration of G(Y) and G(Z) were performed in collaboration with Dr Rachel Troke and Joy Shillito.

Chapter 4: Human studies were performed in collaboration with Dr Ben Field and Dr Tricia Tan with assistance from Dr Rachel Troke, Dr Ed Chambers, Dr Vicky Salem, Dr Alex Viardot, Dr Kevin Baynes, Dr Akila De Silva and Ali Alsafi. Assistance with radioimmunoassays was provided by Dr Rachel Troke and Dr Ben Field.

Chapter 5: Human studies were performed in collaboration with Dr Rachel Troke, Dr Jaimini Cegla, Dr George Tharakan, Dr Ben Jones and Dr Tricia Tan, with assistance from Dr Julia Wilde, Dr Nassim Parvisi, Dr Mohammed Hussain and Dr Chung Thong-Lim. Assistance with radioimmunoassay was provided by Dr Rachel Troke and Dr Jaimini Cegla.

In-house radioimmunoassays (RIA) used in this thesis were established and are maintained by Professor Mohammad Ghatei.

All glucagon and GLP-1 analogue custom-made peptides were designed by Professor Steve Bloom. All intellectual property relating to these peptides belong to Imperial College London.

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ABBREVIATIONS

α -MSH	α -Melanocyte Stimulating Hormone
ABC	Avidin-biotin peroxidase complex
AC	Adenylate cyclase
ACC	Acetyl-CoA carboxylase
ACC1	Acetyl-CoA carboxylase 1
ACC2	Acetyl-CoA carboxylase 2
AcN	Acetonitrile
ACTH	Adrenocorticotrophin hormone
AgRP	Agouti-Related Protein
AHA	Anterior Hypothalamic Area
AMPK	Adenosine mono-phosphate protein kinase
ANOVA	Analysis of Variance
AP	Area postrema
ARC	Arcuate Nucleus
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown Adipose Tissue
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BMI	Body Mass Index
BMR	Basal metabolic rate
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CART	Cocaine- and Amphetamine-Regulated Transcript
CAT	Carnitine-acylcarnitine translocase
CB1	Cannabinoid 1 receptor
CCK	Cholecystokinin
CCK-1	Cholecystokinin-1 Receptor
CCK-2	Cholecystokinin-2 Receptor
cdNA	complementary deoxyribonucleic acid
CeA	Central nucleus of the amygdala
c-fos-ir	c-fos immunoreactivity
CHO	Chinese Hamster Ovary
CLAMS	Comprehensive Laboratory Animal Monitoring System
CNS	Central Nervous System
cNTS	Caudal Nucleus Tractus Solitarius
CO ₂	Carbon dioxide
CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
CRH	Corticotrophin releasing hormone
CTA	Conditioned Taste Aversion
D2	2 iodothyronine deiodinase
<i>db</i>	Diabetes gene
DIO	Diet-Induced Obesity
DMN	Dorsomedial Nucleus
DMSO	Dimethyl sulfoxide
DMV	Dorsal motor nucleus of the vagus nerve
DPP-IV	Dipeptidyl Peptidase IV
DPX	Dibutyl phthalate xylene

DVC	Dorsal vagal complex
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EE	Energy expenditure
ELISA	Enzyme-linked Immunosorbent Assay
EMG	Electromyography
F-6-P	Fructose-6-phosphate
FADH	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FFA	Free fatty acid
FGF-21	Fibroblast growth factor-21
fMRI	Functional magnetic resonance imaging
FT3	Free Tri-iodothyronine
FT4	Free Thyroxine
G-6-P	Glucose-6-phosphate
G-6-Pase	Glucose-6-phosphatase
GABA	Gamma-Aminobutyric Acid
GCG	Glucagon
GCGR	Glucagon receptor
GDP	Guanosine diphosphate
GHRH	Growth Hormone Releasing Hormone
GHS-R1	Growth Hormone Secretagogue Receptor 1
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-Like Peptide-1
GLP-1-ir	Glucagon-Like Peptide-1 immunoreactive
GLP-1R	Glucagon-Like Peptide-1 Receptor
GLP-2	Glucagon-Like Peptide-2
GLP-2R	Glucagon-Like Peptide-2 Receptor
GPCR	G-protein coupled receptor
GRPP	glicentin related pancreatic polypeptide
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamo-pituitary-adrenal
HPLC	High Performance Liquid Chromatography
HPT	Hypothalamo-pituitary-thyroid
HRP	Horseradish peroxidase
HSL	Hormone Sensitive Lipase
ICV	Intracerebroventricular
IgG	Immunoglobulin
im	Intramuscular
ip	Intraperitoneal
IP	Intervening Peptide
IP-1	Intervening Peptide-1
IP-2	Intervening Peptide-2
IP3	Inositol 1,4,5-triphosphate
ipGTT	Intraperitoneal glucose tolerance test
iv	Intravenous
LH	Lateral hypothalamus
LHA	Lateral Hypothalamic Area
LiCl	Lithium chloride
MALDI-MS	Matrix-assisted Laser Desorption Ionisation-mass spectroscopy
MCH	Melanin concentrating hormone
MC1R	Melanocortin-1 Receptor

MC2R	Melanocortin-2 Receptor
MC3R	Melanocortin-3 Receptor
MC4R	Melanocortin-4 receptor
ME	Median Eminence
MEMRI	Manganese-enhanced magnetic resonance imaging
MPO	Medial Preoptic Area
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NA	Noradrenaline
NADH	Nicotinamide adenine dinucleotide
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
ns	Not significant
NTS	Nucleus Tractus Solitarius
NuA	Nucleus accumbens
O ₂	Oxygen
<i>ob</i>	<i>obese</i> gene
Ob-R	Leptin receptor
OLI	Oxyntomodulin-Like Immunoreactivity
OXM	Oxyntomodulin
OX-R1	Orexin receptor 1
PAC1	Pituitary adenylate cyclase-activating peptide type 1 receptor
PACAP	Pituitary adenylate cyclase-activating peptide
PBN	Parabrachial nucleus
PBS	Phosphate buffer solution
PC	Prohormone convertase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PET-CT	Positron emission tomography-computed tomography
PGC1- α	Peroxisome proliferator-activated receptor- γ co-activator-1 α
PHI	Peptide histidine isoleucine
PIS	Patient information sheet
PKA	Protein kinase A
PMSF	Phenylmethanesulfonylfluoride
PNS	Peripheral nervous system
POMC	Proopiomelanocortin
PP	Pancreatic Polypeptide
PPAR	Peroxisome proliferator-activated receptor
PTH	Parathyroid hormone
PTH1R	Parathyroid hormone receptor 1
PVN	Paraventricular Nucleus
PYY	Peptide YY
RAMP	Receptor activity-modifying protein
REE	Resting Energy Expenditure
RER	Respiratory Exchange Ratio (Respiratory Quotient)
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RQ	Respiratory quotient
rRPN	Rostral raphe pallidus nucleus
rtPCR	Real-time polymerase chain reaction
RYGB	Roux-en-Y Gastric Bypass
s/c	Subcutaneous
SCh	Suprachiasmatic nucleus

SEM	Standard Error of the Mean
SNS	Sympathetic nervous system
SON	Supraoptic Nucleus
SPSS	Solid phase peptide synthesis
STAT3	Signal Transducer and Activator of Transcription-3
T3	Tri-iodothyronine
T4	Thyroxine
TFA	Trifluoroacetic Acid
Tg	Triglycerides
TH	Thyroid hormone
TMB	Tetramethylbenzidine
TRH	Thyrotrophin-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
UCP-1	Uncoupling protein 1
UCP-2	Uncoupling protein 2
UCP-3	Uncoupling protein 3
UK	United Kingdom
US	United States of America
VAS	Visual Analogue Scale
VCO ₂	Rate of carbon dioxide production
VIP	Vasoactive intestinal peptide
VMN	Ventromedial nucleus
VO ₂	Rate of oxygen consumption
VPAC1	Vasoactive intestinal peptide 1 receptor
VPAC2	Vasoactive intestinal peptide 2 receptor
VTa	Ventral tegmental area
WAT	White adipose tissue
WHO	World Health Organisation
Y1R	PYY receptor subtype 1
Y2R	PYY receptor subtype 2
Y4R	PYY receptor subtype 4
Y5R	PYY receptor subtype 5

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CHAPTER 1:

GENERAL INTRODUCTION

1.1 Introduction

The World Health Organisation estimates that worldwide over one billion adults and over forty two million children under the age of five are overweight ^{1,2}. In the UK, a quarter of all adults are obese³ and 30% of children are classified as overweight or obese⁴. Obesity reduces life expectancy by approximately nine years and accounts for almost 600 deaths a week in England alone⁵. When homeostatic mechanisms controlling food intake are poorly adapted to the unique modern environment of plenty, both excess energy intake and decreased exercise results in weight gain. Obesity is an increasing health and socioeconomic burden that is associated with type 2 diabetes mellitus, hyperlipidaemia, hypertension, cardiovascular disease and certain forms of cancer⁶.

1.2 Regulation of appetite and body weight

The control of food intake is complex and consists of neural and hormonal signals between the gut and central nervous system (CNS). In addition, hormones such as leptin indicate 'energy reserves' within adipose tissue and are strongly implicated in feeding behaviour and energy expenditure (EE). The brain is responsible for the interpretation of signals from the periphery and for modifying feeding behaviour depending on energy requirements and the 'wanting' of food. Even prior to eating, gut hormones are released which begin the process of meal termination and feelings of satiation. Gut hormones act within key brain areas such as the hypothalamus and brainstem which contain intricate neuronal networks and connections related to energy homeostasis, regulation of food intake and glucose homeostasis.

1.2.1 Central regulation of energy balance

1.2.1.1 Hypothalamus

Early lesioning studies led to a dual-centre hypothesis, in which the ventromedial nuclei (VMN) were responsible for the inhibition of food intake and the lateral hypothalamic areas (LHA) stimulated appetite⁷. However, it is now clear that nuclei within the hypothalamus are closely integrated within complex networks that signal to areas within the brain involved in the regulation of appetite. Interconnecting hypothalamic nuclei such as the arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial nucleus (DMN), VMN and LHA are implicated in the regulation of food intake, EE and

glucose homeostasis (Figure 1.1). These nuclei also project to other important areas within the brain including the brainstem, reward centres such as the amygdala and nucleus accumbens, and the prefrontal cortex which is involved in conditioned taste aversion (CTA).

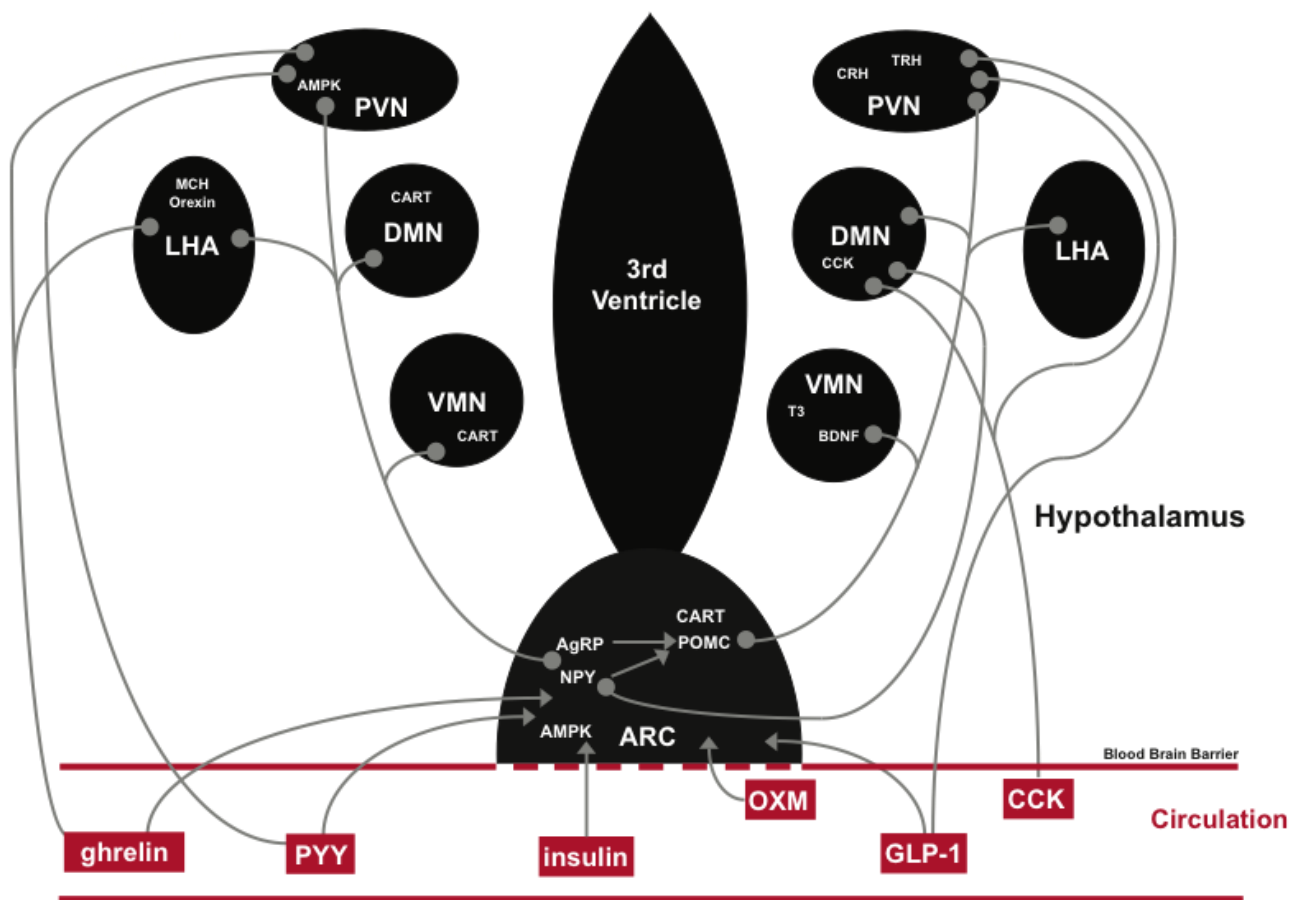


Figure 1.1. Schematic diagram outlining hypothalamic circuits involved in the regulation of food intake. Circulating hormones act on the ARC where orexigenic NPY/AgRP and anorexigenic POMC/CART neurones are present. Abbreviations: arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamic area (LHA), brain-derived neurotrophic factor (BDNF), melanin concentrating hormone (MCH), cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), oxyntomodulin (OXM), peptide YY (PYY), agouti related protein (AgRP), neuropeptide Y (NPY), pro-opiomelanocortin (POMC), cocaine- and amphetamine-related transcript (CART), adenosine mono-phosphate protein kinase (AMPK), thyrotrophin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), tri-iodothyronine (T3). Reproduced from Simpson et al (2009)⁸.

Arcuate Nucleus (ARC)

The ARC is a key hypothalamic nucleus in the regulation of appetite. In mice, subcutaneous injection of monosodium glutamate induces necrosis in the ARC with subsequent development of obesity⁹. Anatomically related to the median eminence, the ARC is not fully insulated from the circulation by the blood brain barrier (BBB) and hence, is strategically positioned to integrate a number of peripheral signals controlling food intake^{10,11}. Two major neuronal populations in the ARC are prominently implicated in the regulation of feeding. One population is localised more medially in the ARC, increases food intake and co-expresses neuropeptide Y (NPY) and agouti-related protein (AgRP)¹². The second population of neurones co-expressing cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC), inhibits food intake and tends to cluster more laterally in the ARC¹³. Neuronal projections from these two populations then communicate with other hypothalamic areas involved with appetite regulation such as the PVN, DMN and LHA¹⁴⁻¹⁷.

Melanocortin peptides, including α -MSH, released from ARC POMC neurones bind to downstream MC4-Rs to inhibit food intake^{18,19}. The MC4-R is highly expressed in the hypothalamus, most notably the PVN²⁰. AgRP is the endogenous antagonist at the MC3-R and MC4-R²¹. Whereas intracerebroventricular (ICV) administration of α -MSH to rats reduces food intake, this effect is inhibited by the simultaneous ICV administration of AgRP²². The majority of CART neurones in the ARC also contain POMC mRNA²³. Animal studies have shown that ICV administration of CART inhibits food intake²⁴, whereas ICV injection of CART antiserum increases food intake^{24,25}. This suggests that CART peptide is an endogenous inhibitor of feeding.

Within the hypothalamus, NPY has been implicated as an important physiological regulator of body weight through its effects on food intake and EE²⁶. NPY/AgRP neurones have extensive projections within the hypothalamus including the PVN, DMN and LHA which appear to be the main targets for the orexigenic effects of NPY²⁷⁻³². Approximately 20% of ARC NPY neurones innervate the PVN and DMN^{29,30}. Stimulation of this pathway leads to increased food intake through direct stimulation of Y1 and Y5 receptors in addition to AgRP antagonism of MC3 and MC4-Rs in the PVN³³. Furthermore, local release of NPY within the ARC inhibits POMC neurones³⁴. ICV injection of NPY potently stimulates food intake in rats³⁵. Repeated daily injections of NPY results in chronic hyperphagia and increased weight gain in rats³⁶. In support of the critical role of the NPY/AgRP system in energy balance, ablation of ARC NPY/AgRP neurones in adult mice reduces food intake and body weight³⁷⁻⁴⁰.

Paraventricular Nucleus (PVN)

Microinjection of almost all known orexigenic peptides into the PVN, including NPY and AgRP stimulate feeding⁴¹⁻⁴³. NPY/AgRP and POMC neurones from the ARC communicate with PVN neurones containing corticotrophin releasing hormone (CRH) and thyrotrophin releasing hormone (TRH)^{44,45}. Both CRH and TRH have been implicated in the control of energy balance, by contributions to both food intake and EE^{46,47}. Therefore, in energy balance, a key role for the PVN is to convey information from the ARC to other brain areas involved in appetite regulation.

Lateral Hypothalamic Area (LHA)

The LHA is another key downstream target of neuronal projections from the ARC and contains the orexigenic neuropeptides melanin concentrating hormone (MCH) and orexins. Lesioning of the LHA reduces body weight^{7,48}. NPY, AgRP and α -MSH immunoreactive terminals are extensive in the LHA and are in contact with MCH and orexin-expressing cells^{49,50}. MCH immunoreactive fibres project to the cortex and spinal cord, consistent with a potential role in appetite control and EE^{51,52}. Interestingly, work by another group found that a subpopulation of MCH neurones in the LHA co-express CART⁵³ and mainly project to the brainstem⁵⁴. In contrast, MCH fibres lacking CART have been found to project to the forebrain, suggesting MCH may modulate food intake and EE through two separate neuronal projections depending on the presence of CART⁵⁴.

Dorsomedial Nucleus (DMN)

Destruction of the DMN results in hyperphagia and obesity, although less dramatically than VMN lesioning⁵⁵. The DMN contains a large number of NPY terminals^{29,56,57} and α -MSH terminals originating in the ARC⁵⁸. α -MSH fibres also project from the DMN to the PVN, terminating on TRH-containing neurones⁵⁹. In the DMN, α -MSH fibres are in close apposition to NPY neurones⁶⁰. It has been postulated that α -MSH suppresses NPY gene expression in the DMN indirectly via separate inhibitory inter-neurones, possibly through GABA-ergic pathways⁶⁰. In diet-induced obese (DIO) mice⁶¹, obese agouti mice⁶² and MC4-R knockout mice⁶², NPY mRNA expression is increased in the DMN, whereas it is reduced in the ARC. This difference in NPY response is again highlighted by the finding that NPY levels in the DMN, in contrast to the ARC and PVN, are not elevated during fasting⁶³. It is thought that lack of leptin signalling on NPY neurones in the DMN may partly account for this since leptin deficient *ob/ob* mice show increased NPY mRNA in the ARC but not in the DMN⁶².

Cholecystokinin (CCK) has also been implicated in the DMN modulation of NPY signalling and hence food intake⁶⁴. CCK-1 receptors and NPY co-localise in DMN neurones and administration of CCK into the DMN downregulates NPY gene expression and inhibits food intake in rats⁶⁵. Furthermore, Otsuka Long Evans Tokushima Fatty rats, a CCK-1 receptor knockout model, exhibit hyperphagia and increased NPY mRNA expression in the DMN ⁶⁶.

Ventromedial nucleus (VMN)

Lesions of the VMN result in rapid onset hyperphagia and obesity⁴⁸. The VMN has a large population of glucoresponsive neurones that respond to blood glucose levels and numerous histamine, dopamine, serotonin, and GABA neurones that respond to feeding-related stimuli⁶⁷. The VMN receives NPY, AgRP and POMC neuronal projections from the ARC. Brain-derived neurotrophic factor (BDNF) is highly expressed in the VMN and is important during development for neuronal survival⁶⁸. Lateral ventricle administration of BDNF reduces food intake and body weight⁶⁹. Previous studies have implicated ARC POMC neurones in activating VMN BDNF neurones to decrease food intake⁷⁰. The VMN has also recently been described as the site of a novel hypothalamic appetite regulatory circuit involving tri-iodothyronine (T3). Administration of T3 peripherally increases food intake in rats and activates neurones in the VMN⁷¹.

1.2.1.2 Brainstem

In comparison to hypothalamic circuits controlling food intake, much less is known about those in the brainstem. Extensive reciprocal neuronal projections exist between brainstem and hypothalamic feeding circuits to provide an alternative pathway through which circulating satiety factors can communicate with the hypothalamus^{72,73}. The vagus nerve is the major neuroanatomical link between the gastrointestinal (GI) tract and the brain. Cell bodies of afferent fibres of the abdominal vagus nerve are located in the nodose ganglia, which project onto the brainstem. Here, the dorsal vagal complex (DVC), consisting of the dorsal motor nucleus of the vagus nerve (DMV), the area postrema (AP), and the sensory nucleus of the tractus solitarius (NTS), interfaces with hypothalamic and higher centres^{73,74}. In addition, the parabrachial nucleus (PBN) receives information related to feeding including taste, gastric distension and hepatic vagal afferents⁷⁵⁻⁷⁸.

Transection of all gut sensory vagal fibres results in increased meal size and meal duration^{79,80}. Increased gastric volume, nutrient content of the small intestine and gut

hormones such as CCK all dose dependently decrease meal size and increase c-fos expression in the NTS⁸¹⁻⁸⁴. The anorectic response to CCK^{85,86}, glucagon-like peptide-1 (GLP-1)⁸⁷ and peptide tyrosine tyrosine (PYY)^{87,88} are attenuated following vagotomy. Within the brainstem, vagal afferent neurones have been shown to express a variety of receptors including CCK-1R and CCK-2R (at which both CCK and gastrin act)⁸⁹, the leptin receptor Ob-R which is involved in the regulation of energy intake⁹⁰, the PYY receptor subtype Y2R, at which PYY acts to reduce food intake⁸⁸, GLP-1⁹¹ and GLP-2R⁹², growth hormone secretagogue receptor (GHS)-R1 at which ghrelin acts⁹³ and the orexin receptor, OX-R1⁹⁴. In addition, the AP and a significant portion of the caudal medial NTS contains fenestrated capillaries which are thought to allow direct contact with circulating factors in the bloodstream^{95,96}. Therefore, the vagotomy studies show that these hormones regulate food intake indirectly via the vagus nerve. Alternatively they may act directly at the AP by binding to their respective receptors via an incomplete BBB ⁹⁶.

Extensive projections of NTS neurones have been localised to parasympathetic and sympathetic preganglionic neurones in the spinal cord, and important cardiovascular, respiratory and gut motility control centres in the medullary reticular formation^{97,98}. NTS projections are also found in hypothalamic nuclei such as the PVN, DMN and LHA^{73,99-102} and amygdala¹⁰³. The majority of NTS neurones to the hypothalamus are noradrenergic^{101,102} and terminate in the PVN and supraoptic nucleus (SON) ⁹⁹⁻¹⁰². Although the exact role of these NTS projections in influencing food intake is unclear, terminal fibres have been identified on CRH¹⁰⁰ and TRH¹⁰⁴ containing neurones in the PVN. Recent evidence also suggests a role for prolactin-releasing peptide neurones in the NTS and their projections to the DMN following refeeding in fasted rats¹⁰⁵. In addition to noradrenergic projections to the PVN, somatostatin¹⁰⁶, CART¹⁰⁷ and POMC¹⁰⁸ containing neurones within the NTS have also been shown to project to the PVN. Brainstem POMC neurones demonstrate signal transducer and activator of transcription-3 (STAT-3) activation in response to leptin administration¹⁰⁹ and administration of leptin into the DVC suppresses food intake¹¹⁰. Finally, approximately 25% of NTS neurones projecting to the PVN contain preproglucagon derived peptides¹¹¹. Therefore, multiple pathways exist between appetite regulating areas in the hypothalamus and brainstem, highlighting the importance of these regions in energy homeostasis.

NTS neurones also receive descending projections from the hypothalamus (predominantly PVN and LHA) and central nucleus of the amygdala (CeA) ¹¹². Within the

brainstem itself, extensive CCK, galanin and CRH immunoreactive neurones project from the NTS and AP to the PBN^{98,99,113}. Vagal gastric projections also relay to the PBN through the caudal NTS¹¹⁴. Acute gastric distension activates c-fos in all areas of the brainstem (NTS, AP and DMV)⁸⁶ and increased electrical activity is seen in the PBN in response to gastric balloon dilatation⁷⁸. Therefore, signals from the periphery have pivotal roles in transmitting information via afferent vagal fibres to the caudal brainstem or directly to the hypothalamus to modify appetite.

1.2.1.3 Reward pathways

Reward pathways in the brain involved in feeding behaviour include the nucleus accumbens, ventral striatum, ventral tegmental area (VTA), prefrontal cortex and hippocampus. CTA and lesioning experiments demonstrate that the orbitofrontal cortex and amygdala are important in learning and experiencing food. Furthermore the orbitofrontal cortex receives sensory inputs such as taste, smell, sight and feel of food in the mouth as well as chemical composition¹¹⁵. Neuroimaging studies in humans have shown that pleasant and unpleasant odours activate different regions of the orbitofrontal cortex and cingulate gyrus¹¹⁶. If a meal is eaten to satiety, the signal from the orbitofrontal cortex decreases and the feeding behaviour changes from acceptance of food to rejection. This ability to switch from 'likeability' of a food to rejection has been associated with leptin signalling¹¹⁷. The VTA is the origin of the dopaminergic cell bodies of the mesocorticolimbic dopamine system and widely implicated in drug and natural reward circuitry of the brain. Direct administration of leptin into the VTA decreases food intake in rats¹¹⁸. Individuals with congenital leptin deficiency are hyperphagic and obese¹¹⁹. Leptin treatment in these individuals not only ameliorates their obesity but also enables them to discriminate between the 'liking ratings' of food in fed and fasted states¹¹⁷.

The mesolimbic dopaminergic system is particularly important in feeding behaviour. Following ingestion of highly palatable food, there are increased dopamine levels in the nucleus accumbens (NuA)¹²⁰. Leptin decreases dopaminergic neuronal firing in *ex vivo* VTA slices¹²¹. Ghrelin increases dopamine levels in the NuA¹²² and direct injection of ghrelin into the NuA and VTA promotes feeding¹²³. Endocannabinoids also increase food intake via the cannabinoid CB1 receptor. CB1 receptors are highly expressed in the reward centres and modulate dopaminergic signalling in these regions^{124,125}. Blocking CB1 receptors using antagonists, such as rimonabant, inhibits food intake and results in weight loss in rodents and humans^{126,127}. However, rimonabant has now been withdrawn as a treatment for obesity due to psychiatric complications¹²⁸. It is therefore

important when developing anti-obesity treatments, to be aware of the interaction of gut hormones with reward centres of the brain and potential side effects these may cause.

1.2.2 Peripheral regulation of energy balance

1.2.2.1 The gut-brain axis

Anticipation of a meal, mechanical stimulation due to the presence of food in the stomach and gut nutrient content, stimulate secretion of gut hormones which activate signalling pathways from the gut to the brainstem and hypothalamus (the gut-brain axis) to terminate food consumption. In addition to direct effects via the circulation, gut hormones may also affect food intake via the vagus nerve as previously described. These signalling pathways are summarised in figure 1.2. Such 'anorectic' hormones include PYY, pancreatic polypeptide (PP), CCK, OXM and GLP-1. Circulating levels of these hormones rise following a meal and are proportional to the caloric intake and composition of a meal¹²⁹⁻¹³². In contrast, ghrelin is an 'orexigenic' hormone and initiates hunger prior to a meal.

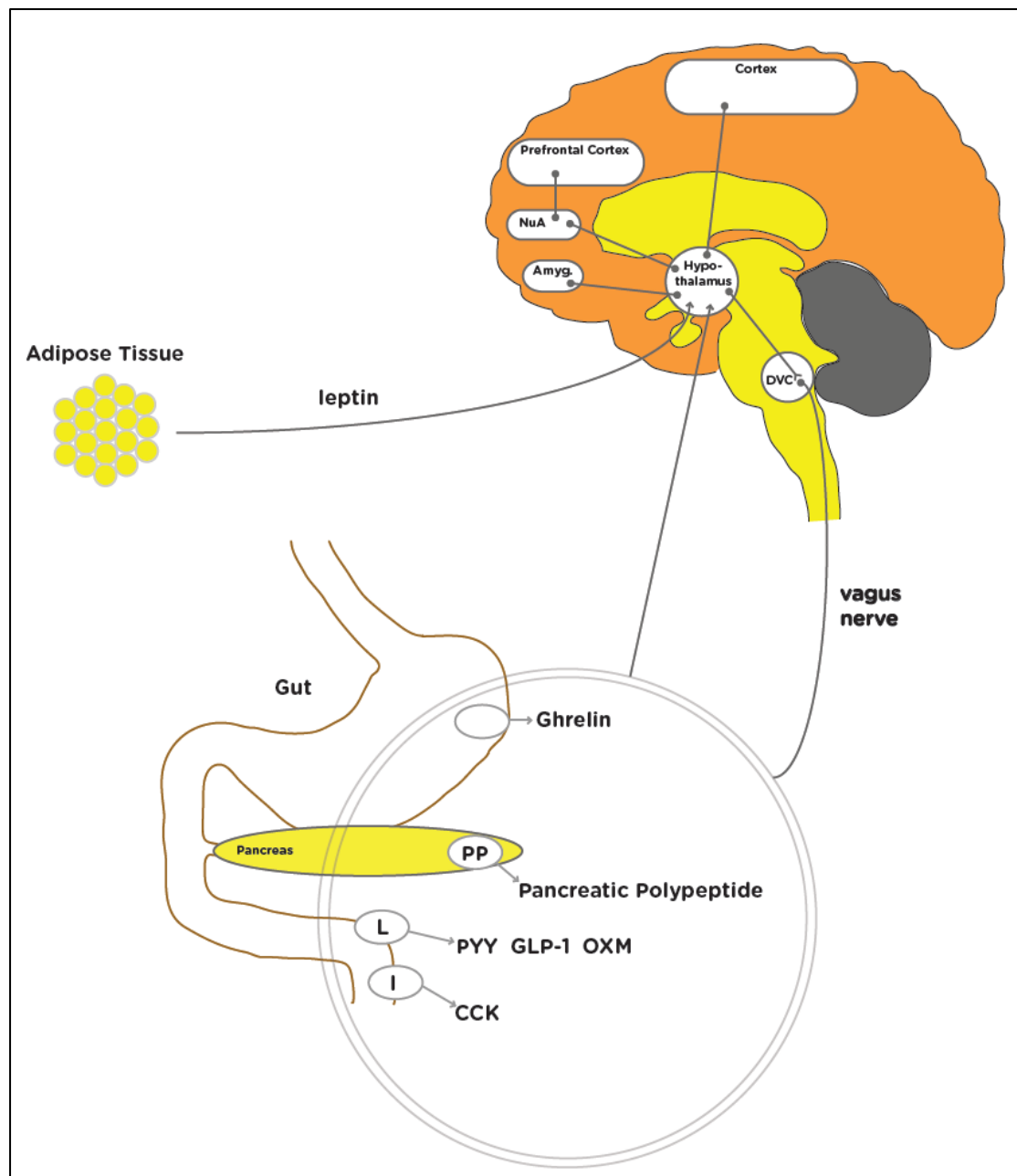


Figure 1.2 Signalling pathways between the gut and central nervous system. Gut hormones acting via vagal afferents act on the NTS in the brainstem which in turn signals to the hypothalamus. Some gut hormones may also act directly on hypothalamic nuclei via the circulation. Projections exist between hypothalamic nuclei and the pre-frontal cortex, involved in conditioned taste aversion, as well as reward centres such as the amygdala and nucleus accumbens. Abbreviations: Dorsal vagal complex (DVC), peptide tyrosine tyrosine (PYY), cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), amygdala (Amyg.), nucleus accumbens (NuA), pancreatic polypeptide (PP). (Reproduced from Simpson et al 2010)¹³³.

1.2.2.2 Secretin family peptides

Peptide hormones within the secretin family are expressed in endocrine cells of the pancreas, GI epithelium and neurones in the brain. They exert several biological functions including regulation of growth, food intake, glucose homeostasis, gut transit and digestion and EE¹³⁴. The secretin family comprises glucagon, GLP-1, GLP-2, glucose-dependent insulintropic peptide (GIP), growth hormone releasing hormone (GHRH), secretin, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP). These hormones are structurally related (see figure 1.3) and their active site is contained within the N terminal amino acids. They exert their actions through the evolutionarily distinct G protein coupled receptor (GPCR) 'class B' or 'secretin' family. These receptors consist of a large N-terminal extracellular domain, which plays an important role in ligand binding, and seven transmembrane helices with intra and extracellular loops followed by an intracellular C terminal domain. In addition, there are variations of these receptors as a result of post-translational processing or interaction with receptor activity-modifying proteins (RAMPs). Three RAMPs have been identified, the presence of which can alter the structural confirmation of the receptor and thus ligand binding. The calcitonin-like receptor belongs to the class 'B' GPCR family. In the presence of RAMP 1, this receptor binds to and is activated by calcitonin gene related peptide. However, in the presence of RAMP 2 or RAMP 3, it is activated by adrenomedullin¹³⁵. RAMP2 also interacts with the glucagon receptor and the PTH1 receptor, and RAMP3 interacts with the secretin receptor¹³⁶. The significance of these RAMP and receptor pairings are unknown although their presence suggests alternative receptor morphology and potential agonists.

Following binding of secretin family peptides to the GPCR class B family of receptors, these hormones activate adenylate cyclase and increase cAMP through downstream signalling pathways¹³⁷. Although each hormone binds to its own respective receptor, at high doses, they can bind to other receptors within the family. For example, PACAP binds to several GPCRs including PAC1 (highest affinity to PACAP), VPAC1 and VPAC2 (VIP receptors) but can also bind to the secretin receptor at high doses¹³⁸. Due to their similarity within the secretin family, these hormones exert similar biological effects in the gut including inhibition of gastric acid secretion, increased insulin production and delayed gastric emptying. This thesis focuses on two members of the secretin family, glucagon and GLP-1, and the regulation of food intake, EE and glucose homeostasis.

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

GLP-1, along with glucagon, GLP-2, glicentin, OXM and other peptides are all encoded by the preproglucagon gene. The preproglucagon gene is widely expressed in the CNS, intestinal L cell and pancreatic α cells¹³⁹ and encodes a larger precursor called proglucagon. The post translational products depend on the tissue-specific presence of prohormone convertase (PC). In α cells, the pancreatic action of PC2 produces glucagon¹⁴⁰ whereas PC 1/3 action in intestinal L cells and the CNS produces GLP-1, GLP-2 and OXM¹⁴¹ (Figure 1.4).

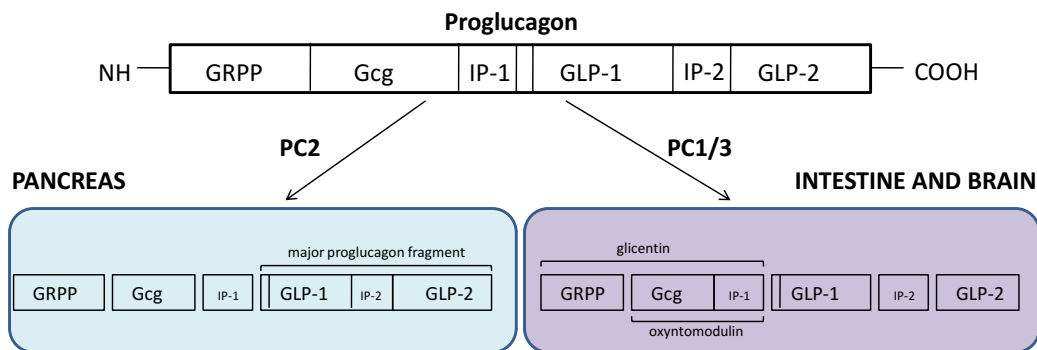


Figure 1.4 Post translational products of the preproglucagon gene. Products are dependent on site-specific presence of prohormone convertase (PC) 2 and 1/3. Abbreviations: glicentin related pancreatic polypeptide (GRPP), intervening peptide (IP), glucagon (Gcg), glucagon-like peptide-1 (GLP-1), glucagon-like peptide 2 (GLP-2).

GLP-1 is produced by the post translational processing of proglucagon by PC1 and 3 in the intestinal L cells and CNS. Within the L cells of the intestine, GLP-1 is released into the circulation but is rapidly degraded by the action of dipeptidyl peptidase IV (DPPIV)¹⁴² resulting in a half life of just a few minutes. In the brain, preproglucagon mRNA expression is exclusively seen in the caudal region of the NTS and overlaps with vagal afferent terminals from the gut^{143,144}. GLP-1 receptors are found in the PVN, ARC, DMN and SON of the hypothalamus and the AP, NTS and DMV of the brainstem¹⁴⁵⁻¹⁴⁸. GLP-1 immunoreactive (-ir) fibres are more widespread and have been identified in the hypothalamus, particularly the PVN, DMN, SON with lower densities seen in the ARC and VMN and the CeA^{143,149}. Retrograde labelling has identified a large number of GLP-1-ir neurones that project from the NTS to the PVN, DMN and SON^{143,144,150}.

GLP-1R are also extensively found outside the CNS including α , β and δ cells of the pancreatic islets, liver, lung, heart, kidney, stomach and intestine^{151,152}. GLP-1 and

exendin-4 (a GLP-1R agonist) administered ICV or subcutaneous (s/c) increase blood pressure and heart rate in rats although this may be a dose dependent effect ¹⁵³. In contrast, increasing evidence suggests beneficial cardiovascular effects following GLP-1 administration in patients with type 2 diabetes ^{154,155}.

In humans, intravenous (iv) infusion of GLP-1 reduces food intake in both lean and overweight volunteers^{156,157} and those with type 2 diabetes¹⁵⁸. Due to the short half life of circulating GLP-1, long acting analogues such as exenatide and liraglutide have been developed. Both exenatide and liraglutide cause weight loss in patients with type 2 diabetes ^{159,160}. Both GLP-1 analogues are now licenced for the use in patients with type 2 diabetes due to the effects on weight loss and improved glycaemic control¹⁶¹. GLP-1 lowers blood glucose mainly through its incretin effect and glucose dependent stimulation of insulin from pancreatic β cells¹⁶². GLP-1 also inhibits glucagon release¹⁶³ and delays gastric emptying¹⁶⁴. The precise mechanism of GLP-1 induced inhibition of glucagon is unclear however it has been postulated that GLP-1 binds to the GLP-1R on pancreatic α cells and inhibits glucagon release through a direct mechanism¹⁶³. Alternatively, glucagon may be inhibited indirectly by GLP-1 induced secretion of insulin, which itself reduces circulating glucagon. However, GLP-1 is able to suppress glucagon release from pancreatic islets in patients with type 1 diabetes that have no residual β cell insulin secretion¹⁶⁵.

The GLP-1 analogues, exenatide and liraglutide not only reduce food intake and cause weight loss as discussed previously, but they also improve glucose control through the release of insulin and inhibition of glucagon¹⁶⁶. Similarly, DPPIV inhibitors such as sitagliptin, prolong GLP-1 action and reduce glucose in patients with diabetes¹⁶⁷. Finally, GLP-1 agonists stimulate β cell proliferation and inhibit apoptosis through the release of transcription factors, thereby increasing β cell mass¹⁶⁸.

1.2.2.4 Glucagon

Glucagon is a 29 amino acid peptide secreted by the α cells of the pancreas and was first discovered by Kimball and Murlin in the 1920s¹⁶⁹. Fifty years later, the glucagon receptor (GCGR) was identified¹⁷⁰. Cloning of the receptor cDNA in 1993 identified it as belonging to the class B GPCR family¹³⁴. Although there is a high degree of homology between GLP-1 and glucagon and their receptors, these peptides bind with a high degree of selectivity to their respective receptors¹⁷¹. To date, only one GCGR has been identified and this is predominantly expressed in the liver and kidney¹⁷². Lower levels of GCGR

mRNA are found in the heart, lung, adipose tissue, spleen, small intestine, adrenal glands, endocrine pancreas and brain¹⁷². In the brain, GCGRs have been detected in the olfactory tubercle and bulb, hippocampus, anterior pituitary, amygdala, septum, medulla, thalamus and hypothalamus¹⁷³.

Expression of GCGR in extra-hepatic tissues suggests a broader role of glucagon beyond glucose homeostasis. During feeding, gut hormones known to regulate appetite, including GLP-1, PYY and PP, are released and signal to the brain to initiate meal termination. In rats, circulating glucagon also increases within the first few minutes of ingestion, peaks at the end of a meal and gradually returns to premeal levels within 10 minutes¹⁷⁴. This pattern is also seen during sham feeding¹⁷⁵ suggesting cephalic neural stimuli, rather than an effect of gut nutrients or mechanical gastric distension, account for the rise in glucagon. In humans, glucagon is also secreted at the time of meal ingestion^{176,177}. However, obese non-diabetic subjects show slower glucagon release following a meal compared to lean controls¹⁷⁸. This is in contrast to the response seen in patients with type 2 diabetes in whom glucagon production in response to hypoglycaemia is normal or reduced, yet the secretion of glucagon in response to meals is typically exaggerated¹⁷⁹.

Peripherally administered glucagon, whether given by the s/c, intraperitoneal (ip), iv or intramuscular (im) route in animals and humans, decreases food intake¹⁸⁰⁻¹⁸⁷. In contrast, administration of glucagon antibodies increases meal size and duration in rats^{183,188}. In obese Zucker rats, higher doses of ip glucagon are required compared to lean controls to elicit the same anorectic response¹⁸⁹. Consistent with this, repeated ip glucagon reduces the ability of the hormone to reduce food intake in rats¹⁹⁰. These findings suggest a state of 'glucagon resistance' in obese animals or tachyphylaxis in response to repeated administration, both situations requiring higher doses to elicit an anorectic effect.

The best characterised role of glucagon is in the regulation of glucose homeostasis via the activation of liver enzymes controlling gluconeogenesis and glycogenolysis. Glucagon release is stimulated by low circulating levels of glucose, certain FFAs and amino acids^{191,192}. In contrast, insulin, GLP-1 and somatostatin have an inhibitory effect on glucagon secretion¹⁹³⁻¹⁹⁶. Hypoglycaemia causes the rapid release of glucagon from pancreatic α cells via parasympathetic and sympathetic nerves that convey signals from the VMN to the islets of Langerhans¹⁹⁷. In addition, the liver contains afferent and efferent connections from the hypothalamus that are implicated in the control of glucose

homeostasis and the regulation of feeding. Stimulation of the VMN increases liver glycogenolysis and consequently blood glucose¹⁹⁸. In contrast, stimulation of the LHA decreases blood glucose via activation of hepatic glycogen synthesis through the stimulation of vagal parasympathetic nerves. Blood glucose concentration is also influenced by glucocorticoids, adrenaline and hormones such as thyroid hormones, sex hormones and growth hormone all of which can modify gluconeogenic enzymes in the liver. Once glucagon is released by pancreatic α cells, it enters the bloodstream, where its main target is the liver. Circulating glucagon is hydrolysed by DPPIV resulting in a short half life of 1-2 minutes¹⁹⁹. Clearance of glucagon also occurs at the liver and kidney²⁰⁰.

Glucagon antagonists have been developed in an attempt to reduce hepatic glucose output associated with raised glucagon levels seen in patients with type 2 diabetes²⁰¹. Glucagon antagonism in rodent models of obesity, decreases blood glucose²⁰²⁻²⁰⁴ and long term (18 weeks) inhibition of the GCGR using a monoclonal antibody normalises blood glucose in DIO mice²⁰⁵. However, these studies show no significant changes in food intake or body weight. Furthermore, concern has been expressed regarding the administration of glucagon antagonists in patients with type 2 diabetes as native glucagon protects against hypoglycaemia²⁰⁶. Lastly, glucagon antagonists stimulate hepatic lipogenesis and increase cholesterol levels²⁰⁷, additional effects that are undesirable in patients with diabetes.

The effects of glucagon on glucose homeostasis are well documented however less is known about its role in EE and thermogenesis. Glucagon secretion is stimulated by cold acclimatisation²⁰⁸ and peripheral administration of glucagon in rats increases oxygen consumption and body temperature²⁰⁹. In humans, glucagon increases EE by 15% when co-infused with somatostatin²¹⁰. However, the mechanism through which glucagon increases EE is unknown. Two explanations have been proposed²¹¹, firstly an increase in thermogenesis by brown adipose tissue (BAT) and secondly, futile substrate cycling whereby energy-consuming cyclical metabolic pathways are stimulated but with no net change in product formation in the liver or other tissues²¹².

In vitro studies suggest that glucagon may increase EE via stimulation of oxygen consumption and heat production in BAT^{213,214}. In rats, glucagon administration increases whole body oxygen consumption, body temperature, blood flow as well as temperature and mass of BAT^{215,216}. Many of these effects are attenuated by propranolol, a non-selective β adrenergic receptor blocker, as well as denervation of BAT, suggesting

a role for the sympathetic nervous system in increased EE following glucagon administration^{217,218}. Longer-term studies also implicate BAT in glucagon-induced thermogenesis. Glucagon infusion via continuous osmotic mini pumps for 5 days (at both physiological and pharmacological doses) increases guanosine diphosphate (GDP) binding to brown fat mitochondria²¹⁶. Importantly however, most studies investigating the role of glucagon and EE administer pharmacological doses of glucagon which could activate the hypothalamic-pituitary-adrenal axis and hence a 'stress response'. A physiological role for glucagon in the regulation of EE remains to be determined.

1.2.2.5 Gut hormones

1.2.2.5.1 Oxyntomodulin

Like GLP-1, OXM is secreted from intestinal L cells post-prandially. No specific receptor has been identified for OXM. However, OXM acts as an agonist at both the GLP-1R and GCGR²¹⁹⁻²²¹. In rodent and human studies, OXM has been shown to reduce food intake and body weight, increase EE and improve glucose control^{220,222,223}. In addition, following gastric bypass surgery, obese patients exhibit a two fold rise in OXM, GLP-1 and PYY levels following an oral glucose load compared to controls who had similar body weight loss due to dieting²²⁴. These findings suggest that these changes in circulating gut hormone levels are due to the surgical procedure rather than weight loss and account for the improved glucose profile seen so rapidly following surgery. It has been proposed that the effects of OXM on food intake occur mainly via the GLP-1R. Food intake and c-fos activation in the ARC following peripheral OXM were blocked by the prior administration of exendin₍₉₋₃₉₎²²⁵. Furthermore, GLP-1R knockout mice do not demonstrate reduced food intake following centrally administered OXM²¹⁹. Peripheral OXM also significantly increases c-fos expression in the PVN, AP and NTS. This is abolished in GLP-1R but not GCGR knockout mice²¹⁹. This suggests that the anorectic effect of OXM relies on the presence of a functional GLP-1R. However, both GLP-1 and OXM increase heart rate in mice. This effect is abolished in GLP-1R knockout mice following GLP-1 administration but not OXM²²⁶. Therefore, OXM may have effects that are independent of the GLP-1R.

Kosinski et al, have developed a OXM agonist that differs from OXM by only one residue, yet has no significant activity at the GCGR²²⁷. Chronic treatment with OXM in mice, produces significant weight loss compared to the GLP-1R agonist, suggesting a beneficial weight lowering effect through GCGR agonism²²⁷. Studies in GLP-1R and GCGR knockout mice have also demonstrated the important contribution of GCGR agonism in EE and

weight loss^{228,229}. Repeated administration of a dual GCGR and GLP-1R agonist results in weight loss and reduced food intake in wild-type mice, however this effect is attenuated in GCGR knockout mice²²⁸. Similarly, administration of a dual glucagon and GLP-1 receptor agonist for one month to DIO mice, significantly decreases food intake, body weight and fat mass and increases EE compared to controls²²⁹. These effects are still evident following administration in GLP-1R knockout mice²²⁹. Therefore OXM provides a unique model of dual receptor agonism, causing weight loss through a reduction in food intake via the GLP-1 receptor and increased EE via the GCGR.

1.2.2.5.2 Peptide tyrosine tyrosine (PYY)

PYY is a member of the pancreatic polypeptide (PP) fold family of peptides released by L-cells in the gut into the circulation following a meal. Members of the PP-fold family include NPY, PYY and PP which share a common tertiary structure called the PP-fold and are involved in food intake regulation. PYY is a 36 amino acid peptide and cleaved at the N-terminus by DPPIV to create the truncated form PYY₃₋₃₆. Full length PYY binds to the GPCRs Y1, Y2 and Y5. PYY₃₋₃₆ has highest affinity for the Y2 receptor and is able to cross the BBB freely by a non-saturable mechanism²³⁰. The Y2 receptor is an inhibitory presynaptic receptor highly expressed on ARC NPY neurones¹². NPY neurones also release GABA which causes tonic inhibition of POMC neurones²³¹. Therefore the anorectic action of PYY occurs through inhibition of NPY neurones and reduction of GABA-mediated inhibition of POMC neurones.

PYY is released into the circulation following a meal, with levels rising to a plateau after 1-2 hours and remaining elevated for up to 6 hours¹²⁹. The rise in PYY levels is proportional to caloric intake and meal composition, so that fatty meals result in a higher rise compared to protein or carbohydrate meals²³². PYY₃₋₃₆ reduces food intake and body weight in rodents and humans^{233,234} and improves insulin sensitivity in rodent models of DIO²³⁵. In obese subjects, the rise in PYY following a meal is blunted resulting in impaired satiety and hence greater food intake²³⁶.

In addition to a hypothalamic effect of PYY₃₋₃₆, there is evidence that PYY₃₋₃₆ also acts at the brainstem. Y2 receptors are found in important brainstem regions involved with appetite control such as the AP and NTS^{88,237}. Peripheral administration of PYY₃₋₃₆ causes c-fos activation in these areas²³⁸ and lesioning of the brainstem-hypothalamic neuronal pathways or vagotomy abolishes the anorectic effects of peripheral PYY₃₋₃₆^{87,88}. It therefore appears that PYY₃₋₃₆ can also exert an anorectic effect via the vagus nerve to the brainstem, which in turn has connections to the hypothalamus.

1.2.2.5.3 Pancreatic polypeptide (PP)

PP is secreted by PP cells in the pancreatic islets of Langerhans following a meal and circulating levels are proportional to calories ingested. Peripheral injection of PP in mice²³⁹, dogs²⁴⁰ and humans²⁴¹ dose-dependently reduces food intake. Transgenic mice which overexpress PP are lean and demonstrate reduced food intake²⁴². In human studies, iv infusion of PP causes a significant reduction in hunger and food intake²⁴³. These anorectic effects are thought to be principally mediated through the Y4 receptor since the effect is abolished in Y4 receptor knockout mice²⁴⁴. Ip administration of PP causes c-fos activation in the AP and NTS of the brainstem and ARC, PVN, DMN, VMN and LHA of the hypothalamus²⁴⁵. Y4 receptors are highly expressed in all these regions²⁴⁶.

1.2.2.5.4 Cholecystokinin

CCK was the first gut hormone shown to have an effect on food intake²⁴⁷. CCK is released from I cells of the small intestine and levels rise rapidly following a meal, reaching a peak within 15 minutes²⁴⁸. Peripheral administration of CCK reduces food intake in rodents and humans^{247,249}. Although there are two CCK receptors: CCK1 and CCK2, the anorectic effect appears to be mediated through the CCK1 receptor. CCK 1 receptor knockout rats are obese and ip injection of a CCK1 antagonist results in weight gain²⁵⁰. However, feeding studies to date have been conflicting and although CCK infusions in rodents decreases meal size, some reports have found a compensatory increase in meal frequency²⁵¹. The CCK2 receptor may also have a minor role since CCK2 receptor knockout mice are hyperphagic and have increased body weight compared to wild types²⁵². However, the anorectic effect following peripheral administration of CCK is retained in CCK2 receptor knockout mice²⁵³ and both agonists and antagonists at the CCK2 receptor show no effect on food intake^{254,255}.

Ip administration of CCK increases c-fos expression in the DMN and CART neurones in the PVN^{256,257}. Direct administration of CCK into the DMN decreases food intake²⁵⁸ and down regulates NPY gene expression^{64,250}. The anorectic effect of CCK is abolished following vagotomy²⁵⁹ suggesting that vagal afferent fibres signalling to the DVC in the brainstem are responsible for the reduction in food intake²⁶⁰.

1.2.2.5.5 Ghrelin

Unlike other gut hormones, ghrelin initiates hunger prior to a meal and increases food intake^{261,262}. Ghrelin is produced by the stomach and is an endogenous ligand for the GHS-R1 receptor. Ghrelin-ir neurones are found adjacent to the 3rd ventricle in the brain and between the DMN, VMN, PVN and ARC in the hypothalamus²⁶³. Furthermore, ghrelin

neurons have terminals on hypothalamic NPY/AgRP, POMC and CRH neurons in addition to orexin fibres in the LHA²⁶³⁻²⁶⁵. It appears that the orexigenic effect of ghrelin is mediated predominantly through NPY/AgRP ARC neurons. Peripheral administration of ghrelin stimulates c-fos expression in ARC NPY/AgRP neurons and increases hypothalamic NPY mRNA expression²⁶¹. Ghrelin-induced feeding is abolished by central administration of NPY and AgRP antibodies and antagonists in rats²⁶¹ and in NPY and AgRP null mice²⁶⁶.

In addition to effects on ARC NPY/AgRP neurons, ip administration of ghrelin also stimulates c-fos expression in the PVN²⁶⁷. Direct injection of ghrelin into the PVN stimulates food intake²⁶⁸. This effect is attenuated by pre-treatment with a melanocortin agonist²⁶⁸ or s/c rimonabant, an endocannabinoid CB1 receptor antagonist²⁶⁹. In addition, ghrelin does not induce an orexigenic effect in CB1 receptor knockout mice²⁷⁰. This suggests that the orexigenic effect of ghrelin is dependent on the endocannabinoid system, with possible modulation of anorectic neurotransmitters such as α -MSH.

1.2.2.6 Leptin

Leptin is secreted by adipocytes and circulates at concentrations proportional to fat mass. Leptin administration in rodents and humans decreases food intake and body weight²⁷¹⁻²⁷³. However large amounts of adipose tissue resulting in high circulating levels of leptin, leads to leptin resistance and a lack of expected anorectic effect²⁷⁴. Rodents and humans lacking leptin are obese and hyperphagic, although this can be ameliorated by leptin administration²⁷⁵⁻²⁷⁷. Leptin is able to cross the BBB and bind to the leptin receptor (Ob-Rb) in the hypothalamus²⁷⁸. The predominant effects of leptin on food intake and EE are mediated in the ARC, causing activation of anorectic POMC neurons and inhibition of orexigenic AgRP/NPY neurons^{34,231}. Leptin also decreases MCH and orexin and increases CRH gene expression in the hypothalamus²⁷⁹⁻²⁸¹ and leptin administration appears to modulate ghrelin signalling in the ARC²⁶². Although reduction of food intake and body weight by leptin is largely attributed to its hypothalamic actions, administration of leptin into the 4th ventricle or DVC of the brainstem, have similar anorectic effects²⁸². In addition, direct injection of leptin into the NTS increases STAT3 phosphorylation (transcription activator downstream of the leptin receptor) in the ARC and VMN²⁸². Since vagotomy in rats does not attenuate the anorectic response to peripherally administered leptin²⁸³, it is unclear what the role of leptin in the brainstem may be. Peripheral administration of leptin increases c-fos activation in the NTS²⁸³, where vagal afferent fibres terminate. It has been suggested that leptin may interact with anorectic gut hormone pathways signalling via the vagus

nerve. The leptin receptor is co-expressed with the CCK-1 receptor in the rat nodose ganglion²⁸⁴ and a subset of gastric vagal afferents which are unresponsive to leptin alone, become leptin sensitive after prior treatment with CCK²⁸⁵. Furthermore, the anorectic response following ICV leptin administration, is blocked by ip devazepide, a CCK1 receptor antagonist²⁸⁶. These findings suggest that leptin acting at the brainstem to reduce food intake requires intact CCK signalling²⁸⁶. Conversely, in rodents, central or peripheral leptin pretreatment enhances CCK-induced anorexia^{272,287,288} suggesting a synergistic mechanism between leptin and CCK in the control of food intake at the level of the brainstem.

1.2.2.7 Insulin

Insulin also acts in the brain as an adiposity signal. Like leptin, it is able to cross the BBB and reduces food intake and increases EE ²⁸⁹. Insulin inhibits NPY/AgRP neurones in the ARC¹⁷. However, the anorectic effects of insulin may also reflect an interaction with the melanocortin system. Insulin receptors are present on ARC POMC neurones and ICV administration of insulin increases ARC POMC mRNA expression²⁹⁰. Furthermore, the anorectic actions of insulin are blocked by melanocortin antagonists^{17,290}.

1.3 Regulation of energy expenditure and body weight

EE is the amount of work required by the body to sustain vital functions such as thermogenesis, physical activity, cardiac function and respiration. Total EE can be divided into three main components. Firstly, the basal metabolic rate (BMR) which is the minimum energy required by the body at rest to ensure function of cells and tissues. This represents approximately 60-75% of total EE and depends largely on body weight and body composition. Secondly, physical activity on average accounts for 15-30% of total daily EE. Finally, energy is also expended through adaptive thermogenesis in response to cold, food and physical activity. All three components of EE are tightly regulated, predominantly by central circuits in the brainstem and hypothalamus in addition to peripheral tissues including skeletal muscle and BAT.

1.3.1 Central regulation of energy expenditure

1.3.1.1 Cold induced thermogenesis

For an organism to maintain a constant core body temperature despite changes in environmental temperature, it must be able to adapt its heat production accordingly.

Following exposure to cold, autonomic and hormonal responses activate a number of heat saving mechanisms including cutaneous vasoconstriction to conserve heat in the body core, piloerection, and shivering. However, animals are able to adapt to prolonged cold exposure and as the initial heat produced by shivering in skeletal muscle declines²⁹¹ other thermogenic mechanisms begin to take effect in tissues such as BAT and skeletal muscle^{292,293}.

Thermoreceptors in the skin transmit sensory information via the spinal dorsal horn to the lateral PBN in the pons and subsequently to the medial preoptic area (MPO) of the hypothalamus²⁹⁴. Inhibition of MPO neurones completely blocks the activation of BAT thermogenesis and the increases in EE and heart rate seen following skin cooling²⁹⁵. Although the precise mechanism is unknown, it is thought that afferent signals from thermoreceptors result in excitation of neurones from the MPO to the DMN and the rostral raphe pallidus nucleus (rRPn) in the brainstem²⁹⁵. Inhibition of neurones in the DMN blocks cold-evoked excitation of BAT sympathetic nerve activity and thermogenesis²⁹⁶ in addition to shivering thermogenesis²⁹⁷. Since neurones in the DMN have not been found to project directly to sympathetic preganglionic neurones but do project to the rRPn, it is thought that increased BAT thermogenesis is due to excitation of rRPn neurones via the DMN²⁹⁸. The rRPn is located in the brainstem medulla, a key area containing sympathetic premotor neurones. Retrograde tracing techniques indicate that interscapular BAT sympathetic premotor neurones project to the rRPn and PVN²⁹⁹. Stimulation of rRPn neurones using glutamate agonists and GABA_A receptor antagonists, increases BAT sympathetic nerve activity outflow and thermogenesis^{300,301}. The rRPn also has projections to skeletal muscle, and increases in muscle electromyography (EMG) activity and shivering are seen following excitation of rRPn neurones suggesting a role for rRPn neurones in shivering thermogenesis^{302,303}.

Within the inner mitochondrial membrane of BAT adipocytes, a specialized proton transport channel is located, called uncoupling protein-1 (UCP-1)^{304,305}. Under normal circumstances in most tissues, the oxidation of fuel substrates results in mitochondrial ATP production through oxidative phosphorylation. Oxidative phosphorylation is driven by an electrochemical gradient across the mitochondrial inner membrane whereby protons move down the gradient and enter the mitochondrial matrix through ATP synthase. In BAT, the presence of UCP-1 protons bypass ATP synthase and the energy of the electrochemical gradient is dissipated as heat. Two UCP-1 homologues, UCP-2³⁰⁶ and UCP-3³⁰⁷, have also been identified, with 59 and 57% homology to UCP-1, respectively. Both homologues demonstrate proton transport activity suggesting they may play a role

in adaptive thermogenesis. However, despite having a similar action to UCP-1, their distributions are far more widespread. UCP-2 is present in many organs and cell types at varying levels and UCP-3 is predominantly expressed in skeletal muscle³⁰⁸. Furthermore, expression of UCP-2 and UCP-3 is up-regulated during fasting^{309,310}, a state normally associated with reduced EE.

During cold acclimatisation, hypothalamic activation of the sympathetic nervous system (SNS) innervating BAT increases noradrenaline (NA) which binds to β_3 -adrenoreceptors. Adrenergic stimulation activates the production of cAMP, which activates UCP-1 in addition to inducing lipolysis. The resulting increase in FFAs following lipolysis enhances UCP-1 activity. Although the precise interaction between FFAs and UCP-1 is not yet known, two mechanisms have been proposed. Firstly, it is thought UCP-1 may directly transport protons from the intermembrane space to the matrix, with carboxyl groups from FFAs acting as cofactors³¹¹. However, an alternative mechanism suggests that FFAs cross the inner membrane causing proton dissociation and UCP-1 subsequently transports the resulting FA anions back across the membrane^{293,312}. This 'cycling' of FFAs drives uncoupling of oxidative phosphorylation and the dissipation of energy as heat (Figure 1.5).

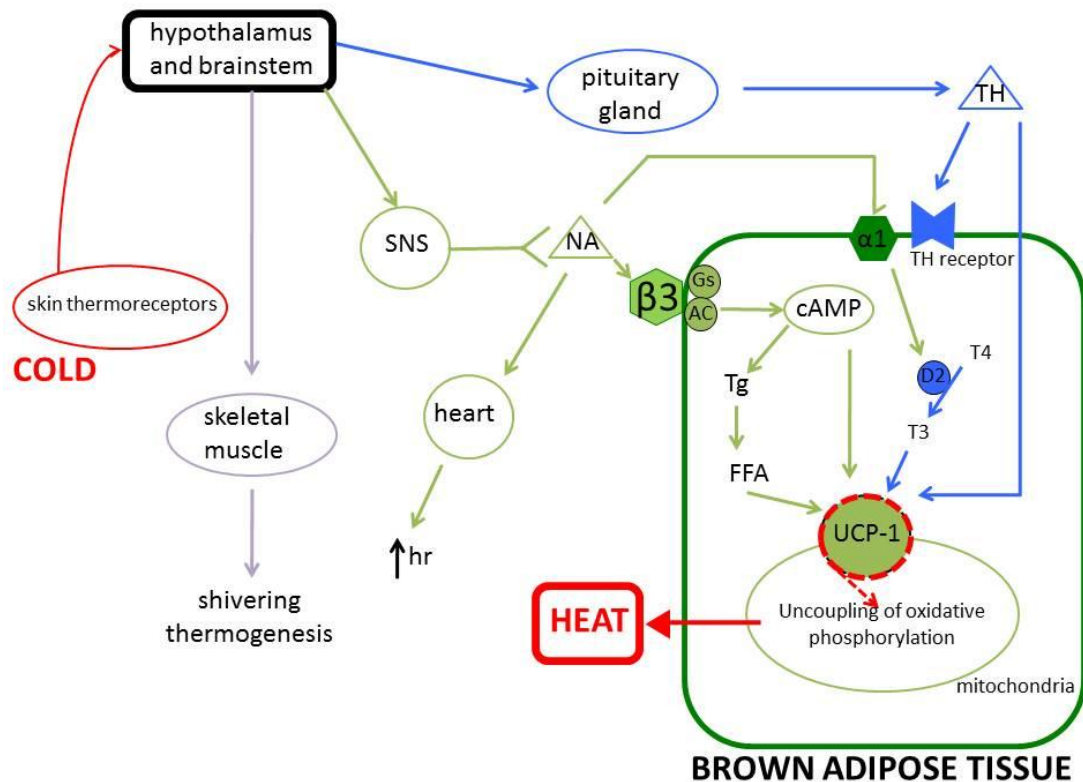


Figure 1.5 Thermogenesis in brown adipose tissue. Signals from the hypothalamus activate the sympathetic nervous system (SNS) resulting in the release of noradrenaline (NA). In brown adipocytes, NA binds primarily to β_3 -adrenoreceptors (β_3). Adrenergic stimulation activates the production of cAMP, which in turn induces lipolysis. The resulting increase in free fatty acids (FFAs) further enhances UCP-1 activity. This results in the uncoupling of oxidative phosphorylation and the dissipation of energy as heat. Abbreviations: α_1 adrenoreceptor (α_1), stimulatory G protein (G_s), adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP), triglycerides (Tg), uncoupling protein-1 (UCP-1), thyroid hormone (TH), 2-iodothyronine deiodinase (D2), thyroxine (T4), tri-iodothyronine (T3), heart rate (hr), free fatty acid (FFA), sympathetic nervous system (SNS), noradrenaline (NA).

1.3.1.2 Sympathetic nervous system (SNS)

The SNS is a well known activator of adaptive thermogenesis and is controlled centrally by the hypothalamus and brainstem. Stimulation of the SNS results in the release of NA from synaptic nerve terminals and binding to β_3 -adrenergic receptors in BAT to promote UCP-1 production. In addition, stimulation of α_1 adrenergic receptors by NA increases thyroid hormone activation of UCP-1 in BAT (see section 1.3.2.2). Finally, SNS stimulation of the heart increases heart rate and blood flow to organs. The role of the SNS in adaptive thermogenesis has been supported by studies using SNS blockers^{313,314}, β -adrenergic receptor agonists^{313,314} and in mice lacking NA³¹⁵. Peripheral administration of hexamethonium, an antagonist at the nicotinic acetylcholine receptor in sympathetic and parasympathetic ganglia, prevents the rise in oxygen consumption following cold exposure in rats³¹⁴. In contrast, atropine, an antagonist at the muscarinic receptors of the parasympathetic nervous system (PNS), did not have this effect, implying a role for the SNS but not PNS in thermogenesis³¹⁴. Similarly, infusion of NA increases oxygen consumption and core body temperature in rats³¹⁶. Furthermore, beta-dopamine hydroxylase knockout mice are unable to synthesise NA or adrenaline and have lower core body temperatures³¹⁵. These findings highlight the importance of the SNS and the effects of catecholamines in thermogenesis.

1.3.2 Peripheral regulation of energy expenditure

1.3.2.1 Diet induced thermogenesis

Diet induced thermogenesis is the short term increase in EE observed in response to food intake and accounts for approximately 10% of total EE. EE rises following ingestion of food but declines during periods of starvation^{317,318}. Heat production arises from the digestion, absorption and storage of food.

1.3.2.2 Brown adipose tissue (BAT)

BAT, in contrast to white adipose tissue (WAT), is involved in the production of heat rather than storage although both are derived from mesenchymal stem cells³¹⁹. Adipocytes in BAT contain large number of mitochondria and high expression of UCP-1. BAT is well recognised to have an important role in the maintenance of body temperature in animals and human neonates^{320,321}. Increased BAT activity in rodents promotes EE, reduces adiposity and protects mice from DIO^{322,323}. Recent studies using positron emission tomography-computed tomography (PET-CT) have shown that BAT is also present in human adults, with reduced activity seen with increasing obesity³²⁴. In

most mammals, BAT is predominantly found in the interscapular region and axillae³²⁵, although in mice it has also been found in small amounts in skeletal muscle³²⁶. In addition, adipocytes in WAT have been shown to differentiate into BAT in response to cold or prolonged β -adrenergic stimulation³²². Furthermore, epididymal WAT adipocytes in rats chronically treated with rosiglitazone (a PPAR gamma agonist) share BAT-type characteristics, show increased UCP-1 expression and are known as 'brite' (brown-in-white) or 'beige' adipocytes³²⁷. Finally, the isolation of 'beige' cells from WAT in mice have been reported which have a distinct gene expression profile from brown or white adipocytes. Despite showing low basal levels of UCP1, they respond to cAMP with raised UCP-1 expression³²⁸.

1.3.2.3 Hypothalamic-pituitary-thyroid axis

The hypothalamo-pituitary-thyroid (HPT) axis has been shown to play an important role in regulating EE. Thyrotropin-releasing hormone (TRH), synthesised in the PVN, reaches target cells in the anterior pituitary via the pituitary portal vein to stimulate the release of thyroid-stimulating hormone (TSH) into the circulation. TSH in turn stimulates the release of thyroid hormone (TH) from the thyroid gland. TH is a key modulator of adaptive thermogenesis. Even a modest increase in TH can result in thermogenesis, whereas the opposite effect is observed when TH levels are reduced³²⁹. However, the precise mechanism through which TH stimulates thermogenesis is not fully understood.

A large number of TH receptors are expressed on BAT tissue³³⁰ and TH appears to directly stimulate transcription of the UCP-1 gene³³¹. UCP-1 gene expression can also be regulated indirectly by 2-iodothyronine deiodinase (D2), an enzyme which catalyses the conversion of inactive TH (T_4 /thyroxine) to the active form (T_3 /tri-iodothyronine)³³². D2 is highly expressed in BAT and is activated by the SNS via α_1 -adrenergic receptors³³³.

1.3.2.4 Skeletal muscle

Unlike rodents and newborns, adult humans have very little BAT suggesting the presence of other thermogenic mechanisms. Skeletal muscle contributes up to 40% of total body mass and accounts for approximately 20-30% of total resting oxygen uptake³³⁴. In addition, up to 50% of adrenaline-induced thermogenesis in humans has been demonstrated to originate from skeletal muscle³³⁵. In humans, mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes and those who are obese^{336,337}.

Despite these findings, the precise mechanism through which skeletal muscle dissipates heat is unknown. UCP-3 expression appears to be mainly limited to skeletal muscle suggesting a role in skeletal muscle thermogenesis. Mice overexpressing UCP3 are leaner and more insulin sensitive³³⁸ despite being hyperphagic³³⁹. Consumption of a high fat diet in rodents increases UCP-3 mRNA levels in skeletal muscle³⁴⁰. Overexpression of UCP-3 in transgenic mice results in mitochondrial uncoupling in skeletal tissue and resistance to DIO when fed a high fat diet³⁴¹. Interestingly, a newly identified protein named irisin, is secreted by skeletal muscle in mice and stimulates UCP-1 expression and BAT-type differentiation in WAT³⁴². Circulating levels of irisin increase in mice and humans following exercise³⁴². In addition, raised levels of plasma irisin in mice fed a high fat diet, increases EE, weight loss and improves glucose control³⁴². These findings suggest that in response to excessive energy intake, increased expression of UCP-3 and production of irisin in skeletal muscle increases EE by stimulating thermogenesis in skeletal muscle, BAT and WAT.

1.4 Summary

Obesity is increasingly prevalent and associated with several disease states that decrease life expectancy. There are no effective pharmacological agents that reduce body weight long-term and those currently on the market, have many unwanted side effects. Body weight is maintained by complex mechanisms involved in the regulation of food intake and EE. Following a meal, gut hormones signal to the brain via the circulation and vagus nerve. 'Anorectic pathways' within the hypothalamus and brainstem signal to other areas within the brain including reward centres, resulting in a feeling of fullness and meal termination. In addition, adiposity signals such as leptin signal to the brain to convey information about energy reserves.

Body weight is also controlled through EE. Following a meal, EE increases to 'burn off' calories consumed. EE is also increased following cold acclimatisation in order to produce heat. Thermoreceptors in the skin signal to key areas in the brain which in turn activate peripheral tissues such as BAT and skeletal muscle to increase thermogenesis. Although the precise mechanisms through which thermogenesis occurs is poorly understood, over the past few decades research has focused on mitochondrial uncoupling proteins that play a major role in energy homeostasis.

GLP-1 is secreted from the gut following a meal and signals to the brain to reduce food intake. Long-acting analogues of GLP-1 are used in patients with type 2 diabetes to improve glucose control and promote weight loss. Glucagon also reduces food intake. However, evidence suggests a predominant role for glucagon in increasing EE. The combination of glucagon and GLP-1 has not previously been investigated and could offer a novel dual mechanism through which weight loss could be sustained. This thesis therefore focuses on the effects of glucagon and GLP-1 independently, and in combination, on food intake, EE and glucose homeostasis.

1.5 Hypotheses

Gut hormones are important in the regulation of energy homeostasis and body weight. This thesis focuses on the role of glucagon and GLP-1 as members of the secretin family of peptides, in the regulation of food intake, EE and glucose homeostasis.

Specifically, I hypothesise that:

- Co-administration of glucagon and GLP-1 in mice acutely reduces food intake to a greater degree than each hormone alone, and that these effects are mediated by specific interaction with satiety centres in the hypothalamus and brainstem.
- Chronic co-administration of novel glucagon and GLP-1 analogues in DIO mice reduces body weight and improves glucose homeostasis, to a greater degree than each analogue alone, and that this effect is due to reduced food intake and increased EE.
- Co-administration of glucagon and GLP-1 reduces food intake, increases EE and maintains normoglycaemia following infusion in overweight human volunteers.

1.6 Aims

In this series of investigations, I aimed to:

- Investigate the acute effects of co-administration of glucagon and GLP-1 in mice, on food intake and c-fos expression in the hypothalamus and brainstem.
- Investigate the chronic effects of co-administration of novel glucagon and GLP-1 analogues on food intake, body weight and glucose homeostasis in DIO mice.
- Investigate the effect of co-administration of glucagon and GLP-1 on food intake, EE and glucose homeostasis in overweight human volunteers.

CHAPTER 2:

THE EFFECTS OF CO- ADMINISTRATION OF GLUCAGON AND GLP-1 ON ENERGY HOMEOSTASIS IN RODENTS

2.1 Introduction

Glucagon and GLP-1 are both products of the preproglucagon gene which is found in the CNS, in intestinal L cells and pancreatic α cells¹³⁹. In the brain, preproglucagon mRNA expression is exclusively seen in the caudal region of the NTS (cNTS) and overlaps with vagal afferent terminals from the gut^{143,144}. Although glucagon opposes the action of GLP-1 on glucose homeostasis, both hormones share an anorectic effect. Peripherally administered glucagon decreases food intake and body weight in both rodents and humans^{181,182,184,187,343}. Conversely, administration of glucagon antibodies increases meal size in rats^{183,188}. GLP-1 receptor agonists are licensed for the treatment of type 2 diabetes mellitus³⁴⁴. In addition to improving glycaemia¹⁵⁹, they reduce appetite, producing modest weight loss^{159,160}.

GLP-1 is released into the circulation following a meal in proportion to the calories consumed¹³². Peripheral administration of GLP-1 in mice and rats reduces food intake³⁴⁵ and increases c-fos-ir in the brainstem and the PVN, but not in the ARC^{87,219,225}. Vagotomy or ablation of the brainstem-hypothalamus pathways attenuates the reduction in food intake seen following peripheral GLP-1 administration^{87,346}. This suggests that peripheral effects are predominantly mediated via the brainstem. However, central administration of GLP-1 to rats also inhibits food intake³⁴⁶ and activates c-fos expression in the ARC, amygdala and PVN^{346,347}. This anorectic effect is abolished in rats with ARC lesions induced by s/c monosodium glutamate¹⁴⁷. In addition, intra-ARC injection of exendin₍₉₋₃₉₎ (a GLP-1R antagonist) diminishes the anorectic effects of centrally but not peripherally administered GLP-1²²⁵. Therefore, the anorectic effect of GLP-1 appears to depend on the mode of administration. Although the precise mechanism of GLP-1 induced anorexia is unknown, GLP-1R mRNA is densely expressed in the ARC and is found in over 60% of ARC neurones that also express POMC mRNA^{348,349}. Furthermore, ICV administration of exendin-4, a GLP-1R agonist, activates α -MSH/POMC neurones in the ARC³⁵⁰. These findings suggest that activation of the melanocortin system in the brain may be responsible for the anorectic effect of GLP-1.

In rats, circulating glucagon also increases within the first few minutes of ingestion, peaks at the end of a meal and gradually returns to pre-meal levels within 10 minutes¹⁷⁴. This pattern is also seen during sham feeding¹⁷⁵ suggesting cephalic neural stimuli, rather than an effect of gut nutrients or mechanical gastric distension, account for the rise in glucagon. In humans, glucagon is also secreted at the time of meal ingestion^{176,177}. Peripherally administered glucagon, whether given by the s/c, ip, iv or im route in

animals and humans, decreases food intake¹⁸⁰⁻¹⁸⁷. In contrast, administration of glucagon antibodies increases meal size and duration in rats^{183,188}. The glucagon anorectic signal appears to reach the brain via the vagus nerve since total subdiaphragmatic vagotomy, selective hepatic vagotomy or lesions in the AP and NTS all prevent its anorectic effect^{181,351-353}. However, the mechanism by which glucagon generates a vagal afferent signal is unknown and GCGRs have not been identified on vagal sensory neurones. This suggests an indirect mechanism perhaps through the release of other hormones. It has been proposed that glucagon reduces food intake indirectly through changes in circulating glucose levels, insulin secretion or another unknown mechanism^{190,354}. The satiety effect of glucagon may depend on its glycogenolytic and hyperglycaemic effects, since glucagon inhibits feeding less potently when liver glycogen is reduced by prolonged fasting³⁵⁴. However, the stress induced by such a prolonged fast and the subsequent hunger may override the anorectic effects of glucagon. Furthermore, hyperglycaemia alone does not reduce food intake since iv glucose infusions do not inhibit feeding in monkeys, dogs or rats³⁵⁵⁻³⁵⁷. In addition, glucagon does not suppress food intake in sham fed animals despite marked hyperglycaemia³⁵⁸. Previously it was thought that the anorectic effect of glucagon was mediated by an increase in insulin secretion¹⁹⁰. However, the anorectic effects of hepatic portal vein glucagon infusion in rats are not affected by co-administration with insulin or insulin antibodies³⁵⁹. Alternatively, glucagon may cause activation of a stress response and stimulation of the SNS or aversive signals such as nausea. CTA studies in response to glucagon administration have not been described, however ip glucagon reduces food intake in rats without affecting the normal behavioural sequence of satiety¹⁸².

Despite these findings, the mechanisms through which GLP-1 or glucagon exert their anorectic effects are still unknown. Historically, the deleterious effect of unopposed glucagon on glucose homeostasis has discouraged study of the mechanism by which glucagon reduces appetite. In particular, the pattern of neuronal activation in the CNS after peripheral administration of glucagon has not been described. The individual anorectic effects of GLP-1 and glucagon are attenuated by vagotomy or by ablation of brainstem-hypothalamic pathways suggesting a peripheral mechanism of action mediated by the vagus nerve^{87,181,346,351-353}. However, like other gut hormones, it is possible that glucagon and GLP-1 also act directly via the circulation and an incomplete BBB at the hypothalamus and brainstem³⁶⁰. Central administration of GLP-1 in rodents also inhibits food intake and GLP-1R mRNA is densely expressed in the ARC^{348,349}.

Similarly, central administration of glucagon in animals, reduces food intake³⁶¹⁻³⁶³ and although much lower levels of GCGR mRNA are detected in the brain compared to the periphery, CNS GCGR mRNA expression is strongest in the hypothalamus and amygdala^{173,364}.

Therefore, my aim was to establish whether glucagon and GLP-1, whilst evolutionarily similar, cause c-fos activation in distinct areas within the brain to exert an anorectic effect. In order to investigate the relationship between the anorectic effects of glucagon and GLP-1, I have studied their individual and combined effects on acute food intake in mice. I used c-fos immunohistochemistry to characterise neuronal activation following the administration of glucagon and GLP-1. My aim was to determine whether these two hormones act via distinct pathways and whether lower doses of each individual hormone could be administered in combination to cause a significant reduction in food intake. In addition, I studied the effect of glucagon and GLP-1 co-administration on circulating glucose. My aim was to determine whether co-administration of low doses of glucagon and GLP-1 was associated with a change in plasma glucose concentration.

2.2 Hypotheses and Aims

2.2.1 Hypotheses

I hypothesised that:

- glucagon and GLP-1 administration decreases food intake in a dose dependent manner.
- co-administration of glucagon and GLP-1 causes a greater reduction in food intake than each hormone alone.
- co-administration of glucagon and GLP-1 ameliorates the rise in glucose observed following glucagon alone.
- peripheral administration of glucagon and GLP-1 activates distinct areas in the brain to reduce food intake.

2.2.2 Aims

To investigate:

- the anorectic effect of peripheral glucagon administration at different doses, in fed and fasted states and at different periods during the light and dark phase of feeding.
- the effects of glucagon and GLP-1 co-administration on food intake and glucose levels at sub-anorectic doses.
- which areas in the brainstem and hypothalamus are activated by peripheral administration of glucagon.
- which areas in the brainstem and hypothalamus are activated by sub-anorectic doses of glucagon and GLP-1 in combination.

2.3 Materials and Methods

2.3.1 Peptide synthesis

Human glucagon and GLP-1 were purchased from Bachem Ltd. (Merseyside, UK).

2.3.2 Animal husbandry and conditions

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/7236). Adult male C57BL/6 mice (Charles River, Margate, UK) weighing 25-30 g were maintained in individual cages under controlled temperature (21-23°C) and lights (12:12 hr light-dark cycle, lights on at 0700). Animals were allowed *ad libitum* access to water and RM1 diet (Special Diet Services, Witham, UK) unless otherwise stated. Animals were regularly handled and acclimatised to ip or s/c injections in order to reduce non-specific stress at the time of the study.

2.3.3 Investigation of the acute effects of glucagon on food intake in mice

Experiments were carried out during the early light phase (0800-1000), after fasting from 1600 the preceding day unless otherwise specified. Randomisation of treatment allocation was stratified by body weight. All mice were injected (maximum volume 50 µl) within a single 30 minute period, with individual injection times noted, then returned to their home cages with a known amount of food. Food was reweighed at 30, 90 and 180 minutes post injection. Doses of glucagon administered are provided for each experiment and are based on previous studies^{185,365}.

2.3.4 Investigation of the acute effects of GLP-1 on food intake in mice

Experimental procedures were performed as described in section 2.3.3. Based on previous studies^{345,366}, mice received s/c GLP-1 at 3, 10, 30, 50, 100 or 600 nmol/kg or saline (n=6).

2.3.5 Investigation of the acute effects of glucagon and GLP-1 co-administration on food intake in mice

Experimental procedures were carried under identical conditions as described in section 2.3.3. Based on the results of the dose response studies (section 2.4.1 and 2.4.2),

each mouse received two s/c injections, one of 30nmol/kg glucagon or saline, and the other of 10 nmol/kg GLP-1 or saline, in quick succession (n=6).

2.3.6 Investigation of the acute effects of glucagon and GLP-1 co-administration on glucose homeostasis in mice

Experiments were carried out during the early light phase (0800-1000) in *ad libitum* fed mice. This was to ensure minimal hepatic glycogen depletion due to an overnight fast. Randomisation of treatment allocation was stratified by body weight. On the basis of results of the dose response studies, each mouse received two s/c injection, saline/saline, saline/glucagon (30 nmol/kg), saline/GLP-1 (10 nmol/kg) or glucagon (30 nmol/kg)/GLP-1 (10 nmol/kg) in combination. All mice were injected (maximum volume 50 µl) within a single 15 minute period, with individual injection times noted, then returned to their home cages and food removed. Tail tip capillary glucose measurements were taken at -30, 0, 15, 30 and 60 minutes post injection and measured using a hand held glucometer (Contour glucometer, Bayer Diabetes Care, UK).

2.3.7 Tissue preparation and c-fos immunohistochemistry

Experiments were carried out during the early light phase (0800-1000) in mice fasted from 1600 the preceding day. Animals were group housed (n=5/cage, weighing 20-25g) and for two weeks prior to experimentation, mice were handled on a daily basis and sham injected on at least three separate occasions. Mice (n=5-6 per group) were injected with a maximum volume of 50 µl s/c of either saline or peptide and immediately returned to their cages. Ninety minutes later, mice were injected with 0.4 mls ip pentobarbitone (Euthatal, Merial Animal Health Ltd, Harlow, UK) prior to decapitation. Whole brains were rapidly removed and immediately placed in formaldehyde solution (Sigma-Aldrich, UK). After 24 hours the brains were transferred to falcons containing 40% sucrose solution (Sigma-Aldrich, UK) in which they remained for 3-4 days. The brains were then snap frozen in isopentane (VWR, UK) which had been cooled in liquid nitrogen (BOC, UK) and the brains stored at -80°C. Coronal sections through the brainstem and hypothalamus were cut at 40 µm using a sled microtome. Free floating sections were stored at -20°C in 24 well plates containing antifreeze solution (0.02M phosphate buffer solution (PBS), glycerol (VWR, UK), ethylene glycol (Sigma-Aldrich, UK)) until c-fos immunohistochemistry was undertaken.

c-fos immunohistochemistry

Free floating sections were transferred to mesh wells in holding trays containing 0.01M PBS (Invitrogen, UK). After being washed three times in 0.01M PBS, slices were placed in methanol (VWR, UK) containing 0.6% hydrogen peroxide for 15 minutes to quench endogenous peroxidases. Following a further three washes in 0.01M PBS, slices were immersed in blocking solution in order to block endogenous proteins, comprising 0.01M PBS with 3% goat serum and 0.25% Triton X (BDH, UK) for 2 hours. These slices were then transferred to wells containing the above blocking solution with 1:20,000 rabbit anti c-fos antibody (Merck Chemicals Ltd, Nottingham, UK) and left to incubate on a shaker overnight. The following day, sections were washed three times in 0.01M PBS with 0.05% Tween (VWR, Lutterworth, UK) (PBST) followed by a 2 hour incubation in secondary antibody solution, comprising 0.01M PBS with 0.3% bovine serum albumin (Sigma-Aldrich, UK), 0.25% Triton-X and biotinylated anti-rabbit IgG (1:400 concentration) (Vector Laboratories, Peterborough, UK). This was followed by a further 3 washes in PBST and then one hour incubation in avidin-biotin peroxidase complex (ABC) solution (Vector Laboratories, UK). This was made up using 1:200 dilution of both avidin and biotin in 0.05M TrisHCl (pH 7.6). Following the one hour incubation, slices were washed three times in PBST before transferring to holding wells containing 0.01M PBS with 1% diaminobenzidine tetrahydrochloride (50mg/ml) (Sigma-Aldrich, UK) and 0.01% hydrogen peroxidase. Sections were closely observed during the staining procedure and transferred to trays containing 0.01M PBS in order to terminate the reaction. Following two washes in PBS, sections were mounted onto polylysine-coated slides and left to dry overnight. The next morning, the sections were dehydrated through a series of graded ethanols and finally xylene (VWR, UK), prior to mounting with DPX and cover slips. The slides were left to dry prior to visualising under a microscope and the number of c-fos positive nuclei counted manually. Data are presented as the mean and standard error of the mean (SEM) number of c-fos positive cellular nuclei across a hypothalamic or brainstem nucleus as defined in a mouse brain atlas³⁶⁷. In the hypothalamus the following nuclei were studied: ARC, PVN, DMN, VMN and LH.

2.3.8 Investigation of the acute effects of glucagon on c-fos immunoreactivity in the brainstem and hypothalamus

Experiments were carried out during the early light phase (0800-1000) in mice fasted from 1600 the preceding day. Animals were group housed (n=5/cage, weighing 20-25g)

and for two weeks prior to experimentation, mice were handled on a daily basis and sham injected on at least three separate occasions. Mice (n=5-6 per group) were injected with a maximum volume of 50 μ l s/c of either saline or glucagon (30, 100, 300, 500 and 750 nmol/kg) and immediately returned to their cages. Ninety minutes later, mice were injected with 0.4 mls ip pentobarbitone (Euthatal, Merial Animal Health Ltd, Harlow, UK) prior to decapitation. Tissue preparation and c-fos immunohistochemistry was subsequently performed as described in section 2.3.7.

2.3.9 Investigation of the acute effects of GLP-1 on c-fos immunoreactivity in the brainstem and hypothalamus

Experimental procedures were performed as described in section 2.3.8. Mice were injected with s/c saline or GLP-1 (3, 30, 50, 100 and 600 nmol/kg).

2.3.10 Investigation of the acute effects of glucagon and GLP-1 co-administration on c-fos immunoreactivity in the brainstem and hypothalamus

Experimental procedures were performed as described in section 2.3.8. Based on results from the dose response studies (section 2.4.1 and 2.4.2), each mouse received two s/c injections, one of 30 nmol/kg glucagon or saline, and the other of 10 nmol/kg GLP-1 or saline, in quick succession (n=6).

2.3.11 Statistical analysis

All data are expressed as mean $\pm$ SEM unless specified. Data from acute feeding and c-fos immunohistochemistry were analysed using one-way ANOVA followed by Dunnett's post test. Data comparing co-administration of glucagon and GLP-1, each hormone alone and saline controls was analysed using one-way ANOVA with Bonferroni multiple comparison test. Data from circulating glucose levels following administration of glucagon and GLP-1 alone and in combination was analysed using two-way repeated measures ANOVA with Bonferroni post test. In all cases, values of $p < 0.05$ were considered statistically significant.

2.4 Results

2.4.1 Investigation of the acute effects of glucagon on food intake in mice

This study was performed in mice fasted from 1600 the preceding day. Mice received s/c glucagon (500, 750 or 1000 nmol/kg) or saline at the onset of the light phase.

Glucagon significantly reduced food intake in fasted mice within the first 30 minutes (mean food intake: 0.50 ± 0.06 [saline] vs. 0.26 ± 0.05 g [500 nmol/kg], $p < 0.05$ vs. saline; 0.12 ± 0.03 g [750 nmol/kg], $p < 0.01$ vs. saline; 0.23 ± 0.11 g [1000 nmol/kg], $p < 0.05$ vs. saline; $n = 6$ per group. Fig. 2.1). There were no significant effects on food intake at subsequent time points.

The experiment was repeated under a series of different conditions to ascertain the optimal protocol to demonstrate the anorectic effect of glucagon. It has previously been postulated that duration of fasting may affect the anorectic response to glucagon^{190,368} and therefore this was also studied. In addition, I wanted to determine the appropriate conditions to undertake c-fos immunohistochemistry studies. The experimental conditions were:

- *ad libitum* fed mice at the onset of the light phase (figure 2.2),
- mice fasted from 0600 on the day of the study and injected midway through the light phase (figure 2.3),
- *ad libitum* fed and mice fasted from 0600, injected at the onset of the dark phase (figure 2.4).

There was no anorectic effect in fed mice following injection of glucagon whether injected at the onset of the light or dark phase (figure 2.2 and 2.4 respectively). In contrast, the anorectic effect of glucagon was seen within the first 30 minutes in mice fasted from 0600 and injected midway through the light phase (mean food intake: 0.25 ± 0.02 [saline] vs. 0.07 ± 0.01 g [500 nmol/kg], $p < 0.01$ vs. saline; 0.13 ± 0.03 g [750 nmol/kg], $p < 0.01$ vs. saline; 0.09 ± 0.02 g [1000 nmol/kg], $p < 0.01$ vs. saline; $n = 6$ per group, fig. 2.3). Similarly, glucagon significantly reduced food intake within the first 30 minutes in mice fasted from 0600 and injected at the onset of the dark phase (mean food intake: 0.47 ± 0.04 [saline] vs. 0.12 ± 0.06 g [500 nmol/kg], $p < 0.01$ vs. saline; 0.10 ± 0.04 g [750 nmol/kg], $p < 0.01$ vs. saline; 0.04 ± 0.01 g [1000 nmol/kg], $p < 0.01$ vs. saline; $n = 6$ per group, fig. 2.4).

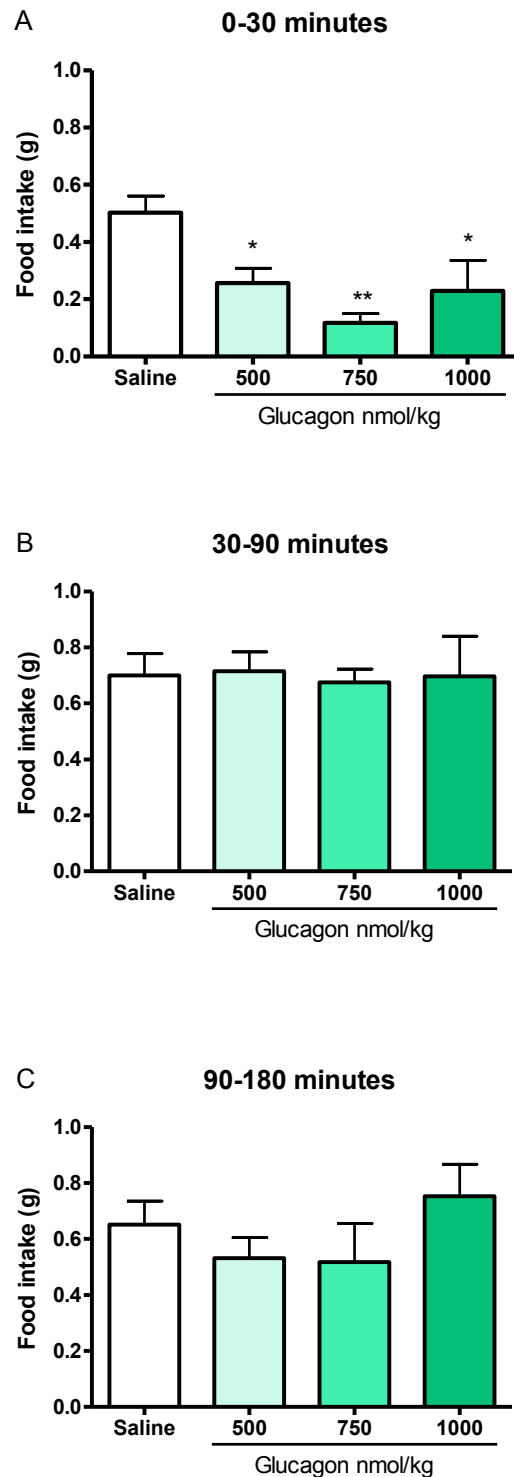


Figure. 2.1 The acute effect of glucagon on food intake in fasted mice at the onset of the light phase. Study performed in mice fasted from 1600 the day before. Mice received s/c injection at the onset of the light phase of saline (controls), glucagon 500, 750 or 1000 nmol/kg. Food intake was measured at 30, 90 and 180 minutes following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. * $p < 0.05$, ** $p < 0.01$ versus controls. $n = 6$ /group.

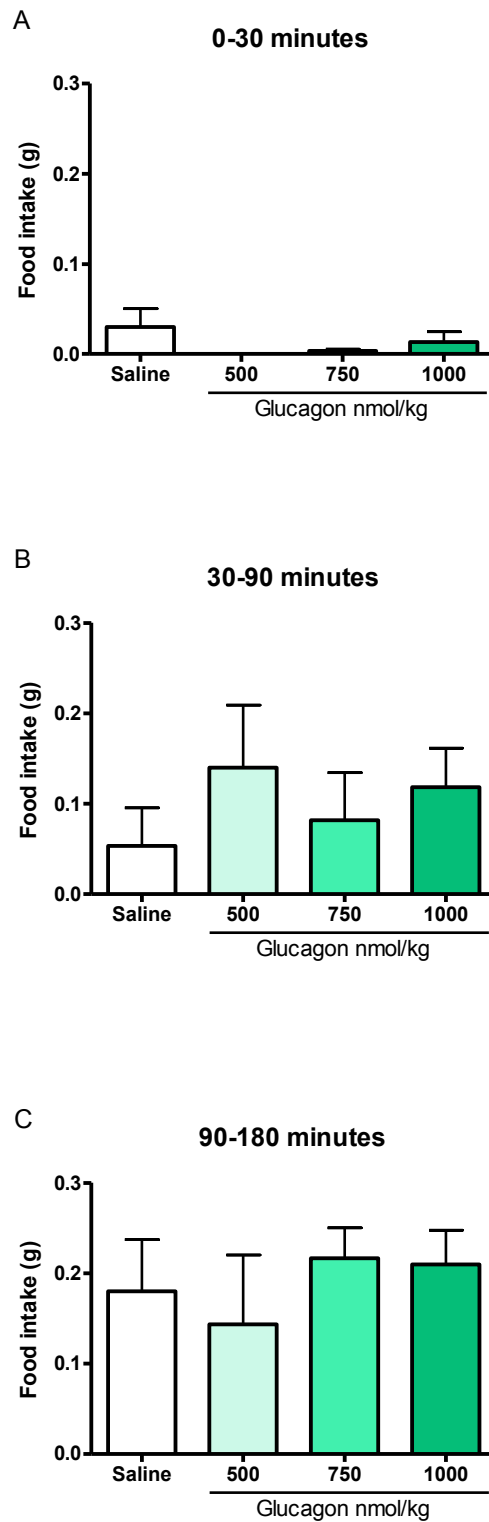


Figure. 2.2 The acute effect of glucagon on food intake in fed mice at the onset of the light phase. Study performed in *ad libitum* fed mice at the onset of the light phase (0800-1000). Mice received s/c injection of saline (controls), glucagon 500, 750 or 1000 nmol/kg. Food intake was measured at 30, 90 and 180 minutes following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. n=6/group.

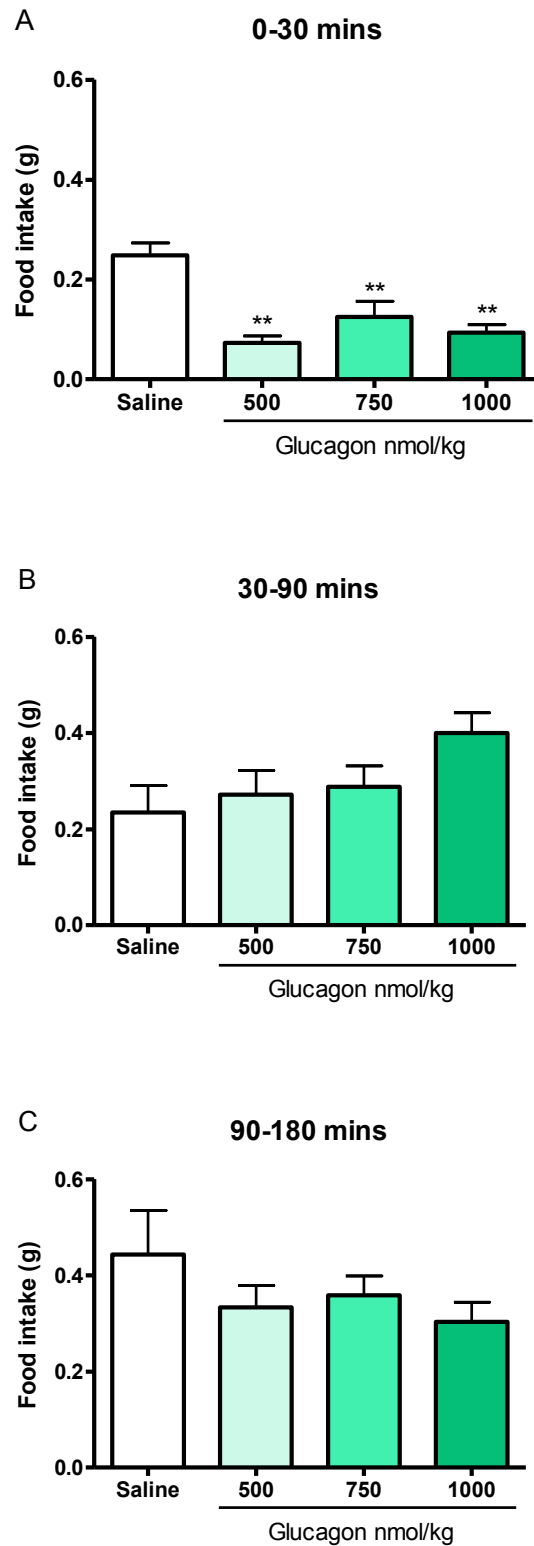


Figure. 2.3 The acute effect of glucagon on food intake in fasted mice midway through the light phase. Study performed in mice fasted from 0600 on the day of the study and injected midway through the light phase (1400). Mice received s/c injection of saline (controls), glucagon 500, 750 or 1000 nmol/kg. Food intake was measured at 30, 90 and 180 minutes following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. ** $p < 0.01$ versus controls. $n = 6$ /group.

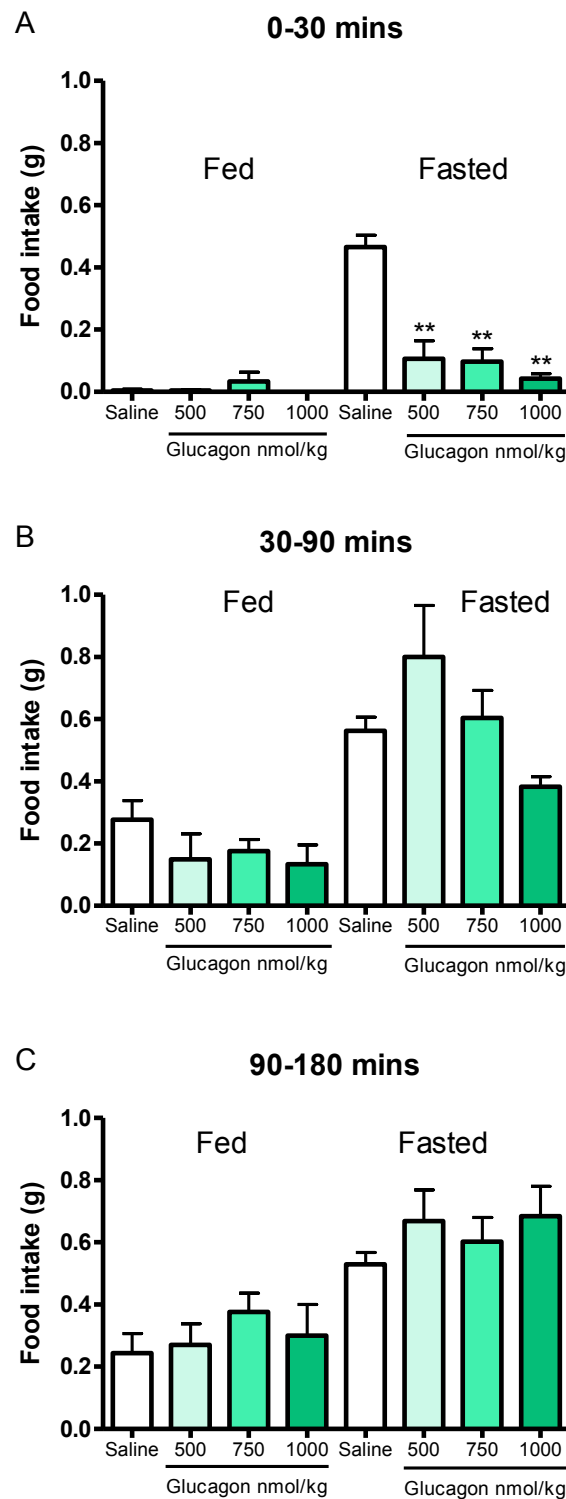


Figure.2.4 The acute effect of glucagon on food intake in fed and fasted mice at the onset of the dark phase. Study performed in *ad libitum* fed and daytime fasted (from 0600 on day of study) mice, injected at the onset of the dark phase (1900). Mice received s/c injection of saline (controls), glucagon 500, 750 or 1000 nmol/kg. Food intake was measured at 30, 90 and 180 minutes following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. ** $p < 0.01$ versus controls. $n = 6/\text{group}$.

In order to determine the minimum effective dose of glucagon in reducing food intake, an acute feeding study was performed following a range of doses of glucagon. Mice were fasted from 1600 the day before and injected at the onset of the light phase (Fig. 2.5). This timing was chosen in order to perform the c-fos immunohistochemistry protocol (section 2.3.7) during the light phase for practical reasons and in order to reduce stress to the mice which could occur if moving animals around during the onset of the dark phase.

Consistent with my previous study, the anorectic effect of glucagon was observed within the first 30 minutes only. The lowest effective dose in reducing food intake was 100 nmol/kg (mean food intake: 0.56 ± 0.04 [saline] vs. 0.30 ± 0.06 g [100 nmol/kg], $p < 0.01$ vs. saline; $n=6$ /group, fig. 2.5A).

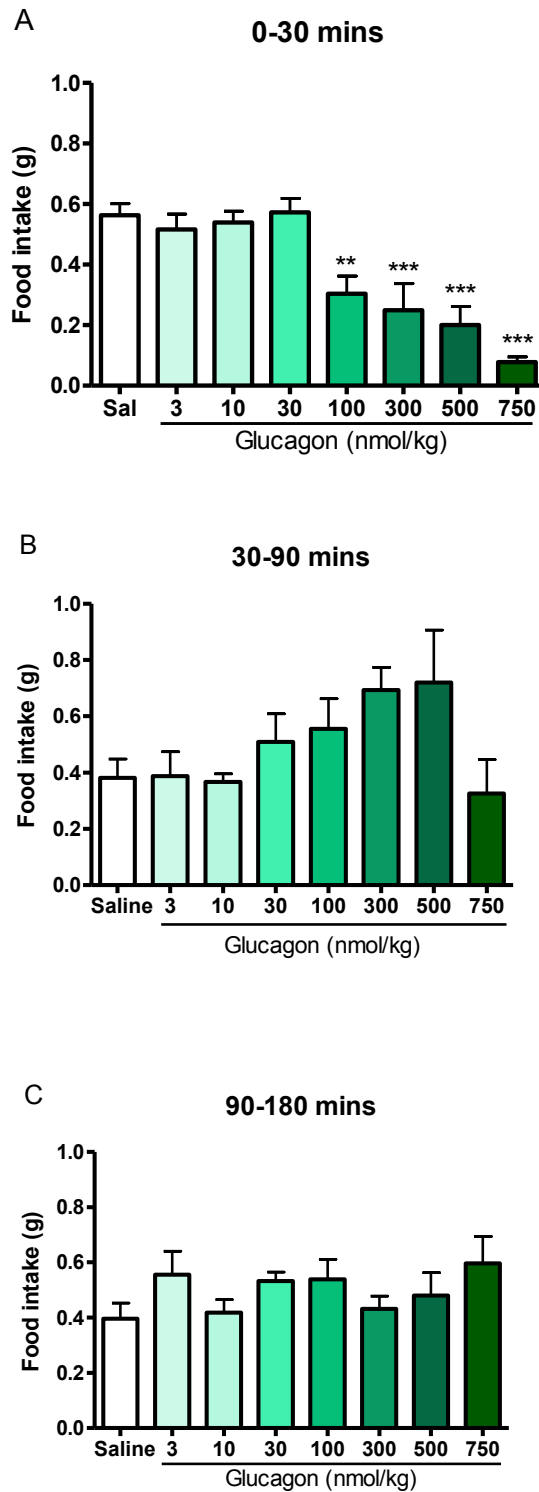


Figure. 2.5 Dose response study of the acute effect of glucagon on food intake. Study performed at the onset of the light phase in mice fasted from 1600 the day before. Mice received s/c injection of saline (controls) or glucagon (3, 10, 30, 100, 300, 500, 750 nmol/kg). Food intake was measured at 30, 90 and 180 minutes following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, *** $p < 0.001$ versus controls. $n = 6/\text{group}$.

2.4.2 Investigation of the acute effects of GLP-1 on food intake in mice

Previous studies have investigated the effects of GLP-1 on food intake under different experimental conditions in rodents ^{345,369,370}. The acute effects of GLP-1 on food intake were investigated under identical experimental conditions as described following glucagon administration in figure 2.5. A similar protocol was used by Neary *et al*, in which an acute anorectic effect was shown following GLP-1 administration at the onset of the light phase in mice fasted for 20 hours³⁴⁵.

S/c GLP-1 decreased food intake within 30 minutes following administration (Fig. 2.6 A). The highest dose investigated (600 nmol/kg) produced an anorectic effect which persisted at 90 minutes post-injection. Following administration of GLP-1 at doses lower than 600 nmol/kg, the anorectic effect was not observed by 90 minutes post injection (Fig. 2.6 B). The lowest effective dose of GLP-1 was 30 nmol/kg (mean food intake: 0.49 ± 0.03 g [saline] vs. 0.23 ± 0.03 g [30 nmol/kg], $p < 0.01$; $n = 6$ /group, fig 2.6 A).

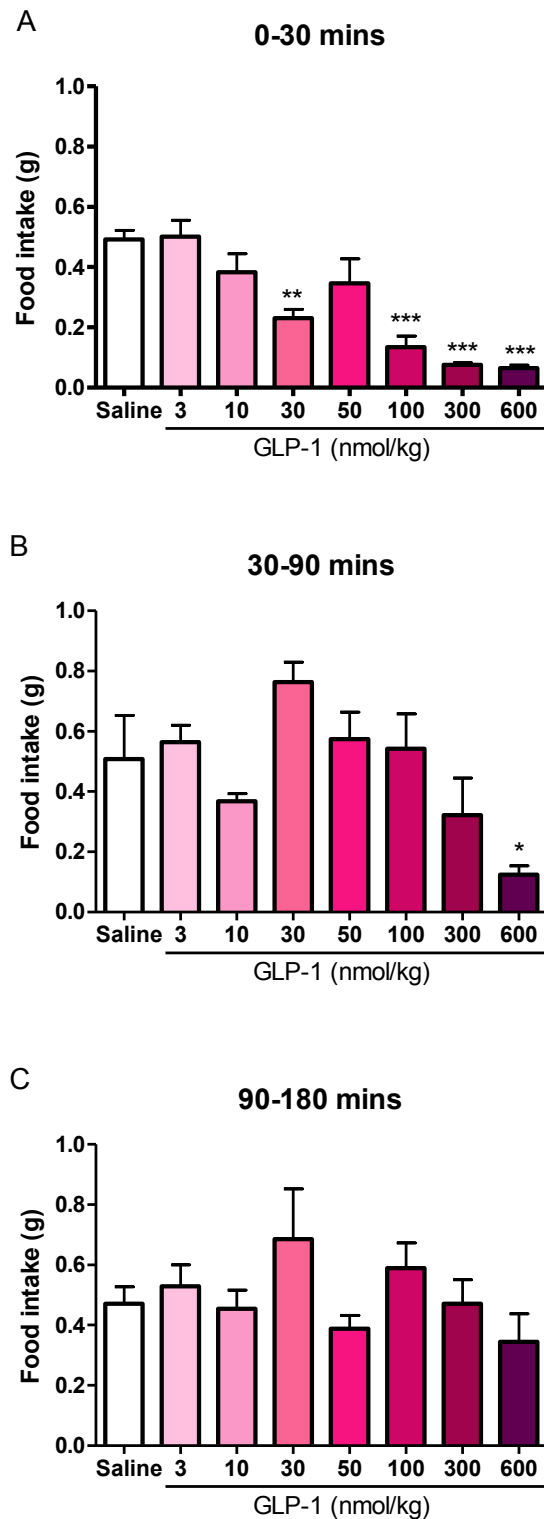


Figure 2.6. Dose response study of the acute effect of GLP-1 on food intake in fasted mice. Study performed at the onset of the light phase in mice fasted from 1600 the day before. Mice received s/c injection of saline (controls) or GLP-1 (3, 10, 30, 50, 100, 300, 600nmol/kg). Food intake was measured at 30, 90 and 180 minutes following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus controls. $n = 6/\text{group}$.

2.4.3 Investigation of the acute effects of glucagon and GLP-1 co-administration on food intake in mice

In order to establish whether co-administration of glucagon and GLP-1 had an additive effect on reducing food intake, a sub-threshold dose of each peptide was administered alone and in combination. Mice were fasted from 1600 the preceding day and injected at the onset of the light phase, as dose response studies following glucagon and GLP-1 administration alone were performed under these experimental conditions. Based on the results from studies 2.4.1 and 2.4.2 these sub-anorectic doses were: glucagon 30 nmol/kg and GLP-1 10 nmol/kg. Higher doses in combination were also studied to investigate anorectic doses as shown in studies 2.4.1 and 2.4.2. The medium dose was chosen to reflect the lowest anorectic dose found. The high dose was used as a positive control (glucagon 750 nmol/kg and GLP-1 300 nmol/kg).

At sub-anorectic doses, administration of glucagon or GLP-1 alone did not significantly reduce food intake compared to saline in the first 30 minutes (fig. 2.7 A). However, co-administration of GLP-1 and glucagon at these doses significantly reduced food intake (mean food intake: 0.39 ± 0.06 [saline] vs. 0.24 ± 0.06 g [GLP-1 10 nmol/kg], $p=0.64$ vs. saline; 0.42 ± 0.07 g [glucagon 30 nmol/kg], $p=1.0$ vs. saline; 0.08 ± 0.06 g [GLP-1 10 nmol/kg and glucagon 30 nmol/kg], $p<0.01$ vs. saline; $n=6$ /group, fig 2.7A).

Higher doses of glucagon (750 nmol/kg) and GLP-1 (30 nmol/kg and 300 nmol/kg) alone, significantly reduced food intake compared to saline controls. At all three dose ranges (glucagon: 30, 100 and 750 nmol/kg; GLP-1: 10, 30 and 300 nmol/kg), the combination of glucagon and GLP-1 significantly reduced food intake to a greater extent than saline controls or glucagon (LD and MD) but not GLP-1 (fig. 2.7A). At later time points, no significant changes in food intake were seen following administration of GLP-1, glucagon or GLP-1 and glucagon in combination at any dose range.

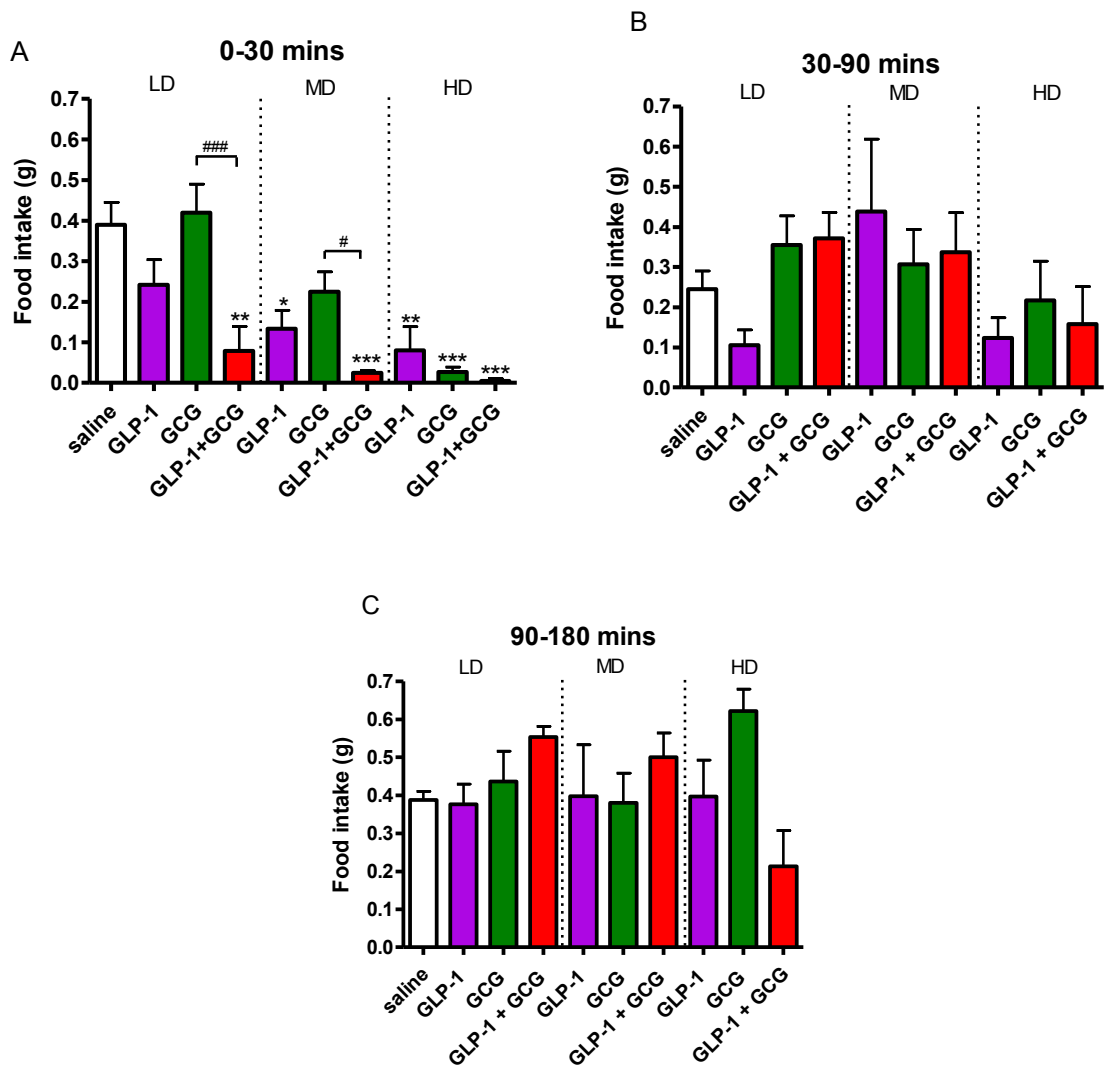


Figure 2.7. Studies of the effects of co-administration of glucagon and GLP-1 on food intake. Study performed at the onset of the light phase in mice fasted from 1600 the day before. All mice received two s/c injections in quick succession of either: saline/saline; saline/glucagon low dose (LD) 30 nmol/kg, medium dose (MD) 100 nmol/kg or high dose (HD) 750 nmol/kg; saline/GLP-1 (10 LD, 30 MD, 300 HD nmol/kg) or glucagon/GLP-1 (at the same doses). Food intake was measured at 30 (Fig. 2.7 A), 90 (Fig. 2.7B) and 180 minutes (Fig. 2.7C) following injection. Columns presented as mean $\pm$ SEM. Data were analysed using one way ANOVA with Bonferroni multiple comparison test * p <0.05, ** p <0.01, *** p <0.001 versus saline (controls); # p <0.05, ### p <0.001 versus glucagon alone. n =6/group.

2.4.4 Investigation of the acute effects of glucagon and GLP-1 co-administration on glucose homeostasis in mice.

On the basis of the feeding studies, the effect of sub-anorectic doses of glucagon and GLP-1 co-administration on circulating glucose levels was studied. At a dose of 30 nmol/kg, glucagon alone significantly increased glucose levels within 15 minutes following injection (mean glucose: 7.2 ± 0.4 [saline] vs. 9.2 ± 0.4 mmol/l [glucagon], $p < 0.001$ vs. saline, $n=8-9$ /group, fig. 2.8). Glucose levels following GLP-1 alone (10 nmol/kg) showed no significant differences to saline controls at any time points.

Co-administration of glucagon and GLP-1 ameliorated the rise in glucose in response to glucagon alone and glucose levels were indistinguishable from saline controls (mean glucose: 9.2 ± 0.4 [glucagon] vs. 7.6 ± 0.3 mmol/l [glucagon and GLP-1 co-administration], $p < 0.01$ vs. glucagon alone, $n=8-9$ /group, fig 2.8).

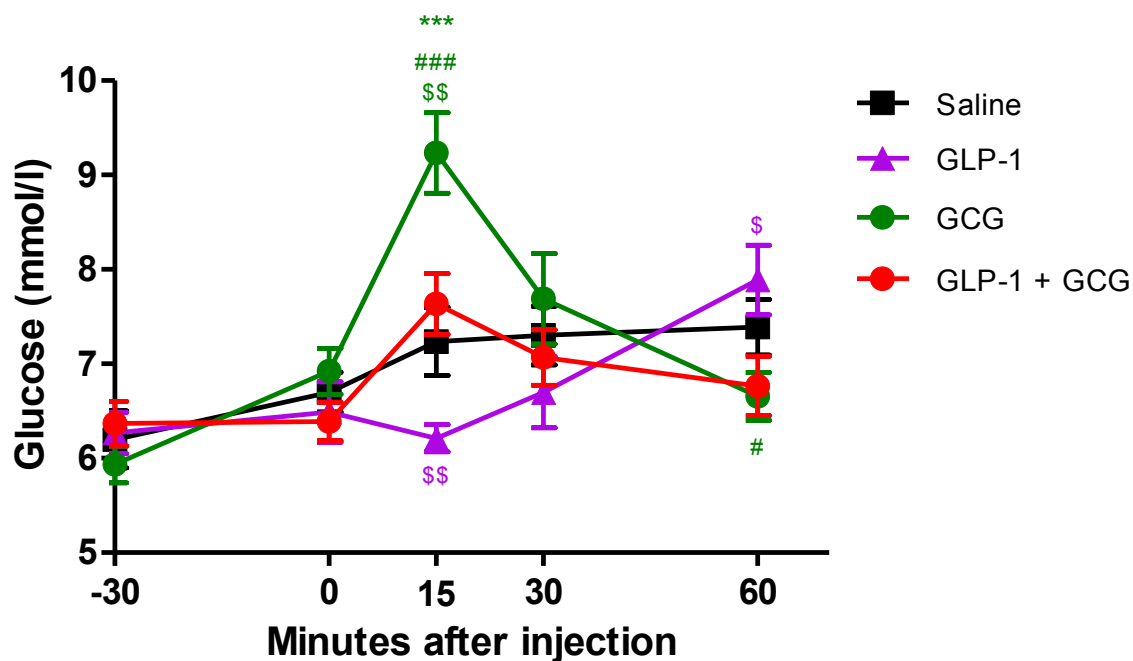


Figure. 2.8 Studies of the effects of co-administration of glucagon and GLP-1 on circulating glucose levels. Experiments were performed during the early light phase (0800-1000) in *ad libitum* fed mice. All mice received two s/c injections in quick succession of either: saline/saline; saline/glucagon (30 nmol/kg), saline/GLP-1 (10 nmol/kg) or glucagon (30 nmol/kg)/GLP-1 (10 nmol/kg). Glucose measurements were taken at -30, 0, 15, 30 and 60 minutes post injection. Data were analysed using two-way repeated measures ANOVA with Bonferroni post test. *** $p < 0.001$ versus saline (controls); # $p < 0.05$, ### $p < 0.001$ versus GLP-1 alone, \$ $p < 0.05$, \$\$ $p < 0.01$ versus glucagon and GLP-1 co-administration. $n = 9$ /group.

2.4.5 Investigation of the acute effects of glucagon on c-fos immunoreactivity in the brainstem and hypothalamus

This study was conducted to determine whether the dose-dependent reduction in food intake seen following glucagon administration in previous feeding studies, correlated with c-fos-ir in the brainstem and hypothalamus.

The lowest glucagon dose to produce a significant increase in AP c-fos-ir was 300 nmol/kg (mean c-fos counts: 7 ± 4 [saline] vs. 81 ± 22 [glucagon 300 nmol/kg], $p < 0.05$ vs. saline, $n = 4-5$ /group, fig. 2.9 A, 2.11A and 2.12). A similar dose-dependent increase in c-fos-ir was seen in the cNTS, following glucagon administration (mean c-fos counts: 3 ± 1 [saline] vs. 108 ± 6 [glucagon 300 nmol/kg], $p < 0.05$ vs. saline, $n = 4-5$ /group, fig. 2.9 B, 2.11A, 2.12). In the PBN there was a significant increase in c-fos-ir at 500 nmol/kg only (mean c-fos counts: 16 ± 1 [saline] vs. 63 ± 10 [glucagon 500 nmol/kg], $p < 0.05$ vs. saline, $n = 3-5$ /group, fig. 2.9 C, 2.11B and 2.12).

There were no significant differences in the hypothalamic nuclei studied in c-fos-ir between saline controls and glucagon at any of the doses administered. In the PVN, there was a non-significant trend towards an increase in c-fos-ir (mean c-fos counts: 13 ± 6 [saline] vs. 27 ± 5 [glucagon 750 nmol/kg], $p = 0.145$ vs. saline, $n = 3-5$ /group; fig. 2.10 B). A dose dependent increase in c-fos-ir occurred in the CeA at a dose of 300 nmol/kg and above (mean c-fos counts: 12 ± 3 [saline] vs. 107 ± 5 [glucagon 300 nmol/kg], $p < 0.01$ vs. saline, $n = 4-5$ /group; fig. 2.10F, 2.11C, 2.12).

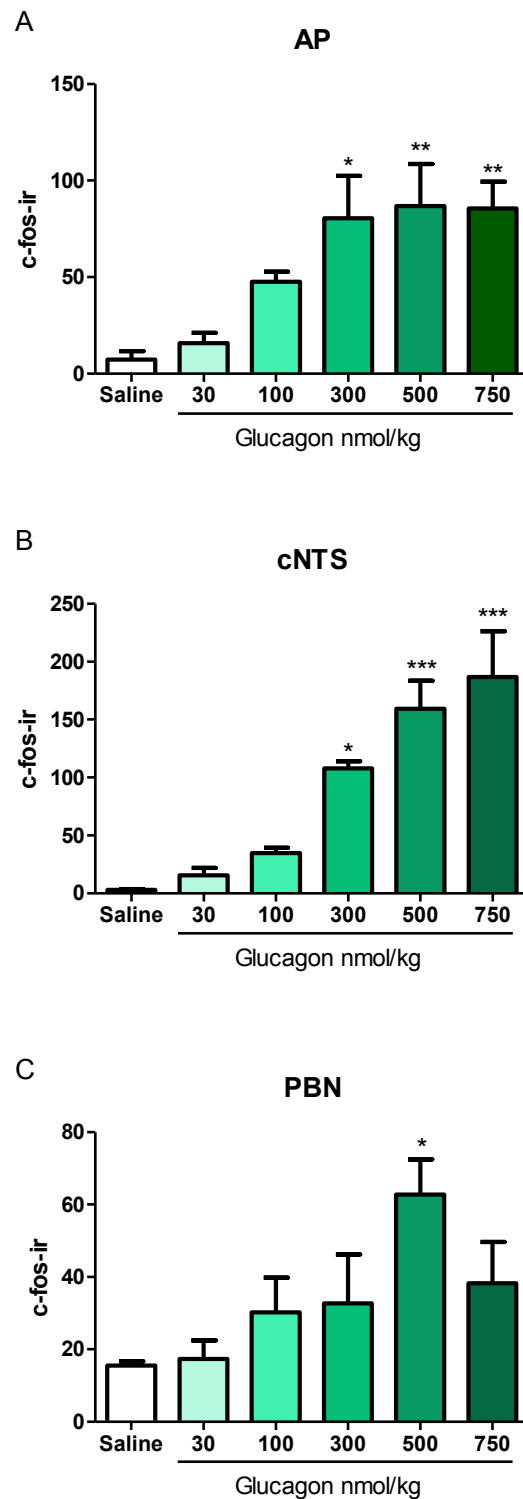


Figure. 2.9 Brainstem c-fos immunoreactivity following increasing doses of glucagon. s/c injections of either saline (controls) or glucagon (30, 100, 300, 500, 750 nmol/kg) were administered at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos immunoreactivity counts are shown in the area postrema (AP) (fig 2.8 A), caudal nucleus tractus solitarius (cNTS) (fig. 2.8 B) and parabrachial nucleus (PBN) (fig 2.8 C) of the brainstem. Data were analysed using one-way ANOVA with Dunnett's post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline controls. $n = 3-5$ /group.

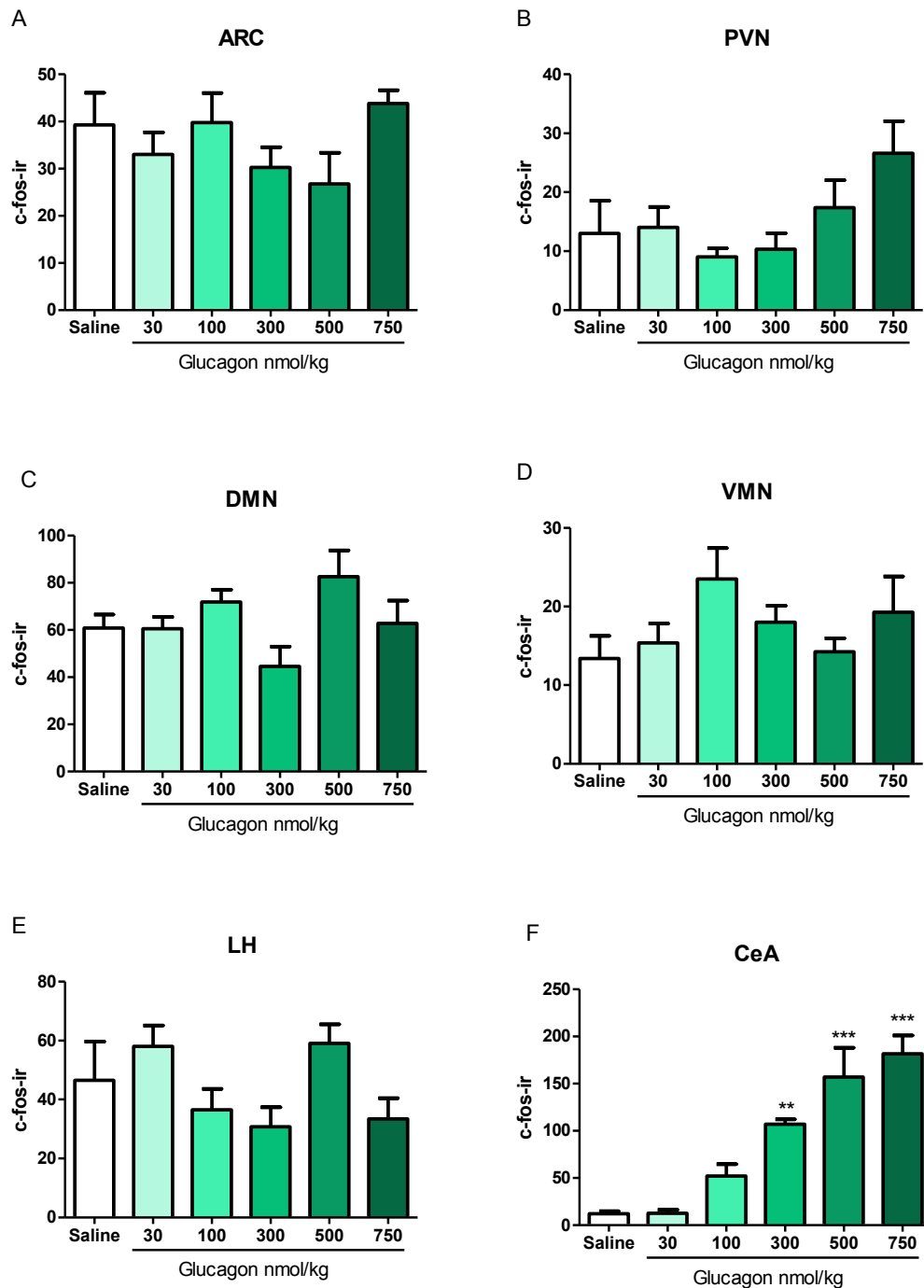


Figure. 2.10 Hypothalamic and central nucleus of the amygdala c-fos immunoreactivity following increasing doses of glucagon. s/c injections of either saline (controls) or glucagon (30, 100, 300, 500, 750 nmol/kg) were performed at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos immunoreactivity counts are shown in the arcuate nucleus (ARC) (fig. 2.9A), paraventricular nucleus (PVN) (fig. 2.9B), dorsomedial nucleus (DMN) (fig. 2.9C), ventromedial nucleus (VMN) (fig. 2.9D), lateral hypothalamus (LH) (fig. 2.9E) and central nucleus of the amygdala (CeA) (fig. 2.9F). Data were analysed using one-way ANOVA with Dunnett's post test. ** $p < 0.01$, *** $p < 0.001$ versus saline controls. $n = 3-5$ /group

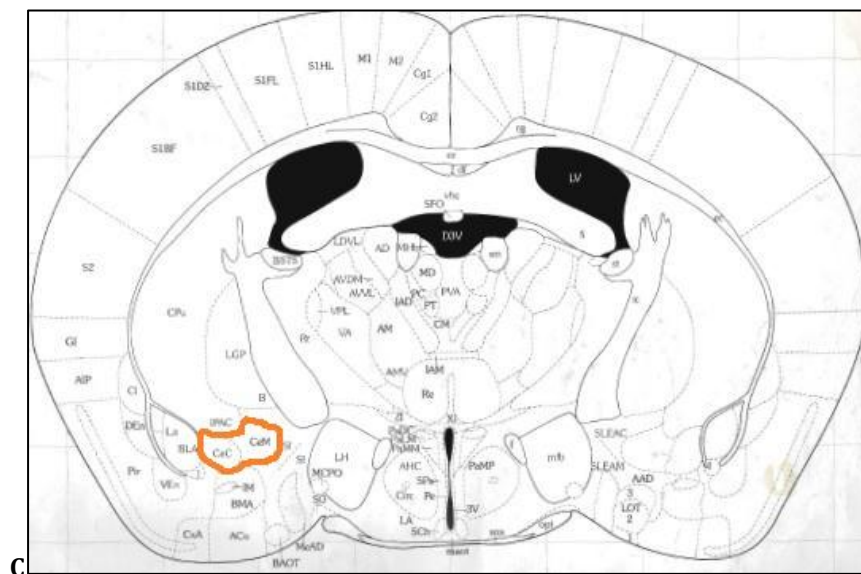
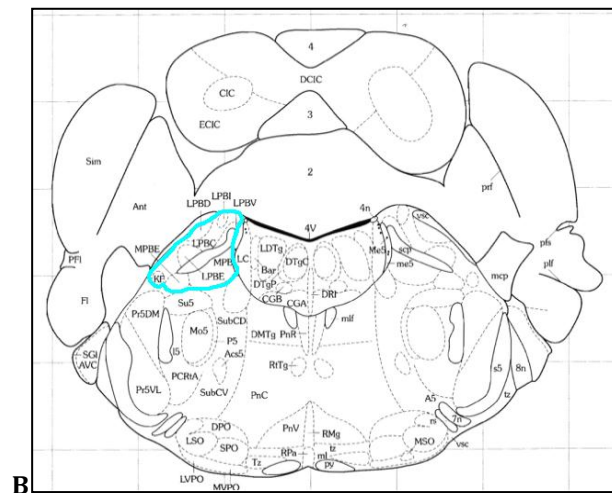
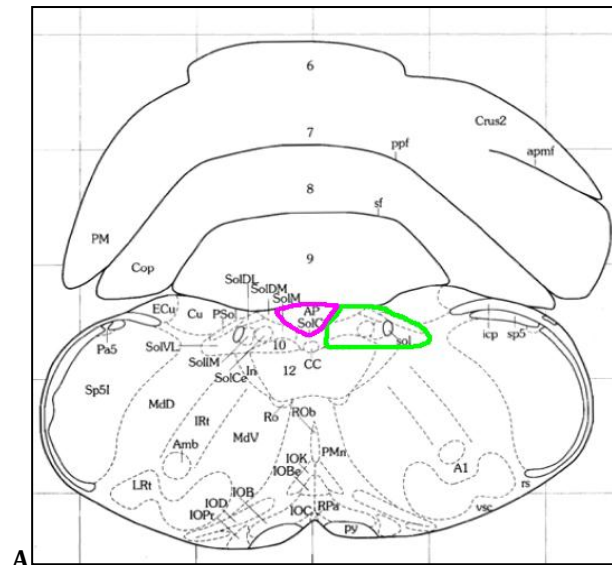


Figure. 2.11 Anatomical diagrams illustrating brain regions studied for c-fos expression. AP and NTS (Fig 2.10A pink and green respectively), PBN (Fig 2.10B blue) and CeA (Fig. 2.10C orange). Reproduced from Franklin and Paxinos mouse brain atlas³⁶⁷.

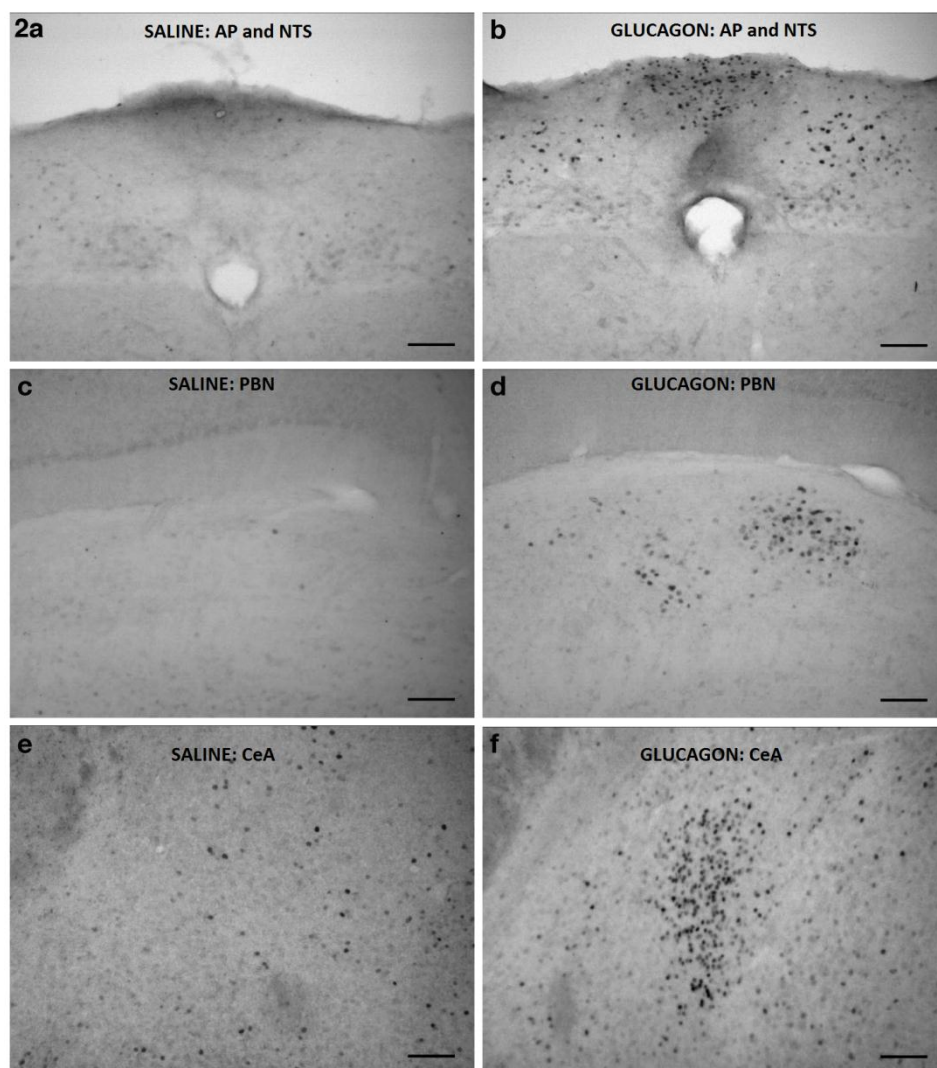


Figure 2.12. Representative photomicrographs of c-fos immunoreactivity 90 minutes after subcutaneous saline or glucagon (750 nmol/kg) administration. a: saline nucleus tractus solitarius (NTS) and area postrema (AP); b: glucagon NTS and AP, c: saline parabrachial nucleus (PBN), d: glucagon PBN, e: saline central nucleus of the amygdala (CeA), f: glucagon CeA. Scale bar=0.1mm.

2.4.6 Investigation of the acute effects of GLP-1 on c-fos immunoreactivity in the brainstem and hypothalamus

GLP-1 increased c-fos-ir in the AP at a dose of 50 nmol/kg and above (mean c-fos counts: 6 ± 2 [saline] vs. 87 ± 14 [GLP-1 50 nmol/kg], $p < 0.001$ vs. saline, $n = 3-5$ /group; fig. 2.13A) and at doses of 30 nmol/kg and greater in the cNTS (mean c-fos counts: 19 ± 6 [saline] vs. 126 ± 17 [GLP-1 30 nmol/kg], $p < 0.05$ vs. saline, $n = 4-5$ /group; fig. 2.13 B). In the PBN, there was a significant increase in c-fos-ir at 100 nmol/kg (mean c-fos counts: 53 ± 13 [saline] vs. 104 ± 16 [GLP-1 100 nmol/kg], $p < 0.05$, $n = 4-5$ /group, fig. 2.13C).

GLP-1 did not significantly increase c-fos-ir in the hypothalamic ARC, PVN, VMN, DMN or LH (fig. 2.14). In the PVN, there was a non-significant trend towards an increase in c-fos-ir (mean c-fos counts: 12 ± 8 [saline] vs. 35 ± 10 [GLP-1 600 nmol/kg], $p = 0.065$ vs. saline, $n = 4-5$ /group; fig. 2.14 B). A dose dependent increase in c-fos-ir was seen in the CeA at a dose of 30 nmol/kg and above (mean c-fos counts: 23 ± 3 [saline] vs. 127 ± 10 [GLP-1 30 nmol/kg], $p < 0.05$ vs. saline, $n = 4-5$ /group; fig. 2.14 F).

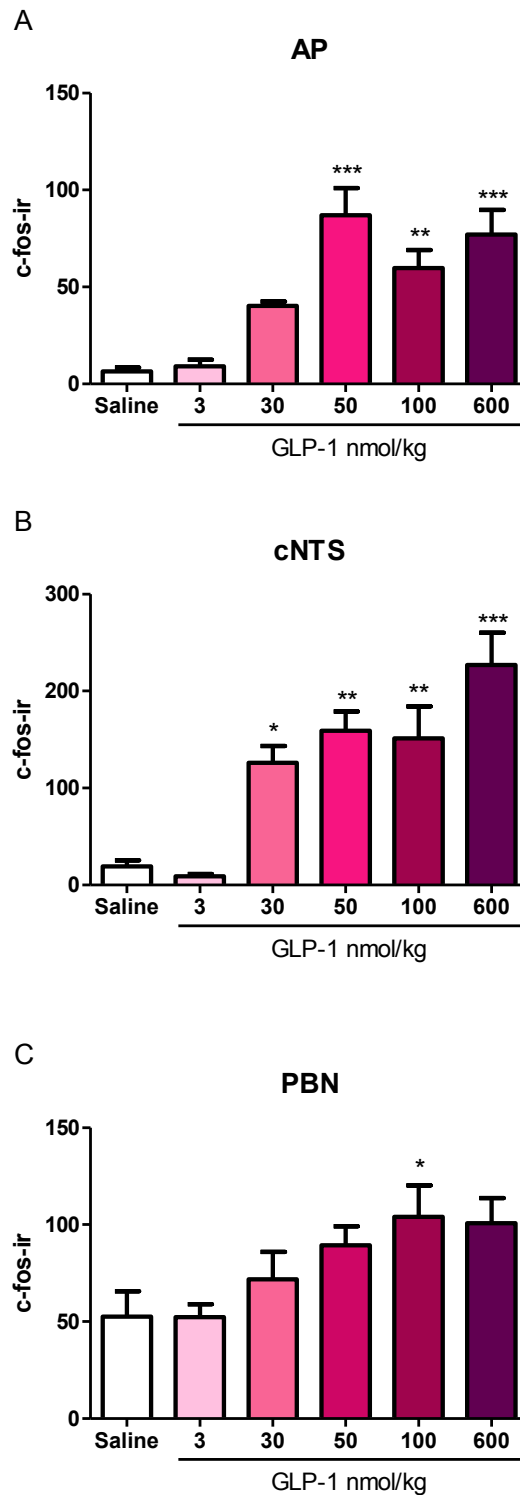


Figure 2.13. Brainstem c-fos immunoreactivity following increasing doses of GLP-1. s/c injections of either saline (controls) or GLP-1 (3, 30, 50, 100, 600 nmol/kg) were performed at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos like immunoreactivity counts are shown in the area postrema (AP) (fig 2.11 A), caudal nucleus tractus solitarius (cNTS) (fig. 2.11 B) and parabrachial nucleus (PBN) (fig 2.11 C) of the brainstem. Data were analysed using one-way ANOVA with Dunnett's post test.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline controls. $n = 3-5$ /group.

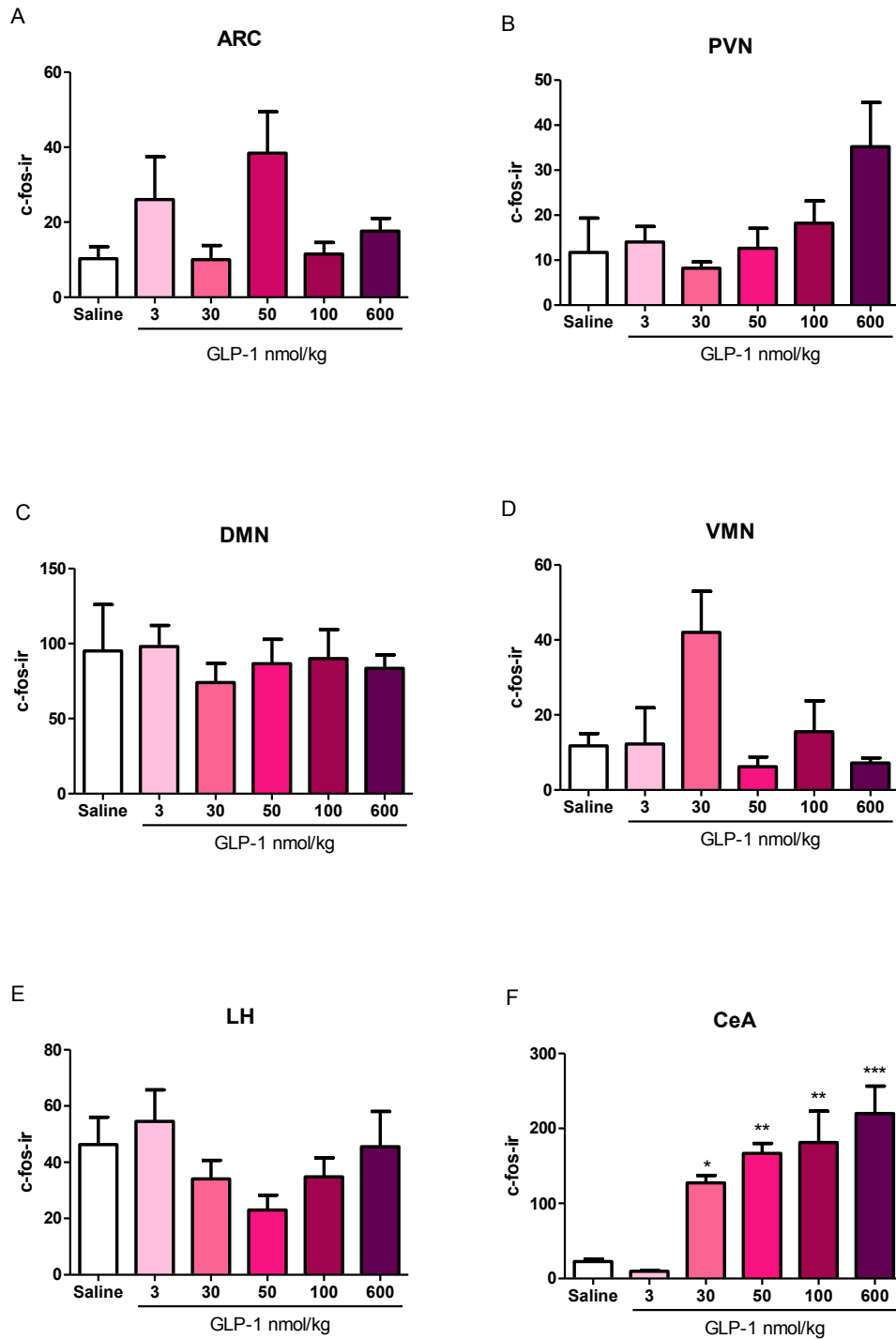


Figure 2.14. Hypothalamic and central nucleus of the amygdala c-fos immunoreactivity following increasing doses of GLP-1. s/c injections of either saline (controls) or GLP-1 (3, 30, 50, 100, 600 nmol/kg) were performed at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos immunoreactivity counts are shown in the arcuate nucleus (ARC) (fig 2.12A), paraventricular nucleus (PVN) (fig. 2.12B), dorsomedial nucleus (DMN) (fig.2.12C), ventromedial nucleus (VMN) (fig. 2.12D), lateral hypothalamus (LH) (fig.2.12E) and central nucleus of the amygdala (CeA) (fig. 2.12F). Data were analysed with one-way ANOVA with Dunnett's post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline controls. $n = 3-5$ /group.

2.4.7 Investigation of the acute effects of glucagon and GLP-1 co-administration on c-fos immunoreactivity in the brainstem and hypothalamus

c-fos-ir was assessed in the brainstem of mice following injection of saline (controls), GLP-1 (10 nmol/kg) or glucagon (30 nmol/kg) either alone or in combination, at the onset of the light phase. These doses were chosen as sub-threshold anorectic doses following the results of study 2.4.3.

In the brainstem, administration of either hormone individually did not significantly alter c-fos-ir in the AP, cNTS or PBN compared to saline controls. However, GLP-1 and glucagon in combination (10 nmol/kg and 30 nmol/kg respectively) significantly increased c-fos-ir compared to saline controls in the AP (mean c-fos counts: 7 ± 3 [saline] vs. 46 ± 9 [GLP-1 and glucagon], $p < 0.01$ vs. saline, $n = 5-6$ /group, fig. 2.15 A, 2.17). GLP-1 and glucagon in combination did not significantly affect c-fos expression in the cNTS or PBN compared to saline controls or compared to each hormone administered individually (fig. 2.15B, 2.15C, 2.17).

In the hypothalamus, administration of sub-anorectic doses of either hormone alone or in combination did not alter c-fos-ir in any of the nuclei studied (fig. 2.16). However, GLP-1 alone and in combination with glucagon, significantly increased c-fos-ir in the CeA (mean c-fos counts: 103 ± 19 [saline] vs. 218 ± 18 [GLP-1 10 nmol/kg], $p < 0.001$ vs. saline; vs. 362 ± 15 [GLP-1 + glucagon], $p < 0.001$ vs. saline, $n = 5-6$ /group, fig. 2.16 F, 2.17).

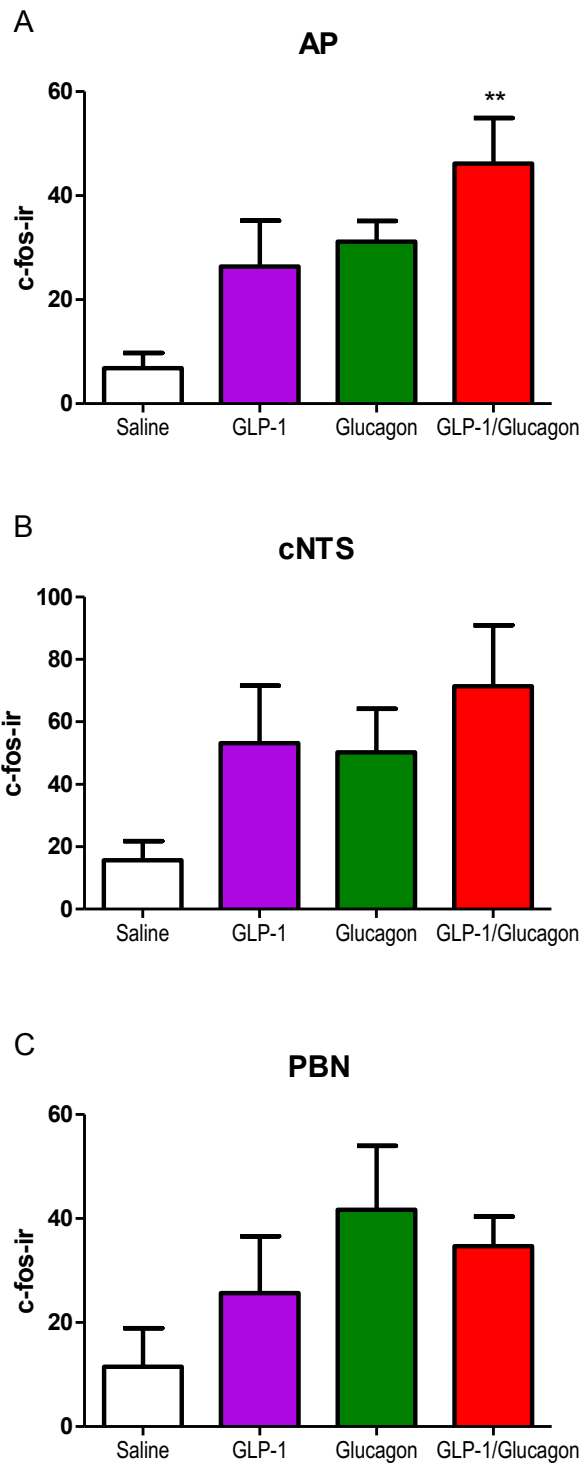


Figure 2.15. Brainstem c-fos immunoreactivity following co-administration of glucagon and GLP-1. Mice were fasted overnight and received s/c injections of either saline (controls), GLP-1 (10 nmol/kg), glucagon (30 nmol/kg) or of both doses together at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos immunoreactivity counts are shown in the area postrema (AP) (fig 2.14A), caudal nucleus tractus solitarius (cNTS) (fig. 2.14B) and parabrachial nucleus (PBN) (fig. 2.14 C) of the brainstem. One-way ANOVA with Bonferroni multiple comparison test. ** $p < 0.01$ versus controls. $n = 5-6$ /group.

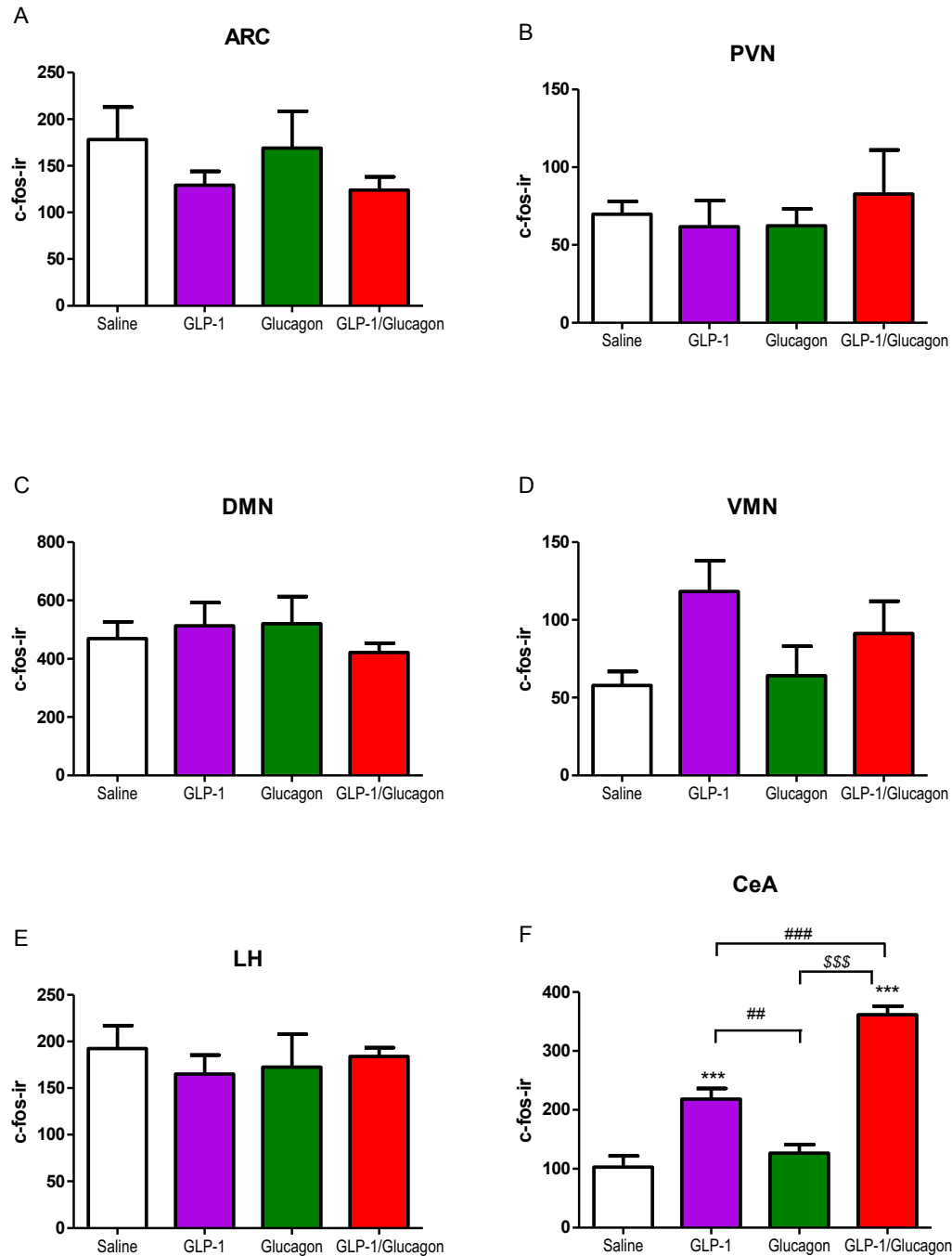


Figure 2.16. Hypothalamic and central nucleus of the amygdala c-fos immunoreactivity following co-administration of glucagon and GLP-1. Mice were fasted overnight and received s/c injections of either saline (controls), GLP-1 (10 nmol/kg), glucagon (30 nmol/kg) or of both doses together at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos immunoreactivity counts are shown in the arcuate nucleus (ARC) (fig. 2.15A), paraventricular nucleus (PVN) (fig. 2.15B), dorsomedial nucleus (DMN) (fig. 2.15C), ventromedial nucleus (VMN) (fig. 2.15 D), lateral hypothalamus (LH) (fig. 2.15 E) and central nucleus of the amygdala (CeA) (fig. 2.15F). Data were analysed with one-way ANOVA with Bonferroni multiple comparison test. *** p<0.001 versus controls, ### p<0.001 versus GLP-1, \$\$\$ p<0.001 versus glucagon. n=5-6/group.

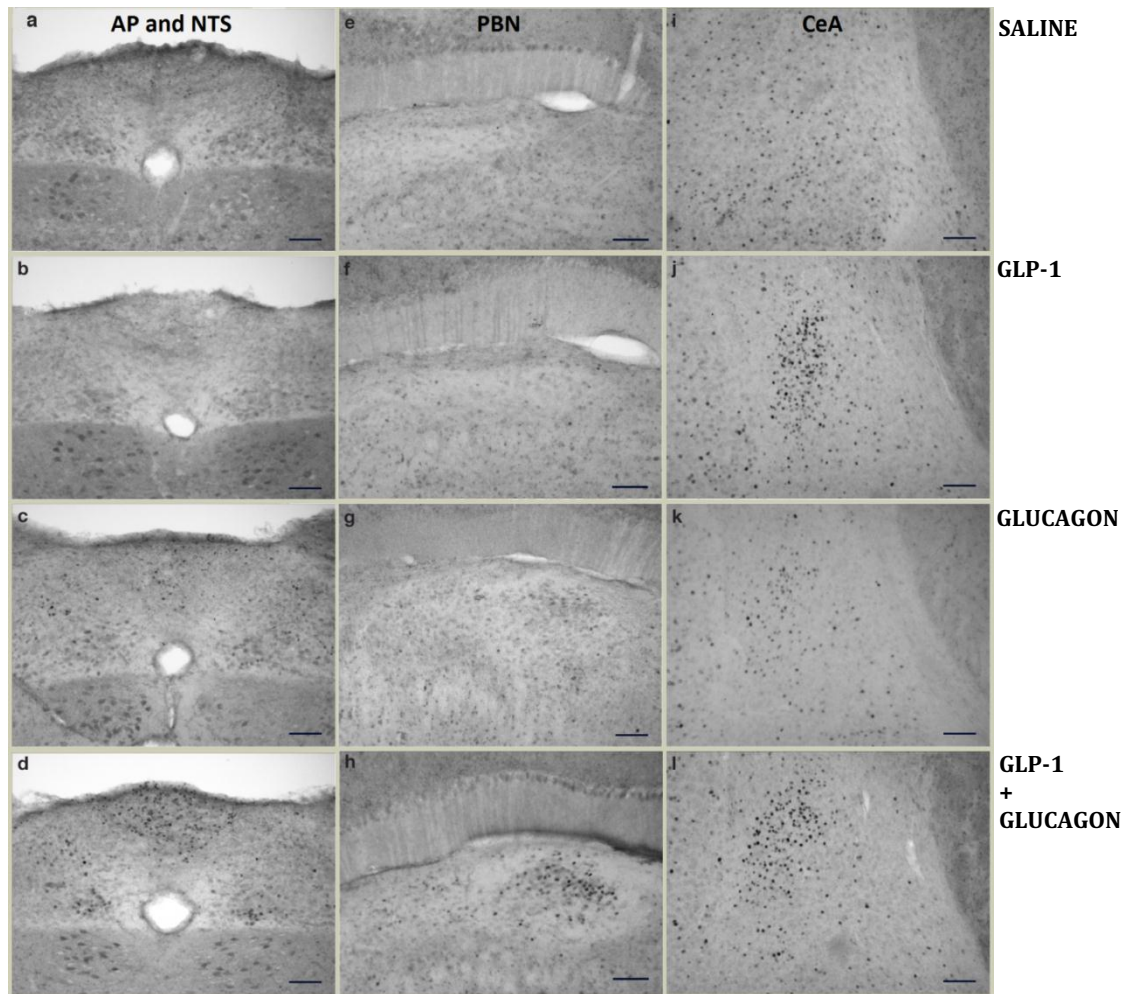


Figure 2.17. Representative photomicrographs of c-fos immunoreactivity 90 minutes after subcutaneous administration of saline, GLP-1, glucagon or GLP-1 + glucagon. a-d: nucleus tractus solitarius (NTS) and area postrema (AP); e-h: parabrachial nucleus (PBN); i-l: central nucleus of the amygdala (CeA). a: Saline b: GLP-1 (10nmol/kg) c: Glucagon (30nmol/kg) d: GLP-1 (10 nmol/kg) + glucagon (30 nmol/kg) e: Saline f: GLP-1 (10nmol/kg) g: Glucagon (30nmol/kg) h: GLP-1 (10 nmol/kg) + glucagon (30 nmol/kg) i: Saline j: GLP-1 (10nmol/kg) k: Glucagon (30nmol/kg) l: GLP-1 (10 nmol/kg) + glucagon (30 nmol/kg). Scale bar =0.1mm.

2.5 Discussion

The individual effects of glucagon and GLP-1 on food intake and c-fos expression in the hypothalamus and brainstem.

The studies described in this chapter investigate the acute effects of glucagon and GLP-1, individually and in combination, on food intake and c-fos activation in the brain. The current work shows that glucagon has an acute anorectic effect on food intake lasting 30 minutes, which is dose responsive. The anorectic effect following glucagon injection is seen in fasted mice, whether administered at the onset of the light phase, midway through the light phase or at the onset of the dark phase. In addition, anorectic doses of glucagon increase c-fos-ir in the AP, cNTS and PBN of the brainstem and the CeA. However, no change in c-fos-ir is seen in the hypothalamic nuclei studied. GLP-1 also reduces food intake in a dose-dependent manner and increases c-fos-ir in the AP, cNTS and PBN of the brainstem and the CeA. Similarly, there are no significant changes in c-fos activation in the hypothalamus following GLP-1 administration.

These results are consistent with previous studies showing that peripheral administration of glucagon^{180-182,184} and GLP-1^{87,156,157} alone causes a significant, acute reduction in food intake. However, in contrast to previously published studies, the anorectic effect of glucagon appears to be irrespective of the time of injection. In rats, following a four hour fast, glucagon significantly reduces food intake by almost 50% when injected at 1200 and 1600 (mid-late light phase), but not at 2000 (dark phase)¹⁹⁰. This raises the possibility that the duration of fasting affects the anorectic response to glucagon. Geary et al³⁶⁸ fail to demonstrate an anorectic effect in fed rats following glucagon injection. However, the same dose significantly reduces food intake following an 8, 12, 18 or 24 hour fast. Similarly, portal vein glucagon infusions in sham vagotomised rats cause a greater reduction in food intake in eight hour fasted rats compared to four hour fasted rats³⁷¹. These results are likely to reflect how hungry the animals are at the time of the study. Animals that have been fasting for a shorter duration will be less hungry and therefore any anorectic effect due to glucagon administration may not be discernable. Similarly, my results support the absence of an anorectic effect following glucagon administration in fed mice³⁶⁸.

Peripheral administration of glucagon, at doses shown to reduce food intake, increases c-fos-ir in the AP, cNTS and PBN of the brainstem and CeA. Currently, there are no published data for c-fos expression in the brain following peripheral administration of

glucagon. My results indicate that peripheral glucagon administration activates c-fos in CNS areas important in the regulation of food intake. Although the central mechanisms underlying the anorectic effect of glucagon are unknown, it is likely that the brainstem plays an important role in terminating food intake in response to glucagon administration. The finding of diminished anorectic response to glucagon in vagotomised animals and those with AP and NTS lesions³⁵³, suggests that a vagal mediated pathway via the brainstem may be the main site of action. It has been hypothesised that glucagon acts on sensory afferents in the liver and/or GI tract and a glucagon satiety signal is relayed from these sites to the brain via the vagus nerve¹⁸¹. In the current study, peripherally administered glucagon induces c-fos-ir in the cNTS, which receives signals directly from the vagus nerve originating in the liver and GI tract¹¹⁴ and thus is consistent with this hypothesis.

The lack of c-fos activation in the hypothalamus seen in the current studies also supports the hypothesis that the brainstem is the main site of action for the anorectic response to glucagon. However, these findings are in contrast to other studies that propose hypothalamic involvement. Administration of glucagon into the 3rd ventricle of rats, potently reduces food intake suggesting a hypothalamic site of action³⁶². In addition, neuronal activation in glucose sensitive neurones in the LH and VMN, is suppressed following electrophoretic application of glucagon³⁶³. The discrepancy between the current results and previous studies could be due to a number of reasons including species differences, doses given, route of administration and peptide degradation *in vivo*. Previous studies were undertaken in rats and glucagon administered centrally rather than peripherally. Earlier studies have shown that c-fos expression following GLP-1 depends on the route of administration. In rats, ICV administration of GLP-1 increases c-fos expression exclusively in the PVN and CeA³⁴⁶, whereas peripheral administration of GLP-1 increases c-fos expression in the ARC only⁸⁷. Similarly, in one study OXM was shown to inhibit food intake in mice following ICV but not ip administration²¹⁹. Species differences can also account for discrepancies in results. Glucagon acutely stimulates adrenocorticotrophin hormone (ACTH) release from the pituitary gland in humans³⁷². In contrast, a decrease in ACTH for the first ninety minutes is seen in response to glucagon in anaesthetised rats³⁷³. Finally, both glucagon and GLP-1 are subject to proteolytic degradation in the circulation^{142,199}. This may explain why vagal mediated activation of c-fos in the brainstem is apparent, yet direct activation of hypothalamic nuclei via the circulation is not seen.

It is possible that the anorectic effect of glucagon and GLP-1 is mediated via a 'stress response' via stimulation of the hypothalamo-pituitary-adrenal (HPA) axis to release corticosterone in rodents or sympathetic activation of the adrenal medulla causing release of catecholamines. Glucagon, GLP-1 and exendin-4 (a GLP-1R agonist) are known to stimulate the HPA axis and SNS in rats, mice and humans³⁷⁴⁻³⁷⁹. The HPA axis modulates food intake initially through the production of CRH in the PVN which modulates NPY³⁸⁰ and leptin signalling³⁸¹ in the hypothalamus. In addition, CRH causes release of ACTH from the anterior pituitary which affects food intake and EE in humans through the stimulated production of cortisol⁴⁶. However, if the anorectic effect seen in the current studies following either glucagon or GLP-1 administration is due to stimulation of the HPA axis, one would expect significant c-fos activation in the PVN³⁸². Ascending neuronal projections exist between the NTS and PVN^{102,383} and noradrenergic terminals from the NTS exist on CRH and TRH containing neurones in the PVN^{104,384}. In my studies, c-fos-ir increases in the PVN in response to high doses of both glucagon and GLP-1, however this fails to reach significance. Furthermore, although the magnitude of rise in c-fos counts in the PVN between saline controls and glucagon or GLP-1 appears significant, absolute c-fos counts are low making interpretation of results difficult. In contrast, the increase in c-fos expression in response to saline and glucagon or GLP-1 in the brainstem and CeA, is of much greater magnitude and suggests a meaningful comparison. Measuring plasma catecholamines and corticosterone during these studies may have been useful in elucidating whether the results reflect activation of a 'stress response'.

Peripheral injection of GLP-1 individually produces a dose related acute reduction in food intake and increases c-fos-ir expression in the AP, cNTS and PBN of the brainstem and CeA. This is consistent with a previous study, in which hepatic portal vein infusions of GLP-1 increases c-fos-ir in the AP, NTS and CeA in rats ³⁸⁵. Further studies of the mechanism through which GLP-1 reduces food intake utilise the prolonged action of exendin-4, which is resistant to proteolytic cleavage and therefore has a longer half-life *in vivo*. Peripheral administration of exendin-4 activates c-fos expression in the PVN, SON and ARC of the hypothalamus and the AP, NTS and PBN in the brainstem of mice ^{219,386,387}. Similarly, a recent study shows that peripheral exendin-4 increases c-fos expression in the AP and NTS and within the enteric nervous system of the GI tract of rats³⁸⁸.

It is thought that exendin-4 decreases food intake through direct activation of GLP-1Rs in the hypothalamus and brainstem via the circulation and an incomplete BBB. However,

the finding that c-fos activation occurs in enteric neurones in the gut in response to exendin-4³⁸⁸, suggests that vagal afferents from these enteric neurones signalling to the brainstem provide another potential mechanism through which exendin-4 and GLP-1 reduce food intake. This is supported by the findings that vagotomy attenuates the anorectic effect of GLP-1 and diminishes c-fos-ir in ARC neurones in response to GLP-1 in rats⁸⁷. In addition, Hayes *et al*¹⁴⁶ use a decerebrate rat model to demonstrate that the brainstem alone is able to elicit the anorectic response to exendin-4. Furthermore, the acute anorectic response to exendin-4 is preserved in AP lesioned rats suggesting that the NTS is responsible for processing the signals mediating the anorectic response³⁸⁹. These studies are consistent with my results that have shown a significant increase in c-fos-ir in the NTS following GLP-1 administration.

The current studies have shown that glucagon and GLP-1 independently increase c-fos-ir in similar areas within the brain, namely the AP, cNTS and PBN of the brainstem and CeA. This was unexpected since OXM, an agonist at both the GCGR and GLP-1R, has been previously shown to activate different areas in the hypothalamus compared to GLP-1³⁶⁶. This suggests that glucagon and GLP-1 also activate distinct regions in the brain.

The PBN receives extensive projections from the NTS⁹⁸ and activation of this pathway may be responsible for the c-fos-ir observed in the PBN. Likewise, the CeA receives projections from both the PBN and NTS^{390,391} and c-fos expression in this nucleus is stimulated by a range of anorectic stimuli including GLP-1^{86,385}. However, the role of the CeA in appetite control is uncertain. Kainic acid lesions of the CeA increase food intake and body weight in rats³⁹². Similarly, CeA administration of the GABA_A antagonist bicuculline, results in increased food intake, whereas the GABA_A agonist, muscimol, decreases feeding³⁹³. These results suggest that the CeA is involved in feeding regulation, possibly through the activation of GABA_A receptors. The CeA appears to be predominantly involved in emotional behaviour, particularly anxiety and conditioned fear³⁹⁴. However, the amygdala also has connections with the NTS and DMV in the brainstem³⁹⁵. Electrical stimulation of the CeA increases pancreatic exocrine secretion and decreases gastric motility, whilst both effects are attenuated by vagotomy^{396,397}. This suggests that an anorectic effect due to activation of the CeA may reflect reduced gastric emptying via the vagus nerve, thereby causing nausea, rather than increased satiety. Therefore, the increase in c-fos-ir in the CeA in response to glucagon or GLP-1 administration observed in my studies, may represent activation of the NTS-CeA pathway. Glucagon and GLP-1 are known to reduce gastric emptying^{164,398} and this may be reflected in my results. Although not investigated in my studies, this could be

measured using solid and liquid-phase gastric emptying techniques and paracetamol absorption studies to assess gut transit time^{399,400}.

The effects of co-administration of glucagon and GLP-1 on food intake and c-fos activation in the brainstem.

The current studies show that co-administration of glucagon and GLP-1 reduces food intake at doses that alone have no significant anorectic effect. Although glucagon and GLP-1 co-administration reduced food intake to a greater extent than GLP-1 alone, this did not reach statistical significance. Administration of lower doses of GLP-1 that did not reduce food intake compared to saline controls would have been useful to investigate this further.

Co-administration of sub-anorectic doses of glucagon and GLP-1 significantly reduce food intake compared to controls and increase c-fos-ir in the AP and CeA. These findings suggest that low doses of each hormone, which alone are not anorectic, can be given in combination to significantly reduce food intake. Clinically, this is of interest due to the common side-effect of nausea and vomiting following GLP-1 administration. Up to two-thirds of patients treated with GLP-1 agonists report nausea, limiting the dose at which these agents can be administered^{401,402}. Co-administration of glucagon and GLP-1 has not been investigated in humans. Understanding the neuronal pathways involved in the anorectic response to glucagon and GLP-1, both independently and in combination, is particularly important since both can cause nausea. If both hormones act through a common pathway to reduce food intake, combination therapy may allow lower doses to achieve anorexia but still cause significant nausea. Although increased c-fos-ir is demonstrated in similar areas of the brain following glucagon and GLP-1 administration, there may be differences in the specific neuronal populations activated by GLP-1 and glucagon within any of the nuclei studied. A further study using immunohistochemistry or another method of characterisation of the neurones activated would help to determine whether there is any difference in the pattern of neuronal activation induced by these two hormones.

A further study of c-fos expression following peripheral OXM would be interesting. OXM is an agonist at both glucagon and GLP-1 receptors²¹⁹⁻²²¹. In mice, peripheral (ip) OXM significantly increases c-fos expression in the PVN, AP and NTS²¹⁹. The current studies show increased c-fos-ir in the AP and CeA only, following peripheral (s/c) co-administration of glucagon and GLP-1. In my studies, sub-anorectic doses of glucagon and GLP-1 are administered to ascertain whether there is an anorectic effect on food

intake following co-administration compared to each hormone alone. The use of such low doses may explain the differences in c-fos expression compared to OXM. Alternatively, these findings may reflect different routes of hormone administration or receptor binding and the activating properties of OXM at the glucagon and GLP-1 receptor compared to the native hormones. The anorectic effect and c-fos-ir distribution seen following peripheral OXM administration is abolished in GLP-1R knockout mice, but not GCGR knockout mice²¹⁹. This suggests that the anorectic effect of OXM is mediated through the GLP-1 receptor alone. Furthermore, in the hypothalamus, peripheral OXM and GLP-1 activate different regions when assessed using manganese-enhanced magnetic resonance imaging (MEMRI)³⁶⁶. Following peripheral injection of OXM in fasted mice, there is reduced signal enhancement in the ARC, PVN and SON, whereas GLP-1 causes a reduction in the PVN only. In the brainstem, GLP-1 and OXM administration individually increases signal intensity in the AP³⁶⁶. Surprisingly, there is no change in c-fos expression in the hypothalamus or NTS of the brainstem following OXM. These results are consistent with my findings following glucagon and GLP-1 co-administration.

In the current studies, glucose levels were measured following sub-anorectic doses of glucagon and GLP-1, independently and in combination. The acute rise in glucose in response to glucagon was attenuated by the co-administration of GLP-1. Although not investigated, this effect was assumed to be secondary to GLP-1-mediated insulin release from pancreatic β cells. These findings suggest that glucagon and GLP-1 co-administration may be of therapeutic value in achieving weight loss due to an additive anorectic effect, without compromising glucose control.

In conclusion, the current studies demonstrate that neurones in the brainstem and amygdala are activated by anorectic doses of glucagon, and that the pattern of activation is indistinguishable from GLP-1. Furthermore low, sub-anorectic doses of glucagon and GLP-1 in combination inhibit food intake and increase c-fos-ir in the AP and CeA. Overall, these findings suggest that GLP-1 and glucagon receptor co-agonism may have a greater satiating effect than agonism of the GLP-1R or GCGR alone. This dual approach in targeting both the glucagon and GLP-1 receptor systems may offer additional benefits in an obesity treatment compared to GLP-1 alone due to increased anorectic effect.

CHAPTER 3:

THE EFFECTS OF LONG-ACTING GLUCAGON AND GLP-1 ANALOGUES ON ENERGY HOMEOSTASIS IN RODENTS

3.1 Introduction

The naturally occurring gut hormone, OXM, is an agonist at both the glucagon and GLP-1 receptor²¹⁹⁻²²¹ and has been shown to have beneficial effects on food intake^{220,222,225}, EE²²³ and glucose homeostasis⁴⁰³. However, using this hormone alone as a therapeutic agent does not allow modification of the extent of co-agonism at each receptor. Study of the effects of glucagon and GLP-1 administered individually and in combination, on food intake, EE and glucose homeostasis may enable identification of the most beneficial ratio of glucagon to GLP-1 receptor agonism to increase EE and reduce food intake, with minimal side effects, whilst improving glycaemic control. In doing so, this approach may lead to new therapies for overweight patients with type 2 diabetes that have advantages above clinically used GLP-1 agonists alone. However, this requires longer term studies to investigate whether the acute effects of these therapies are sustained and lead to significant weight loss.

The previous chapter focused on the investigation of glucagon and GLP-1 alone, and in combination on food intake, glucose homeostasis and activation of key areas within the brain involved with appetite. Both glucagon and GLP-1 have short half-lives of several minutes due to enzymatic breakdown within the circulation by DPPIV^{142,199}. In order to study long term effects of these peptides in animals, this would necessitate frequent and multiple injections in order to achieve raised circulating levels over a 24 hour period, potentially leading to stress and disturbance of normal activity. Previous research groups have developed analogues containing the amino acid sequences of both glucagon and GLP-1 in a single peptide, which bind and activate their respective receptors^{228,229}. Chronic administration of these analogues in DIO mice increases weight loss through a simultaneous reduction in food intake and increased EE, whilst improving glucose and lipid control^{228,229}. All of these findings are of potentially therapeutic benefit to obese patients with diabetes in whom multiple agents are often required to control these aspects. In the Department of Investigative Medicine, Imperial College, our laboratory has designed long-acting glucagon and GLP-1 analogues to investigate the effects on food intake, EE, glucose homeostasis and lipid control. The work outlined in this chapter focuses on the effects of specific analogues on energy homeostasis.

The role of glucagon and GLP-1 in the control of food intake

Peripheral administration of both glucagon and GLP-1 reduces acute food intake as described in chapter 2. GLP-1 analogues have been extensively investigated and cause a sustained reduction in food intake leading to modest weight loss in humans (see Edwards et al, 2012 for review⁴⁰⁴). Similarly, glucagon is anorectic in rodents^{181,182,184,185,351}. However, whether this effect is apparent following repeated administration is unclear. An OXM analogue administered for 14 days in mice causes a sustained reduction in food intake compared to a modified OXM analogue which differs by only one amino acid but does not bind or activate the GCGR²²⁷. This suggests that glucagon does reduce food intake following repeated administration.

The role of glucagon and GLP-1 in the control of energy expenditure (EE)

Studies investigating the role of GLP-1 and its analogues in the control of EE have been conflicting. The administration of exenatide for three months in non-diabetic obese humans, causes weight loss and reduced food intake but no change in weight-adjusted EE⁴⁰⁵. Similarly, once daily administration of the GLP-1R agonist, liraglutide, for four weeks, in overweight patients with type 2 diabetes, does not significantly increase resting EE⁴⁰⁶. In support of this finding, ICV injection of GLP-1 in mice increases activity of sympathetic nerve fibres innervating interscapular BAT and increases expression of genes upregulated by adrenergic signalling and those required for BAT thermogenesis (PGC1- α and UCP-1)⁴⁰⁷. However, in the same study, GLP-1 receptor knockout mice show a normal response to cold exposure, suggesting that GLP-1 receptors are not essential for the thermogenic response to cold acclimatisation. Although circulating catecholamine levels were not measured following ICV injection, the dose of GLP-1 administered may have stimulated a stress response and activation of the SNS, increasing thermogenesis. In healthy volunteers, GLP-1 analogues have been found to increase heart rate and cardiac output^{408,409} and catecholamines during exercise³⁷⁶.

The evidence for increased EE following glucagon administration is more conclusive, although the mechanism is unknown and could also represent a supraphysiological and hence stress response. ICV administration of glucagon in mice also increases activity of sympathetic fibres innervating BAT and genes involved with adrenergic signalling in BAT⁴⁰⁷. In rats, glucagon administration increases whole body oxygen consumption, and body temperature, blood flow to BAT, as well as temperature and BAT mass^{215,216}. Many of these effects are attenuated by propranolol, a non-selective β -adrenergic receptor

blocker, as well as denervation of BAT, suggesting a role for the SNS in increased EE following glucagon administration^{217,218}. Whether these effects of glucagon and increased EE are sustained following repeated administration is unknown. Furthermore, should such an increase in EE be sustained, this may not result in reduced body weight. Compensatory mechanisms such as increased food intake may override any increased EE to maintain stable body weight.

The role of glucagon and GLP-1 in the control of glucose homeostasis

Glucagon and GLP-1 have opposing roles in glucose homeostasis when administered acutely. GLP-1 is known for its incretin effect, whereby it potentiates glucose-stimulated insulin secretion from the pancreas. In contrast, glucagon is best known for its role in gluconeogenesis and glycogenolysis in the liver, thereby increasing circulating glucose levels (see figure 3.1). Both hormones in combination could therefore exploit the beneficial weight lowering effects of glucagon and GLP-1 whilst improving glucose control. These effects would depend on the ratio of GLP-1 and GCGR agonism achieved by this combination.

GLP-1 analogues and their use in clinical practice have been extensively investigated. Increasingly, longer acting preparations are being developed with the intention that effects on weight loss and glucose control will be superior to current preparations. However, weight loss with current GLP-1 analogues is modest and patients with type 2 diabetes frequently require multiple therapies to maintain normal blood glucose levels. The investigation of long-term GCGR agonism has only recently received attention, due to effects on increased EE and potential for weight loss. In contrast, GCGR antagonists have been developed in an attempt to reduce hepatic glucose output attributed with raised glucagon levels seen in patients with type 2 diabetes²⁰¹. Chronic GCGR agonism and the effects on glucose homeostasis and body weight in humans are unknown.

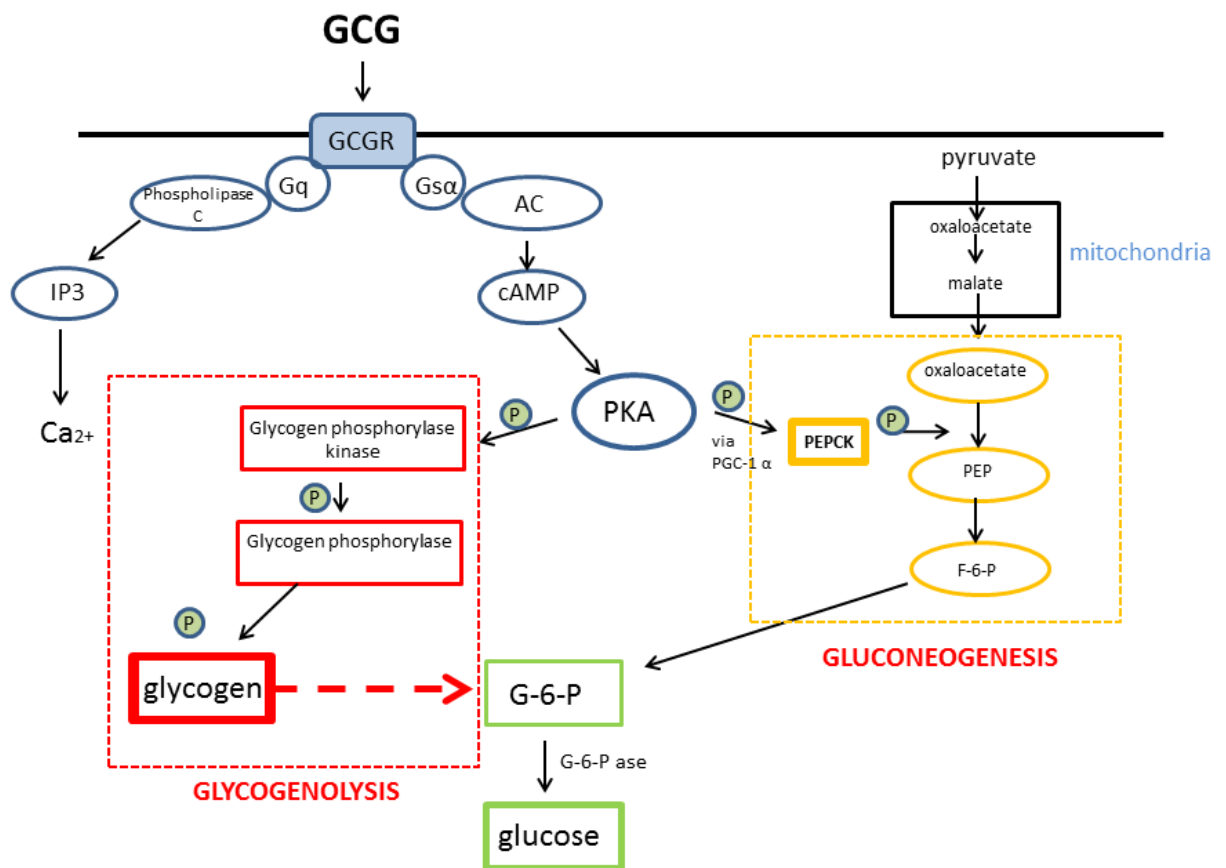


Figure 3.1. Simplified diagram depicting the role of glucagon in glycogenolysis and gluconeogenesis. Glucagon (GCG) signals through its receptor (GCGR) on the hepatocyte cell surface and activates G proteins called Gsα and Gq. Activation of Gq increases intracellular calcium ions via phospholipase C and inositol 1,4,5-triphosphate (IP3). Activation of Gsα, leads to activation of adenylyl cyclase (AC) and increased levels of cAMP with subsequent activation of protein kinase A (PKA). Activated PKA phosphorylates and activates glycogen phosphorylase kinase which in turn activates glycogen phosphorylase. This latter enzyme phosphorylates glycogen promoting its breakdown and the production of glucose-6-phosphate (G-6-P). G-6-Pase then converts G-6-P into glucose. Glucagon also upregulates the G-6-Pase gene via a nuclear transcription factor coactivator called peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). Glucagon also promotes glycogenolysis through the inhibition of glycogen synthase (not shown). During gluconeogenesis, an important rate-limiting step involves the conversion of oxaloacetate to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK). Glucagon increases PEPCK transcription and activity indirectly through PKA activation and upregulation of PGC-1α, which in turn increases transcription of the PEPCK gene. Through a series of enzymatic reactions, PEP is converted to fructose-6-phosphate (F-6-P) and subsequently G-6-P. Glucagon also increases gluconeogenesis by inhibiting glycolysis. Diagram adapted from Jiang and Zhang 2003⁴¹⁰.

The role of glucagon and GLP-1 in the control of circulating lipids

Beneficial effects of GLP-1 and its analogues on circulating lipids have been demonstrated in patients with type 2 diabetes⁴¹¹. However, it is likely that these effects are due, at least in part, to the associated weight loss and improved diabetes control. It is unclear whether GLP-1 has any direct effects on circulating lipids. To address this, one recent study investigated lipid oxidation in healthy volunteers using a pancreatic clamp technique, whereby plasma insulin and glucagon levels were clamped using somatostatin and hormone replacement⁴¹². During a four hour GLP-1 infusion, lipolysis and substrate oxidation remained the same as controls⁴¹². In contrast, a study in which such a clamp technique was not used, demonstrated that GLP-1 reduced postprandial triglyceride levels⁴¹³. However, this was also accompanied by marked changes in other hormones and likely to be due to insulin-mediated inhibition of lipolysis and reduced gastric emptying. In contrast, there is now emerging evidence to suggest a beneficial effect of insulin sensitisers such as GLP-1 analogues and DPPIV inhibitors in non-alcoholic steatohepatitis (NASH). NASH is characterised by insulin resistance and accumulation of fat within the hepatocytes, and can lead to liver cirrhosis. Exendin-4 administration in mice reduces apoptosis and increases lipolysis in hepatocytes in response to ischaemic reperfusion injury⁴¹⁴. Animal studies have demonstrated that GLP-1 protects against NASH by increasing fatty acid oxidation, decreasing lipogenesis and improving hepatic glucose metabolism^{415,416}. It is unknown however whether these effects occur independently of weight loss and improved glycaemic control, both of which also ameliorate NASH.

A role for glucagon in lipolysis has also had conflicting results. *In vitro* and *ex vivo* studies previously suggested a role for glucagon in stimulating an enzyme called hormone sensitive lipase (HSL) in adipocytes, causing the hydrolysis of triglycerides into glycerol and fatty acids⁴¹⁷ (see figure 3.2). In contrast, human *in vivo* studies, have shown very little effect of glucagon infusion on circulating lipid profile. Iv infusion of glucagon to healthy human volunteers shows no change in plasma levels of triglycerides or FFAs⁴¹⁸. Similarly, s/c glucagon infusion in healthy male volunteers does not increase glycerol or FFAs at either physiological or supraphysiological levels⁴¹⁹.

However, there may be a role for glucagon in the regulation of hepatic lipid metabolism. GCGR knockout mice exhibit defects in hepatocyte lipid oxidation and accumulation of excessive lipids⁴²⁰. In addition, s/c glucagon administration to wild-type mice inhibits triglyceride synthesis in the liver and stimulates fatty acid oxidation⁴²⁰.

This chapter focuses on the effects of long-acting glucagon and GLP-1 analogues on acute and chronic food intake, EE and glucose homeostasis in mice. In addition, the effects of these analogues on behaviour and brainstem c-fos activation were studied.

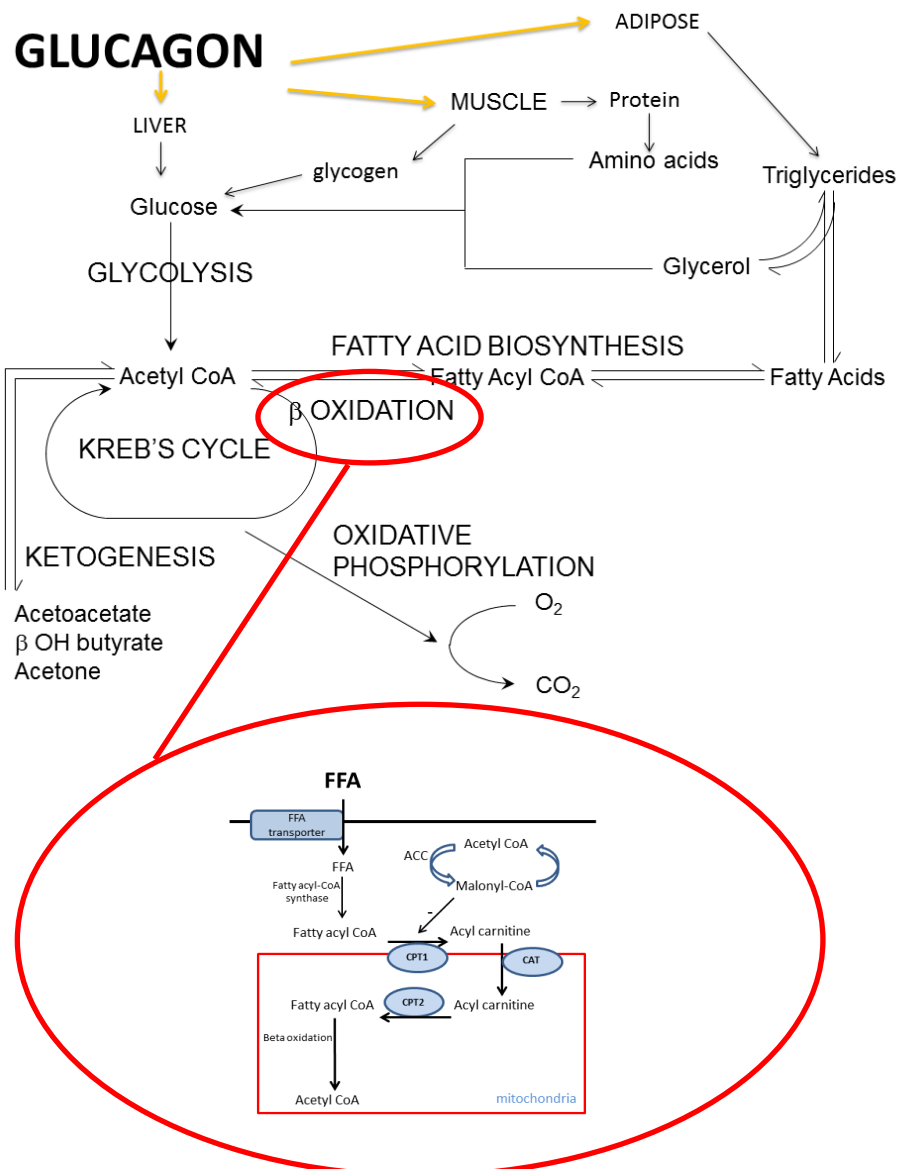


Figure 3.2. The role of glucagon in the control of circulating lipids. Glucagon binds to its receptor in adipocytes, causing increased cAMP and activation of hormone sensitive lipase (HSL). HSL hydrolyses triglycerides into glycerol and free fatty acids (FFAs), which are released into the circulation and transported to the liver. Depending on the size of the FFA, it either diffuses across the cell membrane or is carried by cell surface transporters. Once inside the cell, a CoA group is added to the FFA by fatty acyl-CoA synthase forming long chain acyl-CoA. Carnitine palmitoyltransferase1 (CPT1) converts this long chain acyl-CoA to long chain acylcarnitine, allowing the fatty acid to be transported into the mitochondria via carnitine-acylcarnitine translocase (CAT). Within the inner mitochondrial membrane, CPT2 converts the long chain acylcarnitine back into long chain acyl-CoA which enters the fatty acid beta-oxidation pathway. This pathway involves a series of reactions in which acetyl CoA is cleaved from the fatty acid by co-enzyme A. Acetyl CoA then enters Kреб's cycle to produce NADH and FADH₂ which are used in the electron transport chain to produce ATP and hence energy. Acetyl-CoA carboxylase (ACC) is a key enzyme involved in fatty acid beta oxidation and fatty acid synthesis. ACC catalyses the carboxylation of acetyl-CoA producing malonyl-CoA which can be used as a substrate for fatty acid synthesis. Malonyl-CoA also inhibits CPT1, thereby blocking mitochondrial fatty acid synthesis. In mammals, two isoforms of ACC are found, ACC1 and ACC2. ACC1 maintains regulation of fatty acid synthesis and is highly expressed in adipose tissue. In contrast ACC2 mainly regulates fatty acid oxidation and is found in highly metabolic organs such as skeletal muscle and heart. Both ACC1 and ACC2 are found in the liver where fatty acid oxidation and synthesis is important. Adapted from Fillmore et al 2012 ⁴²¹.

3.2 Hypothesis and aims

3.2.1 Hypothesis

I hypothesised that dual glucagon/GLP-1 analogues and co-administration of glucagon and GLP-1 analogues reduce food intake and increase EE causing weight loss and improved glucose homeostasis in mice.

3.2.2 Aims

To investigate the effect of glucagon/GLP-1 analogues on:

- acute food intake, behaviour and c-fos activation in the brainstem of lean mice
- chronic food intake, body weight, glucose homeostasis and markers of EE in DIO mice

3.3 Materials and Methods

3.3.1 Investigation of the acute effects of a long acting dual glucagon and GLP-1 analogue G(X) on food intake and c-fos immunoreactivity in the brainstem in mice.

3.3.1.1 Peptides

Human glucagon, human GLP-1 and exendin-4 were purchased from Bachem Ltd. (Merseyside, UK). A dual glucagon/GLP-1 analogue, G(X), was designed by Prof. Stephen R. Bloom, Dept Investigative Medicine, Imperial College, London. Analogues were custom synthesised using solid phase peptide synthesis (SPPS) methodology (Bachem Ltd). Peptide purity was determined by reverse-phase high performance liquid chromatography (HPLC) and by Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). Work involving receptor binding assays, cAMP activation and feeding studies of the glucagon/GLP-1 analogue G(X) was undertaken by other members of the research team. These results and the amino acid sequence of G(X) is found in Appendix B. G(X) contains identical amino acid sequences to glucagon from positions 1 to 16 with the exception of a serine to aminoisobutyric acid (AIB) substitution at position 2. AIB is structurally similar to alanine except for a methyl group in place of a hydrogen at the alpha-carbon leading to changes in helix formation and reduced DPPIV cleavage²²⁹. From positions 16-39, amino acid substitutions have been made to resemble exendin-4, including a Trp-cage at the C-terminal (amino acids 32-39). Although evidence has been conflicting, it is thought that the Trp-cage may increase the binding affinity of exendin-4 to the GLP-1R⁴²². However, the EC₅₀ values demonstrate that G(X) binds and activates the GCGR and GLP-1 receptors with low affinity (appendix B). Despite these findings, initial pilot data from other members in the department found that G(X) dose-dependently reduced food intake. Furthermore, the anorectic action persisted for up to eight hours at the highest dose administered and suppressed food intake by almost 85% compared to saline controls over this time period (see appendix B4). However, subsequent studies demonstrated that G(X) was less effective than exendin-4 at reducing food intake when administered at the same dose. Therefore, further analogues were developed that were also biologically active but were specific for the GLP-1R or GCGR (G(Y) and G(Z) respectively (see appendix B).

3.3.1.2 Animals

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402).

Adult male C57BL/6 mice (Charles River, Margate, UK) weighing 25-30 g were maintained in individual cages under controlled temperature (21-23°C) and lights (12:12 hr light-dark cycle, lights on at 0700). Animals were allowed *ad libitum* access to water and RM1 diet (Special Diet Services, Witham, UK) unless otherwise stated. Animals were regularly handled and acclimatised to ip or s/c injections in order to reduce non-specific stress at the time of the study.

3.3.1.3 Investigation of the acute effects of a long-acting glucagon and GLP-1 analogue G(X) on food intake in mice

Experiments were carried out during the early light phase (0800-1000), in C57BL/6 mice fasted from 1600 the preceding day, unless otherwise stated. Body weights of animals were recorded the day before the study in order to randomise mice to each group and calculate weight adjusted doses. Specific experimental details are noted for each study, but in each case the following was performed: a maximal volume of 0.05 ml was injected s/c and mice returned to their home cage with a known amount of food following injection. Food was reweighed at regular intervals post injection.

3.3.1.4 Investigation of the acute effects of co-administration of exendin₍₉₋₃₉₎ and a long-acting glucagon and GLP-1 analogue G(X) on food intake in mice

In order to elucidate how much of the anorectic effect was due to activation of the GLP-1 receptor, G(X) was co-administered with the GLP-1 receptor antagonist exendin-4₍₉₋₃₉₎. Experiments were carried out as described in section 3.3.1.3, however two s/c injections were administered to each mouse. The first injection was either ip vehicle or exendin-4₍₉₋₃₉₎ at 25000 nmol/kg. The dose of exendin₍₉₋₃₉₎ used was based on previous studies^{219,423}. Exendin-4₍₉₋₃₉₎ has previously been shown to bind with at least 10-fold lower affinity at the GLP-1R compared to GLP-1 and this high dose was chosen to ensure that the majority of GLP-1 receptors were blocked⁴²³. A second s/c injection of either vehicle or analogue G(X) (50nmol/kg) was administered 5 minutes later. This time delay was to allow exendin-4₍₉₋₃₉₎ to disperse through the circulation and bind to the GLP-1 receptors before administration of a GLP-1R agonist. Food intake was measured at 0.5, 1.5, 3 and 6 hours post injection.

3.3.1.5 Investigation of behaviour in mice following administration of a long-acting glucagon and GLP-1 analogue G(X)

In order to investigate whether the dual glucagon/GLP-1 analogue G(X) caused adverse effects, a behaviour study was conducted. Experiments were carried out during the early light phase (0800-1000) in mice fed *ad libitum*. Environmental enrichment was removed the day before to ensure adequate visualisation. A maximal volume of 0.2 ml was injected ip and mice immediately returned to their cages. LiCl was used as a positive control as it is known to induce behavioural abnormalities in mice⁴²⁴. Behavioural patterns were monitored continuously for 90 minutes post injection by observers blinded to the treatment group. Behaviour was classified into the following categories: feeding, grooming, drinking, rearing, hunching, locomotion, burrowing, inactive, pica and sleeping. Each mouse was observed for 15 seconds every five minutes. During these 15 seconds, three behaviours lasting five seconds each were recorded giving a total of 36 observations per hour. Data are presented as median (interquartile range).

3.3.1.6 Investigation of c-fos immunoreactivity in the brainstem following administration of a long-acting glucagon and GLP-1 analogue G(X).

Experiments were carried out during the early light phase (0800-1000) in mice fasted from 1600 the preceding day. Animals were group housed (n=5/cage, weighing 20-25g) and for two weeks prior to experimentation, mice were handled on a daily basis and sham injected on at least three separate occasions. Mice (n=5-6 per group) were injected with a maximum volume of 50 µl s/c of either saline, glucagon 750 nmol/kg, GLP-1 100 nmol/kg or G(X) 50 nmol/kg and immediately returned to their cages. Ninety minutes later, mice were injected with 0.4 mls ip pentobarbitone (Euthatal, Merial Animal Health Ltd, Harlow, UK) prior to decapitation. Tissue preparation and c-fos immunohistochemistry was subsequently performed as described in section 2.3.7.

3.3.2 Investigation of the chronic effects of long acting glucagon and GLP-1 analogues G(Y) and G(Z) on food intake and body weight in diet induced obese (DIO) mice.

3.3.2.1 Peptides

In order to investigate the chronic effects of glucagon and GLP-1 separately, two analogues (G(Y) and G(Z)) were designed with specificity towards the GLP-1R or GCGR

respectively. These analogues were designed by Prof. Stephen R. Bloom, Dept Investigative Medicine, Imperial College, London. Analogues were custom synthesised using SPPS methodology (Bachem Ltd). Peptide purity was determined by reverse-phase HPLC and by MALDI-MS. Receptor binding assays, cAMP activation, pharmacokinetic profiles and acute feeding studies of the glucagon and GLP-1 analogues were undertaken by other members of the research team. These results and the amino acid sequences of G(Y) and G(Z) are found in Appendix B. G(Y) contains the same amino acids as glucagon up to position 14, with the exception of Lys→His¹². Subsequent amino acids are based on the structure of exendin-4 with the exception of the Trp-cage which is not included. Previous studies have demonstrated that amino acids in position 2, 3, 10 and 12 in glucagon are key in maintaining GCGR activity⁴²⁵. Therefore substitution of Lys at position 12 may partly explain why G(Y) predominantly activates the GLP-1R and not the GCGR. In addition, the Ser→Glu¹⁶ is likely to impact on the protein structure since serine at position 16 of glucagon is important in helix formation²²⁹. In contrast, G(Z) is identical to glucagon with the exception of three amino acid changes at positions 20, 21 and 27. Both G(Y) and G(Z) have an amidated C-terminal to avoid introducing a charged group which can affect peptide solubility.

The EC₅₀ values demonstrate that G(Y) binds and activates predominantly the GLP-1R, whereas G(Z) is specific for the GCGR (see appendix B). Both peptides show prolonged pharmacokinetic profiles in rats compared to native glucagon or GLP-1. G(Y) significantly decreased food intake compared to saline controls by 30% over 24 hours (appendix B4). G(Z) reduced food intake for the first 4 hours by almost 40% compared to saline controls however cumulative food intake thereafter was not significantly reduced compared to saline controls. The vehicle used was 1:1 zinc chloride: saline solution. At pH <5.5, the charged histidines in the analogue do not interact with the zinc ions in solution, however upon injection (pH of the skin is approximately 7.4), the charged histidines in the peptides bind to the zinc ions. This histidine-zinc binding causes precipitation of the peptide and the formation of a subcutaneous depot⁴²⁶. This results in longer lasting peptide action.

3.3.2.2 Animals

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402). Adult (aged 8 weeks old) male C57BL/6 mice (Charles River, Margate, UK) with an initial mean body weight of 25g were group housed for 8 weeks followed by two weeks singly housed. Individual

cages were maintained under controlled temperature (21-23°C) and lights (12:12 hr light-dark cycle, lights on at 0700). Animals were allowed *ad libitum* access to water and high fat diet (60% kcal from fat, Research Diets, New Jersey). Animals were regularly handled and acclimatised to s/c injections in order to reduce non-specific stress at the time of the study. At the start of the study the mice had a mean body weight of 38g (36-45g), which is approximately 25% more than the average weight of a mouse at 18 weeks old fed a chow diet⁴²⁷.

3.3.2.3 Investigation of the effects of chronic administration of long acting glucagon and GLP-1 analogues on food intake and body weight

Mice were maintained in individual cages as described in section 3.3.2.2. Mice were injected s/c once daily at 1500 for 70 days with either vehicle, G(Y) (5 nmol/kg days 1-5, 10 nmol/kg day 6 onwards), G(Z) 20 nmol/kg days 1-5, 40 nmol/kg days 6-15, 80 nmol/kg day 16 onwards) or a combination of G(Y) and G(Z) at these same doses. Following injection, mice were returned immediately to their cages. Initial doses were chosen based on previous acute feeding studies, with lower doses administered initially and then increased according to response until day 16. Thereafter doses remained the same. Food and body weight were measured every 1-2 days, immediately prior to injection.

3.3.2.4 Investigation of glucose homeostasis following chronic administration of long acting glucagon and GLP-1 analogues

Mice were maintained in individual cages as described in section 3.3.2.2. and injected daily with chosen peptides or vehicle as described in section 3.3.2.3. On day 70, mice underwent an ip glucose tolerance test (ipGTT). Due to the number of mice involved, this was performed on 3 consecutive days so that the test could be undertaken at the same time of day (T=0 at 1300). *Ad libitum* fed mice, were injected with peptide or vehicle at 0700 on the day of the study and then fasted until the ipGTT. Blood samples were collected at -30, 0, +30, +60, +90 and +120 minutes following ip 2g/kg glucose solution. Blood samples were obtained using tail tipping and serum glucose measured using a handheld glucometer. Blood samples were also taken at -30, +30, +60 and +120 minutes following glucose injection for measurement of insulin. These insulin samples were collected in 0.5mls eppendorfs and centrifuged at 10000 rpm for 10 minutes. Plasma was then separated using a pipette and stored in 0.5 ml eppendorfs at -20°C

until assayed. Insulin samples were assayed in singlet using an insulin ELISA (Millipore, UK).

3.3.2.5 Investigation of the effects of chronic administration of long acting glucagon and GLP-1 analogues on markers of energy expenditure (EE) and gluconeogenesis.

Tissue harvesting

Following the ipGTT, mice were maintained as described in section 3.3.2.3 and continued to receive daily s/c injections. On day 73, mice were killed by carbon dioxide (CO₂) asphyxiation and cardiocentesis performed in order to obtain blood samples for later analysis of markers of energy homeostasis. Blood samples were collected into heparinised eppendorfs and centrifuged at 10000 rpm for 10 minutes. Plasma samples were then stored in 2.5 mls eppendorfs at -80°C until further analysis was performed. Immediately following cardiocentesis, the following tissues were harvested, weighed and snap frozen in liquid nitrogen: interscapular BAT, epididymal WAT, and liver. In order to maintain consistency, both epididymal WAT deposits were removed and the same lobe of liver was harvested in each mouse.

Total RNA extraction

Real time quantitative polymerase chain reaction (rtPCR) was used to analyse the expression of various mRNA sequences in liver, BAT and WAT using TaqMan gene expression assays. Genes of interest focused on markers of EE, gluconeogenesis and lipid oxidation. All materials for qPCR were supplied by Applied Biosystems, Warrington, UK, unless otherwise stated.

Tissues were harvested as described above. Total RNA was extracted using the Tri-reagent method according to the manufacturer's protocol. Using a pestle and mortar, samples of liver, BAT or WAT were ground under liquid nitrogen, homogenised in 1 ml Tri-reagent and the suspension transferred to 1.5 ml eppendorfs to incubate at room temperature for 10 minutes. 100 µls of bromo-chloropropane was then added, mixed and incubated for 10 minutes at room temperature. The solution was centrifuged for 10 minutes at 14,000g at 4°C and the upper aqueous phase transferred to an autoclaved eppendorf and stored at -20°C until all samples from each tissue had been similarly processed.

To ensure sample purity and absence of DNA, RNA samples were purified using a Purelink RNA Mini Kit (Invitrogen, Paisley, UK) and Purelink DNase set (Invitrogen, Paisley, UK) and instructions followed according to the manufacturer's protocol. Samples were allowed to thaw and 400 µl 70% ethanol added. Each sample was mixed by hand and added to a spin cartridge, prior to centrifugation at 14000g for 15 seconds at room temperature. The flow-through was discarded leaving RNA bound to the cartridge membrane. The spin cartridge then underwent a series of buffer washes and centrifugation, followed by the addition of Purelink DNase. For each sample the following constituents were added to create the Purelink DNase mixture: 8 µl 10x DNase I reaction buffer, 10 µl resuspended DNase and 62 µl RNase free water. Samples contained within the spin cartridges were allowed to incubate at room temperature for 15 minutes followed by a further buffer wash and centrifugation (14000g for 15 seconds). A final wash using 500 µl buffer containing 60% ethanol, was added to the cartridge and centrifuged at 14000g for 15 seconds. The flowthrough was discarded, leaving the dry membrane with bound RNA which was placed into a recovery tube. RNase-free water was added to the membrane in the following quantities depending on the tissue collected: liver 70 µl, BAT 50 µl and WAT 30 µl. Samples were incubated at room temperature for 1 minute and then centrifuged for 1 minute at 14000g to elute the RNA from the membrane into the recovery tube. Purified RNA was then stored at -20°C until all samples from each tissue had been similarly processed.

Finally, RNA in each sample was quantified using a UV 1101 spectrophotometer (Biochrom, Cambridge, UK). A 1:50 dilution of the RNA purified sample was made (2µl RNA sample added to 98 µl GDW) and the resultant 100 µl solution placed in a disposable quartz cuvette (Brand manufacturers, Germany). The absorbance was read at 260nm and 280 nm and the concentration of RNA calculated using the following formula:

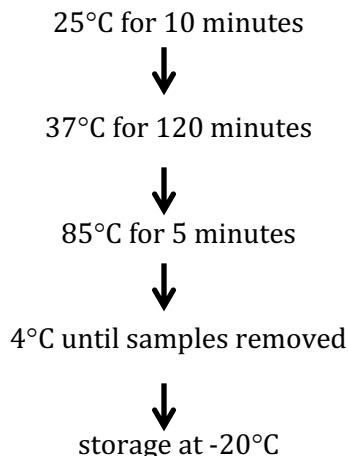
$$\text{Concentration } (\mu\text{g/ml}) = (\text{absorbance}_{260} \times \text{dilution factor}) \times 40$$

The 280nm reading provided an assessment of the degree of purity of the sample. 50 µg/ml of total RNA was required for appropriate amplification using rtPCR. Liver sample RNA content was above this threshold, however, over 50% of BAT and WAT RNA samples were inadequate. For this reason, ethanol precipitation was undertaken on all BAT and WAT samples. The RNA samples were mixed with 1µl of glycogen (5mg/ml, Ambion, UK), 0.1x volume of 2M sodium acetate and 2.5x volume 100% ethanol, and the solution thoroughly mixed by hand. Samples were then incubated at -20°C for at least

one hour. Samples were subsequently centrifuged for 7 minutes at 14,000g. The supernatant was then discarded and the pellet air dried under a fume hood for 2-3 hours. Pellets were re-suspended in the appropriate volume of GDW to give a final concentration of 50 µg/ml and stored at -20°C. Liver samples did not undergo ethanol precipitation but instead were diluted to give a final concentration of 50 µg/ml.

Reverse transcription of mRNA into cDNA

In order to undertake rtPCR, mRNA has to be transcribed into its complementary DNA sequence (cDNA) by reverse transcription. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) was used and the protocol followed according to the manufacturer's instructions. 2X RT Master Mix was prepared using the following components: 2 µls 10XRT buffer, 0.8 µls 25X 100mM dNTP mix, 2 µls 10X RT random primers, 1 µl Multiscribe Reverse Transcriptase and 4.2 µls RNase free water. 500 ng (10 µls of 50µg/ml sample) of each RNA sample was added to 0.5ml eppendorfs containing 10µl 2X RT master mix and pipetted several times to mix. Each eppendorf was transferred to a thermal cycler (PTC-100 7307 Programmable Thermal Controller, MJ Research Inc., US) under the following conditions:



rtPCR assay

During rtPCR, a specific gene is quantified through the use of a reporter-tagged probe, which is complementary to the cDNA of interest. The TaqMan probe anneals specifically to the complementary sequence. When the probe is intact, the proximity of the reporter dye to the quencher suppresses the fluorescence of the reporter. However, addition of DNA polymerase cleaves the probe that is hybridised to the complementary sequence, causing release of the reporter dye from the quencher and thus fluorescence of the reporter. This is illustrated in figure 3.3.

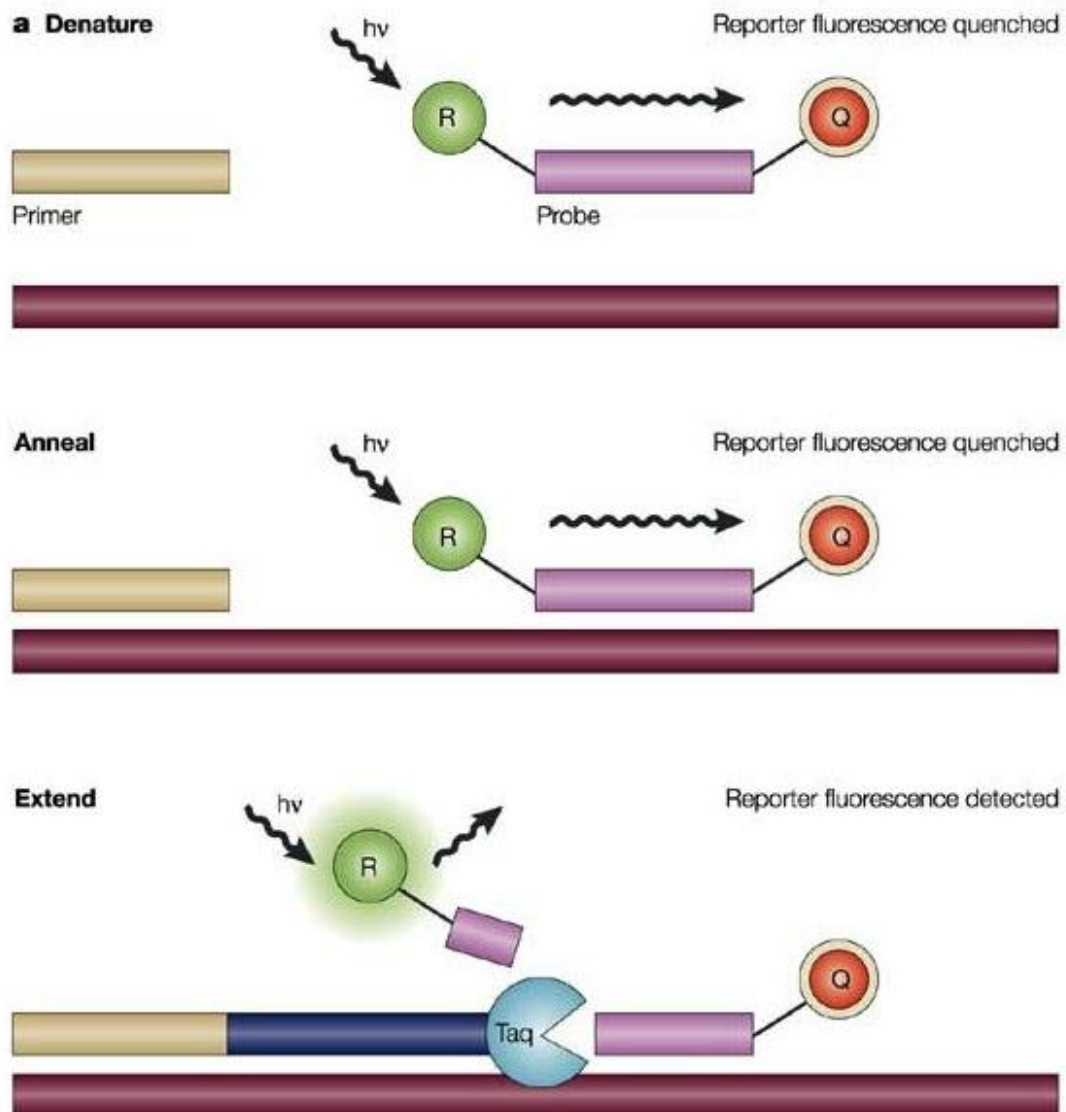


Figure 3.3. Schematic diagram representing the mechanism of real time quantitative polymerase chain reaction (rtPCR). TaqMan probes consist of a fluorophore (6-carboxyfluorescein, FAM) attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end (dihydrocyclopyrroloindole tripeptide minor groove binder, MGB). The PCR cyclers light source under normal conditions excites the fluorophore using Förster-type energy transfer. However, close proximity of the quencher to the fluorophore prevents this excitement. The probe anneals to a region of interest within DNA which has been amplified using a set of primers. As the Taq polymerase extends the primer and synthesises a second strand of DNA the 5' to 3' activity of the polymerase degrades the probe. This releases the fluorophore from the probe, and hence proximity to the quencher, resulting in fluorescence. Therefore, fluorescence detected in rt-PCR is directly proportional to the amount of fluorophore and hence DNA template present in the sample. Figure reproduced from Koch WH (2004)⁴²⁸.

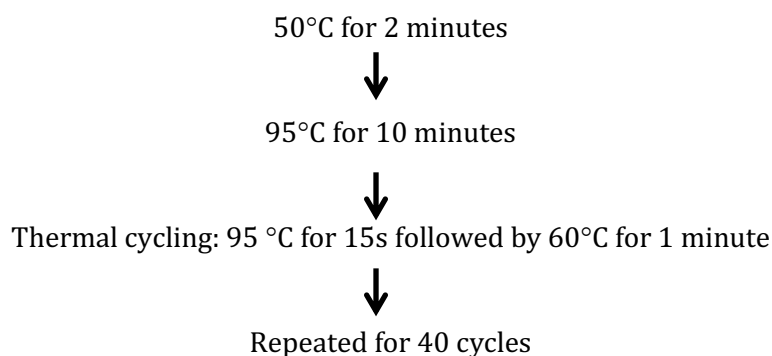
In this study, the following probes were obtained (Applied Biosystems, UK) in order to quantify the expression of the relevant genes shown in table 3.1.

Tissue	Gene of interest (abbreviation)	Full name of gene	Probe no.
Liver	G-6-Pase	Glucose-6-phosphatase	Mm00839363_m1
	PEPCK1	Phosphoenolpyruvate carboxykinase 1	Mm01247058_m1
	ACC1	Acetyl-coenzyme A carboxylase alpha	Mm01304257_m1
	FGF-21	Fibroblast growth factor-21	Mm00840165_g1
BAT	UCP-1	Uncoupling protein-1	Mm01244861_m1
	PPAR γ	Peroxisome Proliferative activated receptor gamma	Mm01184322_m1
	PGC1 α	Peroxisome proliferative activated receptor gamma co-activator 1 alpha	Mm01208835_m1
WAT	UCP-1	Uncoupling protein-1	Mm01244861_m1
	PPAR γ	Peroxisome Proliferative activated receptor gamma	Mm01184322_m1
	HSL	Hormone sensitive lipase	Mm00495359_m1

Table 3.1. Probes used to quantify various genes of interest in liver, brown adipose tissue (BAT) and white adipose tissue (WAT) in DIO mice. The full name and abbreviation of the gene of interest is given, in addition to the probe number. Probes were obtained from Applied Biosystems (UK).

For each rtPCR reaction, the following constituents were added to each well within a 384 well plate: 10 μ l TaqMan Universal PCR Master Mix, 1 μ l 20x TaqMan Gene Expression Probe, 1 μ l cDNA and 8 μ ls GDW. A gene expression assay targeting the 18S ribosomal subunit was used as a control (using 1:5 dilution of sample). Each reaction was mixed and centrifuged for 1 minute at 1000g at room temperature to remove bubbles from within the reaction mixture. Each plate was then loaded into an rtPCR

machine (7900HT Fast Real-Time PCR System, Applied Biosystems, Warrington, UK) and PCR performed under the following conditions:



Each probe was validated prior to experimental procedures, using a randomly selected sample from the relevant tissue and compared to 18S control. The amplification data for each target gene was normalised to the expression of the 18S endogenous control and expressed relative to an internal calibrator (vehicle group sample) using the $2^{-\Delta\Delta CT}$ method (SDS v2.3 software, Applied Biosystems, UK). This method allows data to be expressed as the fold change in gene expression normalised to a reference gene (18S) and relative to an internal untreated control.

Leptin

Leptin was measured using a mouse leptin ELISA kit (Crystal Chem Inc., USA) and the manufacturer's protocol was followed. The kit uses a sandwich-ELISA method (see figure 3.4), in which monoclonal rabbit anti-leptin antibodies are immobilised on the surface wells. Guinea-pig anti-mouse leptin IgG antibody is added to each well which simultaneously bind to the immobilised plate antibodies and mouse leptin in the sample (5 µl added), standard or control solutions. The plate is then incubated overnight at 4°C. The following day, a series of washes remove unbound material. Subsequently, streptavidin-horseradish peroxidase conjugated to anti-guinea pig IgG antibody is added which binds to the guinea pig anti-leptin IgG of the complex immobilised to the microplate well. After excess streptavidin-enzyme conjugate is washed away, a substrate for horseradish peroxidase is added (3,3', 5, 5'-tetramethylbenzidine). The peroxidase reaction is stopped by the addition of 1N sulphuric acid. Leptin concentration was measured spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) by the increased absorbancy at 450 and 630 nm. A standard curve was constructed and sample concentration derived from this (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

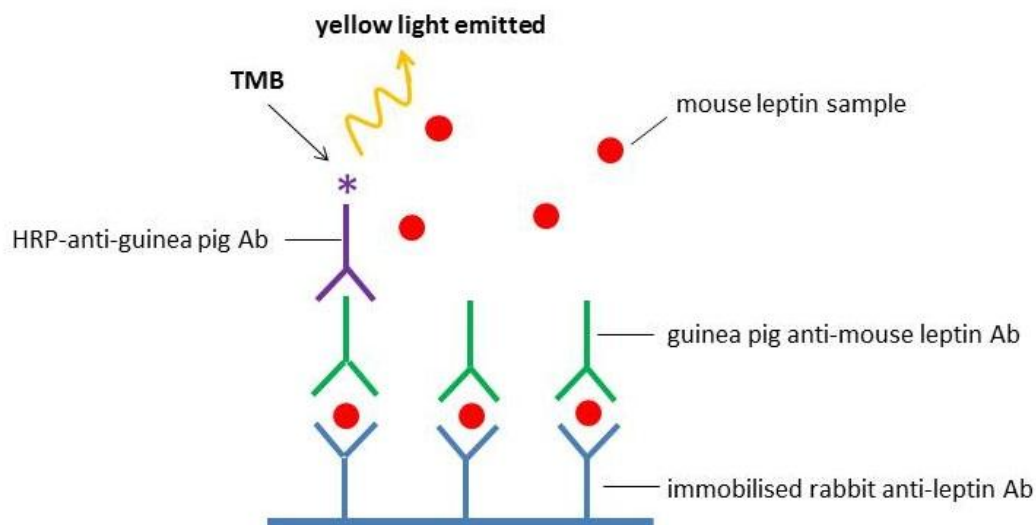


Figure 3.4. Schematic diagram representing mouse leptin ELISA assay protocol. Rabbit anti-leptin antibodies are immobilised on the well plate, to which guinea-pig anti-mouse leptin IgG antibodies and mouse leptin sample are added. HRP (horse radish peroxidase)-conjugated anti-guinea pig IgG antibody is pipetted into the wells. TMB (tetramethylbenzidine) is added and oxidised by the peroxidase present. The reaction is halted by the addition of sulphuric acid, causing the solution to turn yellow depending on the concentration of leptin present, and can be read using a spectrophotometer.

Free tri-iodothyronine (fT3)

fT3 was measured using a fT3 mouse ELISA assay (Cusabio Biotech, China) and the manufacturer's protocol was followed. The kit uses a competitive inhibition enzyme immunoassay technique, in which monoclonal anti-T3 antibodies are immobilised on the surface wells. These antibodies bind to T3 in the sample (50 μ l added), standard or control solutions. In addition biotin-conjugated T3 is added to the well and incubated for 1 hour. During this time, a competitive inhibition reaction occurs between T3 in the samples/standards and biotin conjugated T3 with the pre-coated antibody specific for T3. After washing, to remove unbound material, streptavidin-horseradish peroxidase is added which binds to biotin. After excess streptavidin-enzyme conjugate is washed away, a substrate for horseradish peroxidase is added (3,3', 5, 5'-tetramethylbenzidine). The peroxidase reaction is stopped by the addition of 0.3M HCl. Enzyme activity was measured spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) by the increased absorbancy at 450 nm. A standard curve was constructed and sample concentration derived from this (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

Triglycerides

Triglycerides were measured using a triglyceride quantification kit (Abcam, Cambridge, UK). Standard, sample (50 µl of 1:10 dilution) and controls were added to a 96 well plate. 2µl of lipase was added to each well and incubated for 20 mins to convert the triglycerides to glycerol and FFAs. The glycerol was then oxidised by the addition of a triglyceride reaction mix (OxiRed in DMSO and enzyme mix) and left to incubate for 1 hour. Enzyme activity was measured spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) by the increased absorbancy at 560 nm. A standard curve was constructed and sample concentration derived from this (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

β-hydroxybutyrate

β-hydroxybutyrate was measured using a quantification kit (Abcam, Cambridge, UK). Standard, sample (20 µl) and controls were added to a 96 well plate and the volume adjusted to 50 µls using the buffer provided. A further 50 µls of enzyme reaction mix was added to each well (containing 46 µls buffer, 2 µls hydroxybutyrate dehydrogenase and 2 µls substrate mix). Samples were incubated at room temperature for 30 mins and enzyme activity measured spectrophotometrically by the absorbancy at 450 nm (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA). A standard curve was constructed and sample concentration derived from this (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

3.3.3 Statistics

All data are expressed as mean ± SEM unless specified. Data from acute feeding studies, and c-fos immunohistochemistry were analysed using one-way ANOVA followed by Dunnett's or Tukey's post test as indicated. Data from the behaviour study were analysed using Kruskal-Wallis followed by Mann-Whitney U.

In the chronic studies, data from cumulative food intake, area under the curve (AUC) for glucose and insulin values, tissue mass, mRNA expression and plasma markers of energy homeostasis were analysed using one way ANOVA with Tukey's post test. Data from body weight, glucose and insulin values during the ipGTT were analysed using repeated measures two-way ANOVA with Bonferroni's post test. In all cases, values of $p < 0.05$ were considered statistically significant.

3.4 Results

3.4.1 Investigation of the acute effects of a long acting dual glucagon and GLP-1 analogue G(X) on food intake and c-fos immunoreactivity in the brainstem in mice.

3.4.1.1 Investigation of the acute effects of a long-acting glucagon and GLP-1 analogue G(X) on food intake in mice

50 nmol/kg of G(X) was the lowest effective dose to reduce food intake in the first 30 minutes (mean food intake: 0.41 ± 0.06 [saline] vs. 0.22 ± 0.05 g [G(X) 50 nmol/kg], $p < 0.05$ vs. saline; $n=5$ /group, fig 3.5). At this dose, the anorectic effect lasted three hours and caused a complete abolition of food intake (fig 3.5). Exendin-4 (10 nmol/kg) was used as a positive control at a dose previously shown to decrease food intake^{400,429}.

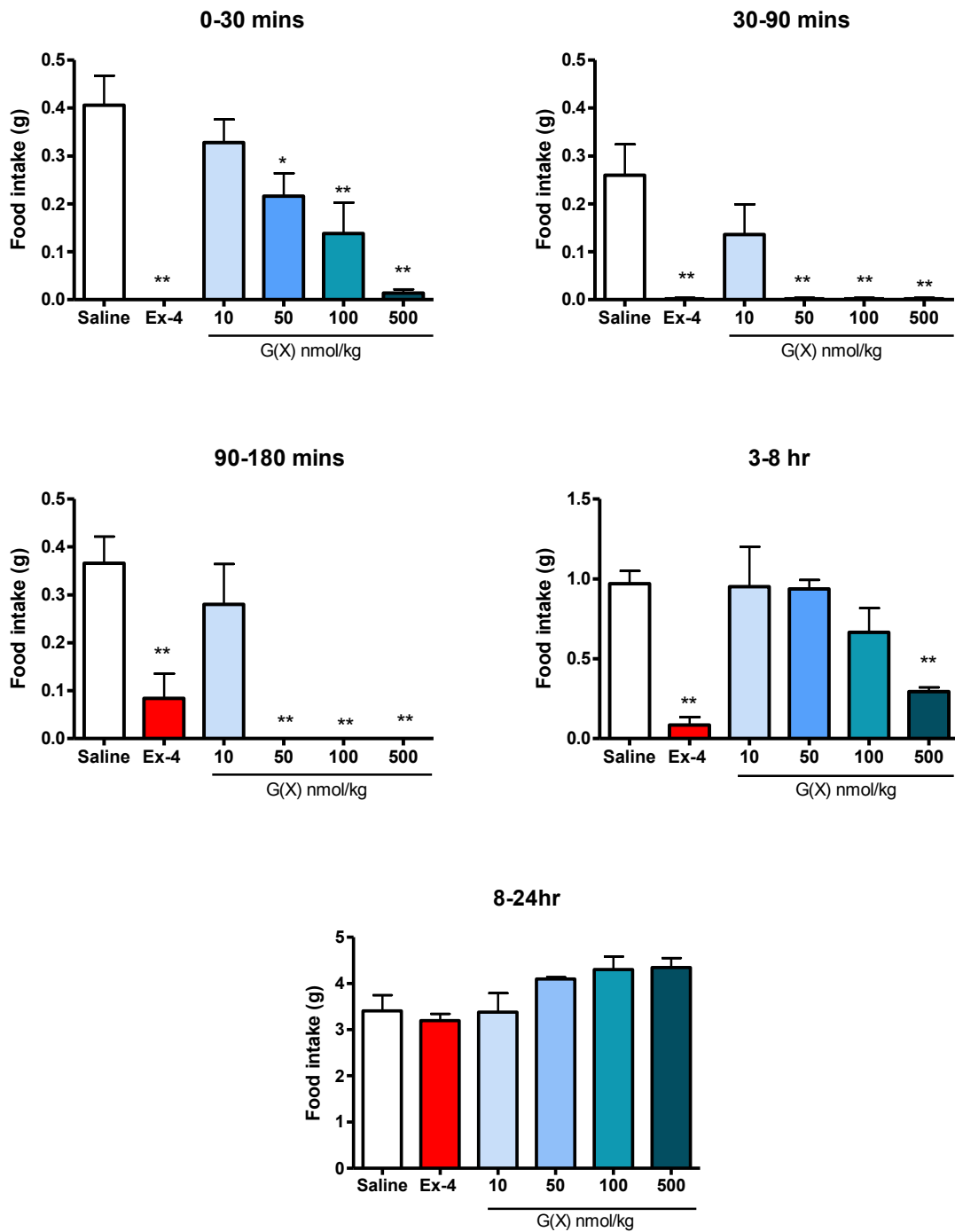


Figure 3.5. Effects of the long-acting glucagon/GLP-1 analogue G(X) on food intake. Food intake was measured following s/c administration of either saline (control), G(X) 10, 50, 100, 500 nmol/kg. Exendin-4 (10 nmol/kg) was used as a positive control. Food intake was measured at 30, 90, 180 minutes, 3 hours, 8 hours and 24 hours following injection. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Dunnett's post test. * $p < 0.05$, ** $p < 0.01$ versus controls. $n = 5$ /group.

3.4.1.2 Investigation of behaviour in mice following administration of a long-acting glucagon and GLP-1 analogue G(X)

A behavioural study was undertaken to ascertain whether the reduction in food intake seen following the administration of G(X) was due to adverse symptoms. *Ad libitum* fed mice were observed for 90 minutes following injection of glucagon, exendin-4 and G(X) at the onset of the light phase. LiCl was used as a positive control as it demonstrates adverse behaviour at high doses⁴²⁴. Exendin-4 was used rather than GLP-1 as it has a longer half life than GLP-1 and has been used in previously published behavioural studies⁴²⁹.

There was no evidence of major adverse behaviour in response to G(X) or glucagon at the doses administered.

0-30 minutes					
Behaviour	Saline	LiCl	Exendin-4 10 nmol/kg	Glucagon 750 nmol/kg	G(X) 50 nmol/kg
Feeding	0 (0-0)	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-0)
Drinking	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Rearing	1 (1-2)	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-2)
Locomotion	4 (4-5)	3 (2-4)	2 (1-6)	5 (3-5)	7 (4-8)
Grooming	5 (4-8)	1 (0-1)*	3 (2-3)	5 (2-6)	2 (2-3)
Burrowing	3 (0-3)	0 (0-0)	0 (0-0)	4 (0-6)	2 (2-3)
Hunching	0 (0-0)	11 (10-12)**	3 (0-9)	0 (0-2)	0 (0-0)
Sleeping	0 (0-3)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Pica	0 (0-2)	0 (0-0)	0 (0-0)	0 (0-0)	3 (0-3)
Inactive	1 (1-2)	2 (1-4)	4 (3-7)*	2 (2-4)	1 (1-3)

30-60 minutes					
Behaviour	Saline	LiCl	Exendin-4 10 nmol/kg	Glucagon 750 nmol/kg	G(X) 50 nmol/kg
Feeding	0 (0-0)	3 (1-5)	0 (0-2)	3 (3-3)	3 (0-3)
Drinking	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Rearing	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Locomotion	0 (0-2)	1 (1-2)	1 (0-1)	5 (2-5)*	0 (0-0)
Grooming	0 (0-0)	4 (2-5)*	0 (0-0)	1 (0-2)	0 (0-1)
Burrowing	0 (0-1)	0 (0-0)	0 (0-0)	5 (3-6)	1 (1-2)
Hunching	0 (0-0)	3 (2-6)	9 (4-12)	0 (0-0)	0 (0-3)
Sleeping	3 (0-8)	0 (0-0)	0 (0-0)	0 (0-0)	7 (0-9)
Pica	5 (0-6)	2 (2-3)	1 (0-3)	5 (1-5)	1 (0-2)
Inactive	1 (0-4)	2 (1-3)	4 (0-6)	0 (0-0)	2 (1-7)

60-90 minutes					
Behaviour	Saline	LiCl	Exendin-4 10 nmol/kg	Glucagon 750 nmol/kg	G(X) 50 nmol/kg
Feeding	0 (0-0)	6 (3-6)*	0 (0-0)	0 (0-0)	0 (0-0)
Drinking	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Rearing	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-0)
Locomotion	0 (0-0)	1 (0-1)	0 (0-0)	0 (0-0)	0 (0-3)
Grooming	0 (0-1)	3 (0-3)	0 (0-0)	3 (3-4)**	0 (0-0)
Burrowing	0 (0-0)	2 (0-3)	0 (0-0)	0 (0-0)	0 (0-0)
Hunching	0 (0-0)	0 (0-0)	3 (3-3)	0 (0-0)	0 (0-0)
Sleeping	9(9-17)	0 (0-0)**	6 (6-9)	12 (12-15)	18(10-18)
Pica	0 (0-3)	4 (3-6)	0 (0-0)	0 (0-1)	0 (0-0)
Inactive	0 (0-0)	3 (3-3)	6 (0-7)	0 (0-0)	0 (0-0)

Table 3.2 Behavioural study in fed mice at the onset of the light phase (0800-1000). ip injections of either saline (controls), LiCl (127 mg/kg), exendin-4 (10 nmol/kg), glucagon 750 nmol/kg or G(X) 50 nmol/kg were performed and mice returned immediately to their cages. Behaviour was recorded for 15 seconds every 5 minutes, continuously for 90 minutes. Data from this study was subdivided into 0-30 minutes, 30-60 minutes and 60-90 minutes. Data presented as median (interquartile range). Adverse behaviour (pica, hunching and inactivity) highlighted in red. Data were analysed using Kruskal-Wallis followed by Mann-Whitney U, *p<0.05, ** p<0.01 versus saline treated control. n=5/group.

3.4.1.3 Investigation of the acute effects of co-administration of exendin₍₉₋₃₉₎ and a long-acting glucagon and GLP-1 analogue G(X) on food intake in mice

In order to establish how much of the anorectic effect of the glucagon/GLP-1 analogue G(X) was due to GLP-1 receptor activation, the GLP-1 antagonist exendin₍₉₋₃₉₎ was administered prior to injection of G(X). Exendin₍₉₋₃₉₎ (25000 nmol/kg) attenuated the anorectic effect of G(X) between 30-90 minutes following administration: (mean food intake: G(X): 0.06 ± 0.01 [G(X) 50 nmol/kg] vs. 0.51 ± 0.07 g [G(X) and exendin₍₉₋₃₉₎ combined], $p < 0.05$ vs. G(X), $n=6$ per group, fig. 3.6).

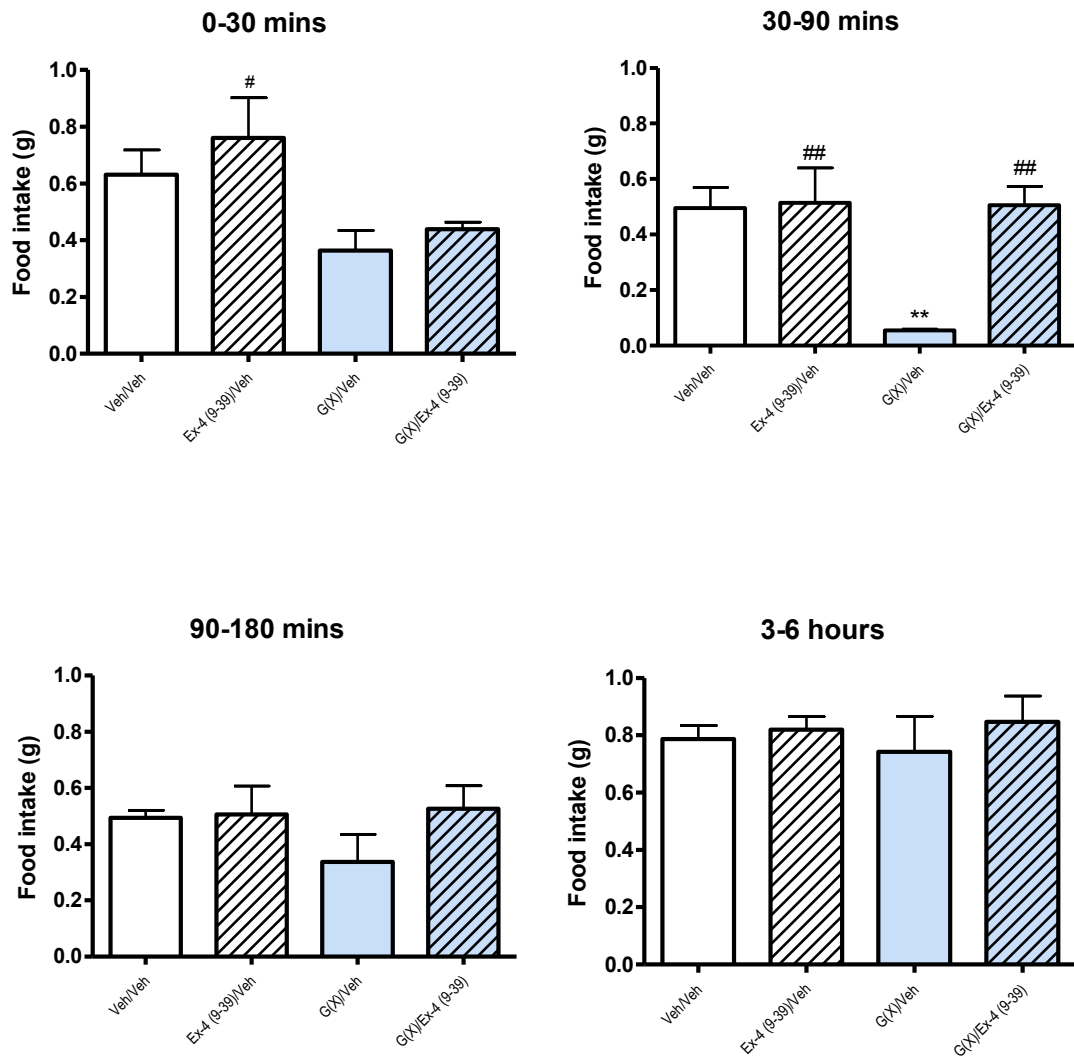


Figure. 3.6. Effect of exendin₍₉₋₃₉₎ on food intake following administration of glucagon/GLP-1 analogue G(X). The study took place at the onset of the light phase (0800-1000) in overnight fasted mice. Exendin₍₉₋₃₉₎ (25000 nmol/kg s/c) or vehicle was injected 5 minutes prior to injection of either vehicle or G(X) (50 nmol/kg). Food intake was measured at 0.5, 1.5, 3 and 6 hours following the second injection. Shaded bars illustrate mice pre-treated with exendin₍₉₋₃₉₎. Results expressed as mean $\pm$ SEM. Data were analysed using one way ANOVA with Tukey's post test. ** $p < 0.01$, versus controls, # $p < 0.05$, ## $p < 0.01$ versus G(X) alone. $n = 6$ /group.

3.4.1.4 Investigation of c-fos immunoreactivity in the brainstem following administration of a long-acting glucagon and GLP-1 analogue G(X).

c-fos-ir was assessed in the brainstem of mice following injection of the glucagon/GLP-1 analogue G(X). The study was performed in fasted mice at the onset of the light phase. Glucagon and GLP-1 were used as positive controls at doses shown to increase c-fos-ir in previous studies. Glucagon and GLP-1 both increased c-fos-ir in the AP and cNTS at the doses administered (mean c-fos counts: AP: 25 ± 4 [saline] vs. 92 ± 14 [GLP-1], $p < 0.05$ vs. saline; vs. 84 ± 18 [glucagon], $p < 0.05$ vs. saline, $n = 5/\text{gp}$; cNTS: 62 ± 22 [saline] vs. 213 ± 22 [GLP-1], $p < 0.05$ vs. saline; vs. 218 ± 43 [glucagon], $p < 0.05$ vs. saline, $n = 5/\text{gp}$, fig. 3.7). Following administration of G(X), there was an increase in c-fos-ir in the AP compared to saline controls, however this did not reach significance. There were no significant differences between saline and G(X) in the cNTS or PBN.

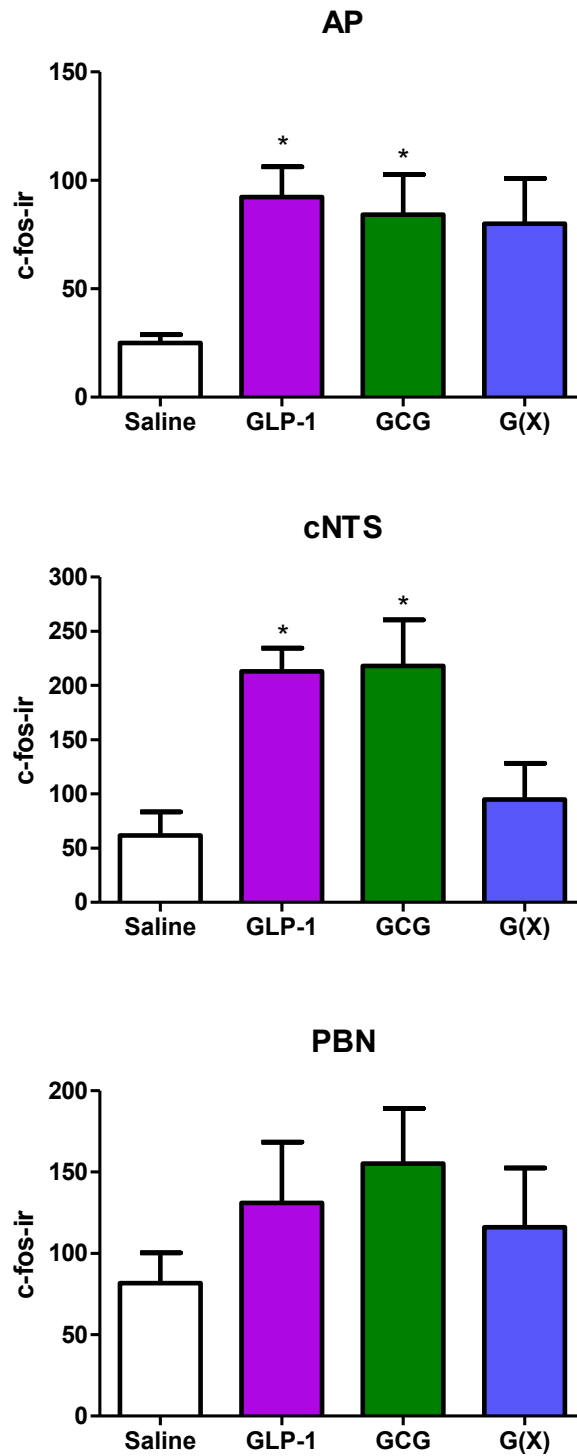


Figure 3.7. c-fos immunoreactivity in brainstem following administration of G(X). Mice were fasted overnight and injected with either saline (controls), glucagon (GCG) (750 nmol/kg), GLP-1 (100 nmol/kg) or G(X) (50 nmol/kg) at the onset of the light phase (0800-1000). Animals were decapitated and brains removed 90 minutes following injection. c-fos-ir is shown in the area postrema (AP), caudal nucleus tractus solitarius (cNTS) and parabrachial nucleus (PBN) of the brainstem. Data were analysed using one-way ANOVA with Dunnett's post test. * $p < 0.05$ versus saline controls. $n = 4-5$ /group.

3.4.2 Investigation of the chronic effects of long acting glucagon and GLP-1 analogues G(Y) and G(Z) on food intake and body weight in diet induced obese (DIO) mice.

3.4.2.1 Investigation of the effects of chronic administration of long acting glucagon and GLP-1 analogues on food intake and body weight

G(X) demonstrated an acute anorectic effect in mice. However, it bound and activated the GCGR and GLP-1R with poor affinity (Appendix B). Therefore, interpretation of these findings and identifying which receptor the anorectic effect was attributable to was difficult. My aim was to investigate the chronic effects of GCGR and GLP-1R agonism separately and in combination on food intake, glucose homeostasis and EE. Therefore, G(Y) and G(Z) were designed to bind and activate predominantly the GLP-1R and GCGR respectively. Both analogues were injected once daily alone and in combination for 70 days. There were no significant differences in cumulative food intake compared to controls (Fig 3.8). Neither G(Y) or G(Z) alone significantly reduced body weight compared to controls. However, the combination of G(Y) and G(Z) significantly reduced body weight compared to saline controls from day 30 onwards despite no change in cumulative food intake (mean body weight change: 0.13 ± 0.45 [vehicle] vs. -2.00 ± 0.46 g [G(Y)+G(Z)], $p < 0.05$ vs. vehicle, $n = 10-12$ /group, fig 3.9). In addition, co-administration of G(Y) and G(Z) significantly decreased body weight compared to G(Y) and G(Z) alone from day 61 onwards.

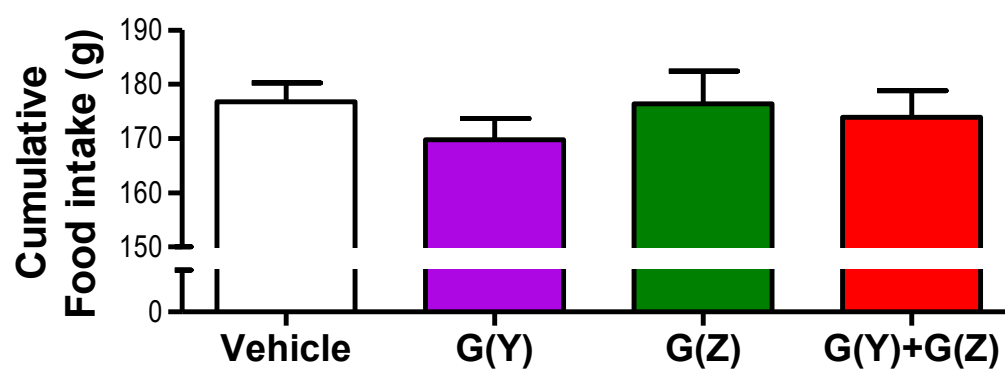


Figure 3.8 Cumulative food intake in DIO mice following chronic administration of G(Y) and G(Z) alone and in combination. Data were analysed using one way ANOVA with Tukey post test. (n=10-12/group).

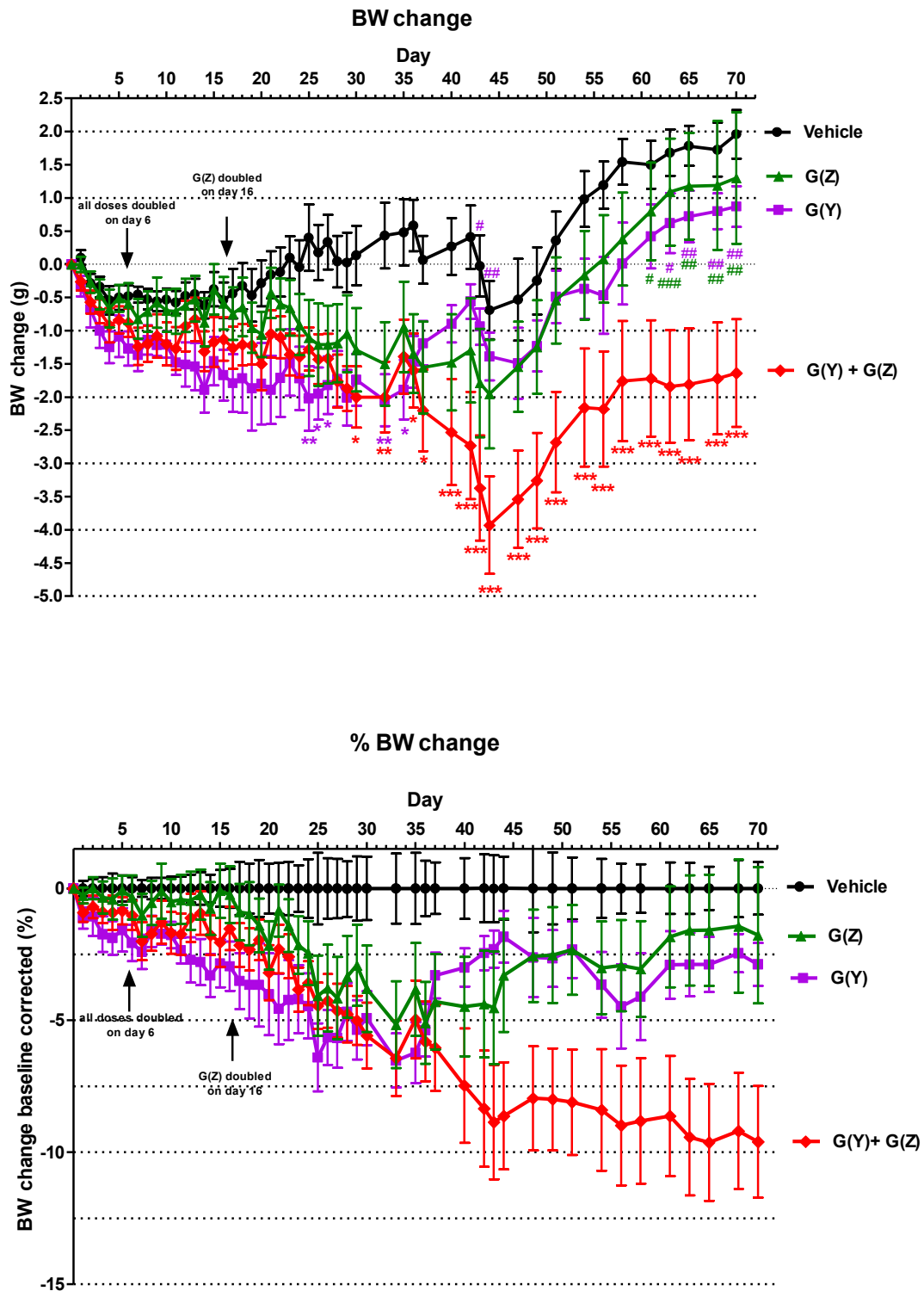


Figure 3.9 Body weight in DIO mice following chronic administration of G(Y) and G(Z) alone and in combination. Mice were injected daily with vehicle/vehicle, G(Y) (5nmol/kg day 0-5, 10 nmol/kg day 6 onwards)/vehicle, G(Z) (20 nmol/kg day 0-5, 40 nmol/kg day 6-15, 80 nmol/kg day 16 onwards)/vehicle or G(Y)/G(Z) at the same doses and body weight (BW) measured every 1-2 days for 70 days. BW and %BW change are shown. Data were analysed using two way repeated measures ANOVA with Bonferroni post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus G(Y) and G(Z) in combination. (n=10-12/group).

3.4.2.2 Investigation of glucose homeostasis following chronic administration of long acting glucagon and GLP-1 analogues.

Fasting plasma glucose values were lowest in the combined G(Y) and G(Z) group and highest in the vehicle group although this did not reach statistical significance (mean glucose: 8.52 ± 0.49 [vehicle] vs. 3.63 ± 0.12 mmol/l [G(Y) + G(Z)], $n=10-12$ /group, fig. 3.10, $t=-30$). Following ip glucose administration, DIO mice injected with vehicle showed a rise in glucose from 8.35 ± 0.6 mmol/l ($T=0$) to 17.7 ± 1.1 mmol/l ($t=30$ mins). Mice administered G(Y) and G(Z) alone showed a slight rise in glucose but all glucose measurements following injection were significantly lower than the vehicle controls. In contrast, plasma glucose did not change following a glucose load in mice co-administered G(Y) and G(Z) (mean AUC: 2291 ± 221 [vehicle] vs. 1362 ± 137 [G(Y)], $p<0.001$; vs. 1192 ± 147 [G(Z)], $p<0.001$; vs. 672 ± 27 [G(Y)+G(Z)], $p<0.001$, $n=10-12$ /group, fig 3.10).

Samples were also collected for serum insulin measurement at $t=0$, 30, 60 and 120 minutes following glucose administration. Fasting insulin was highest in the vehicle and G(Y) groups suggesting insulin resistance whereas it was lowest in the combined G(Y) and G(Z) group (mean insulin: 10.2 ± 1.3 [vehicle] vs. 2.9 ± 0.6 ng/ml [G(Y)+G(Z)], $p<0.001$; vs. 9.4 ± 1.6 [G(Y)], $p>0.05$; $n=10-12$ /group, fig 3.11). G(Z) administration significantly lowered plasma insulin levels compared to vehicle (mean AUC: 1054 ± 144 [vehicle] vs. 480 ± 66 [G(Z)], $p<0.05$, $n=10-12$ /group, fig 3.11).

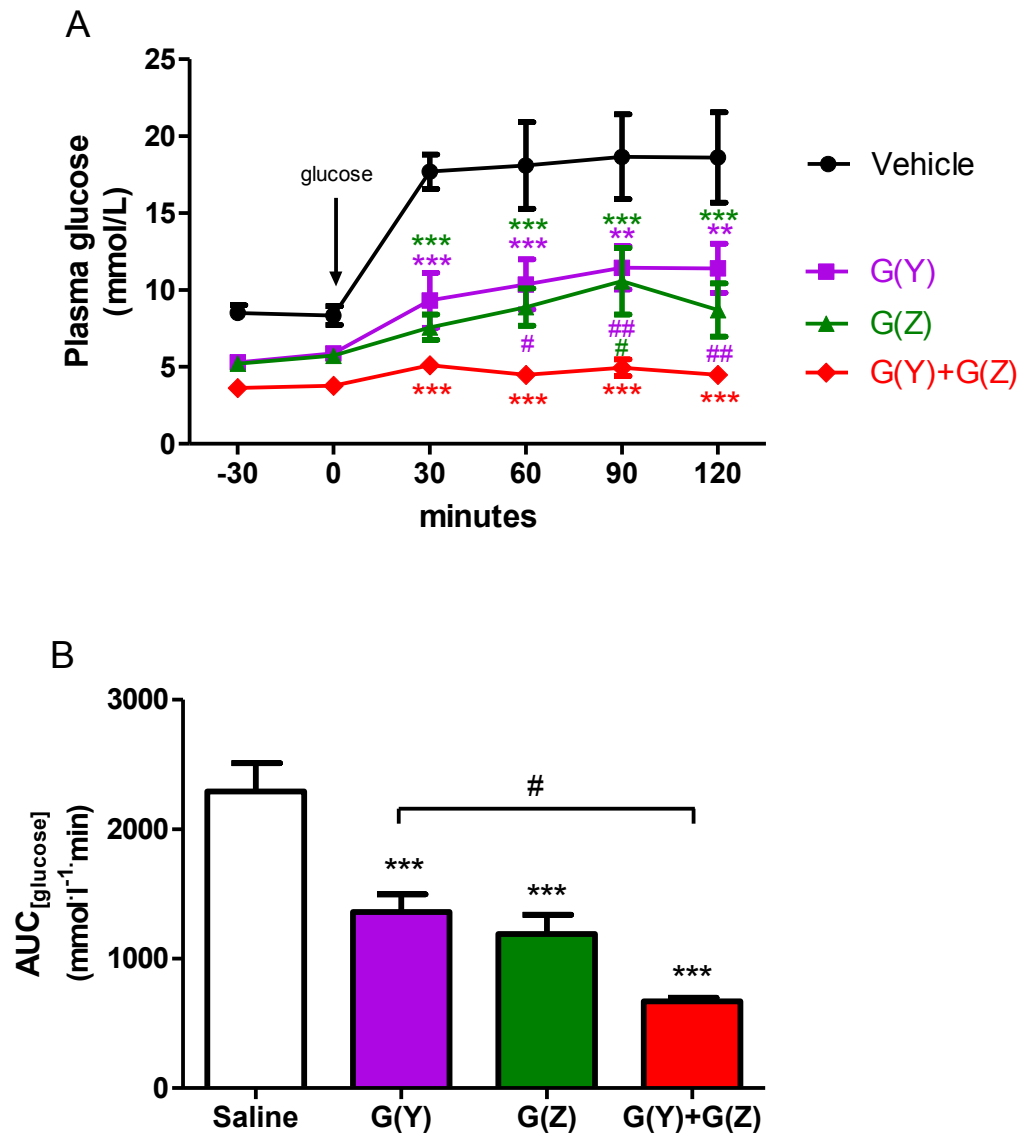


Figure 3.10 Intraperitoneal glucose tolerance test following chronic administration of G(Y) and G(Z) alone and in combination. A: Plasma glucose measurements (mmol/l) and B: Area under the curve (AUC). *Ad libitum* fed mice were injected at 0700 on the day of the study and fasted until the ipGTT at 1300. Blood samples were collected at -30, 0, 30, 60, 90 and 120 minutes following ip glucose administration. Plasma glucose measurements shown in fig. 3.11A: data were analysed using two way repeated measures ANOVA with Bonferroni's post test. ** $p < 0.01$, *** $p < 0.001$ versus saline controls. # $p < 0.05$, ## $p < 0.01$ versus G(Y) and G(Z) in combination. AUC shown in fig. 3.10B: data were analysed using one way ANOVA with Tukey's post test. *** $p < 0.001$ versus saline controls, # $p < 0.05$ versus G(Y) and G(Z) in combination. $n = 10-12$ /group.

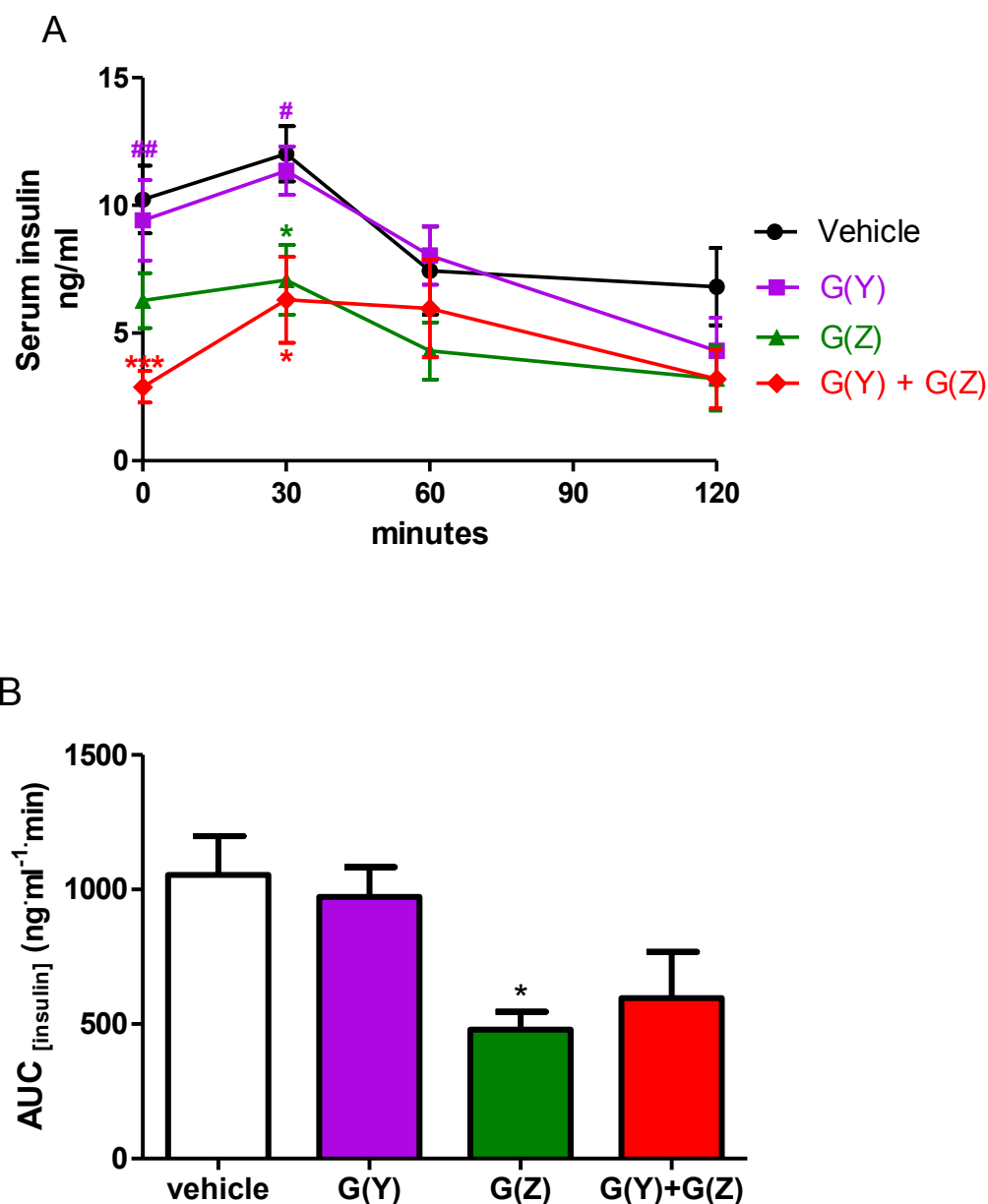


Figure 3.11 Insulin values following intraperitoneal (ip) glucose tolerance test on day 70 following chronic administration of G(Y) and G(Z) alone and in combination. A: Serum insulin measurements (ng/ml) and B: Area under the curve (AUC). Insulin samples were taken at T=0, 30, 60 and 120 minutes following ip glucose. Insulin values and AUC are shown. Insulin measurements were analysed using two way repeated measures ANOVA with Bonferroni's post test. *p<0.05, ***p<0.001 versus saline controls. #p<0.05, ##p<0.01 versus G(Y) and G(Z) in combination. AUC data were analysed using one way ANOVA with Tukey's post test. *p<0.05 versus saline controls. n=10-12/group.

3.4.2.3 Investigation of the effects of chronic administration of long acting glucagon and GLP-1 analogues on markers of energy expenditure and gluconeogenesis.

3.4.2.3.1 The effects of chronic administration of long acting glucagon and GLP-1 analogues on liver, brown adipose (BAT) and white adipose (WAT) tissue mass.

Chronic administration of G(Y) significantly decreased liver weight compared to vehicle (mean liver weight as % of total body weight: 5.8 ± 0.3 [vehicle] vs. $4.2 \pm 0.2\%$ [G(Y)], $p < 0.001$ vs. vehicle, $n = 10-12$ /group, fig. 3.12A). There was no significant difference in liver weight between G(Z) or G(Y)+G(Z) and vehicle controls.

G(Z) alone and in combination with G(Y) decreased BAT mass compared to vehicle whereas G(Y) alone had no effect (mean BAT weight as % of total body weight: 0.86 ± 0.03 [vehicle] vs. $0.67 \pm 0.05\%$ [G(Z)], $p < 0.05$ vs. vehicle; vs. $0.62 \pm 0.03\%$ [G(Y)+G(Z)], $p < 0.01$ vs. vehicle; $n = 10-12$ /group, fig. 3.12B).

G(Y) alone and in combination with G(Z) increased WAT mass compared to vehicle (mean WAT weight as % of total body weight: 2.2 ± 0.1 [vehicle] vs. $2.9 \pm 0.2\%$ [G(Y)], $p < 0.05$ vs. vehicle; vs. $3.0 \pm 0.2\%$ [G(Y)+G(Z)], $p < 0.05$ vs. vehicle; $n = 10-12$ /group, fig 3.12C).

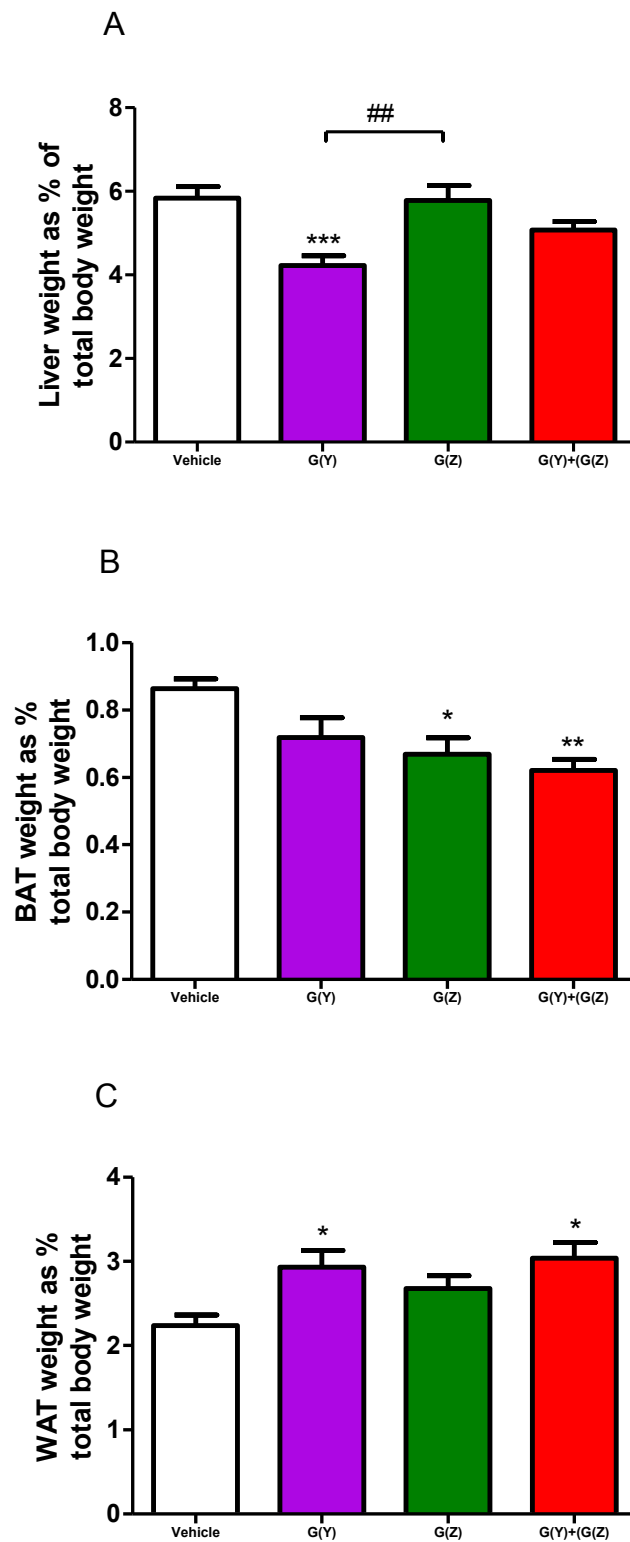


Figure 3.12. Liver (A), brown adipose tissue (B) and white adipose tissue (C) mass following chronic administration of GLP-1 and glucagon analogues. Liver, brown adipose tissue (BAT) and white adipose tissue (WAT) were harvested from DIO mice following chronic administration of GLP-1 and glucagon long-acting analogues alone and in combination. Data were analysed using one-way ANOVA with Tukey post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle; ## $p < 0.01$ vs. G(Y). N=10-12/group.

3.4.2.3.2 The effects of chronic administration of long acting glucagon and GLP-1 analogues on gene expression in liver, brown adipose (BAT) and white adipose tissue (WAT).

There were no significant differences in mRNA expression in liver, BAT or WAT between any groups (figures 3.13, 3.14 and 3.15). In liver, FGF-21 expression was almost 2.5-fold higher following co-administration of G(Y) and G(Z) compared to vehicle however this did not reach statistical significance (mRNA expression: 0.96 ± 0.12 [vehicle] vs. 2.33 ± 0.52 AU [FGF-21], $p=ns$ vs. vehicle, $n=7-10$ /group, fig. 3.13 B).

In WAT, UCP-1 expression following administration of G(Y) increased over 2-fold. However, the number of WAT samples that could be interpreted following rtPCR were low due to inadequate mRNA extraction and this difference did not reach statistical significance (figure 3.15A).

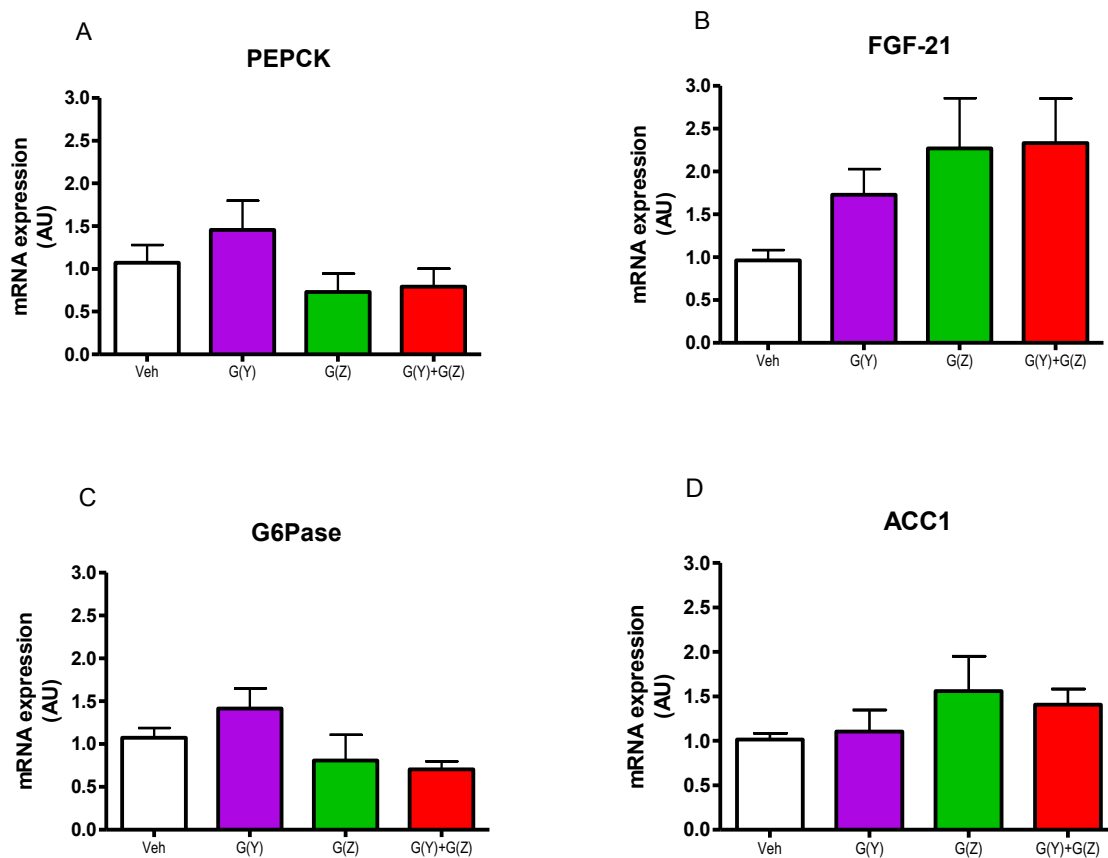


Figure 3.13 mRNA expression in liver following chronic administration of G(Y) and G(Z) alone and in combination in DIO mice. Relative mRNA expression of phosphoenolpyruvate carboxykinase 1 (PEPCK) (A), fibroblast growth factor-21 (FGF-21) (B), glucose-6-phosphatase (G-6-Pase) (C) and acetyl-coenzyme A carboxylase alpha (ACC1) (D) in liver samples. Data were analysed using one-way ANOVA with Tukey's post test. n=7-10/group.

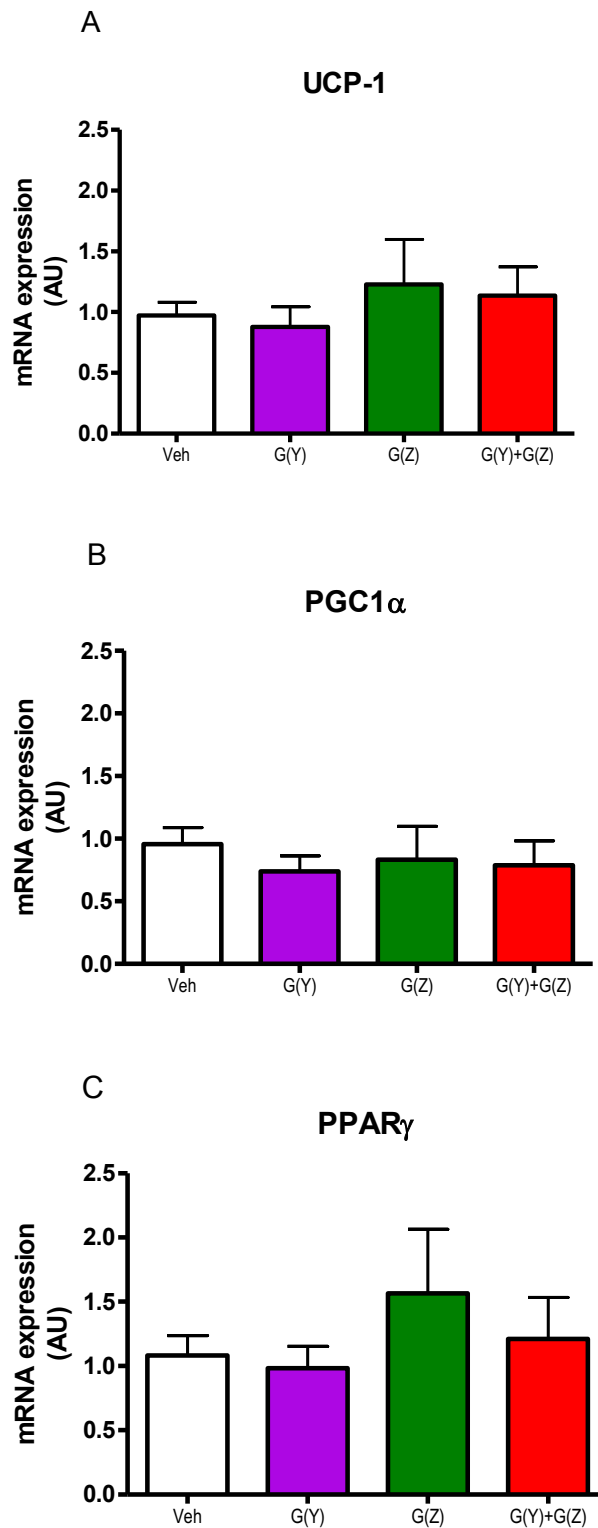


Figure 3.14 mRNA expression in brown adipose tissue following chronic administration of G(Y) and G(Z) alone and in combination in DIO mice. Relative mRNA expression of uncoupling protein-1 (UCP-1) (A), peroxisome proliferative activated receptor gamma co-activator 1 alpha (PGC1 α) (B), peroxisome proliferative activated receptor gamma (PPAR γ) (C) in brown adipose tissue. Data were analysed using one-way ANOVA with Tukey's post test. n=8-10/group.

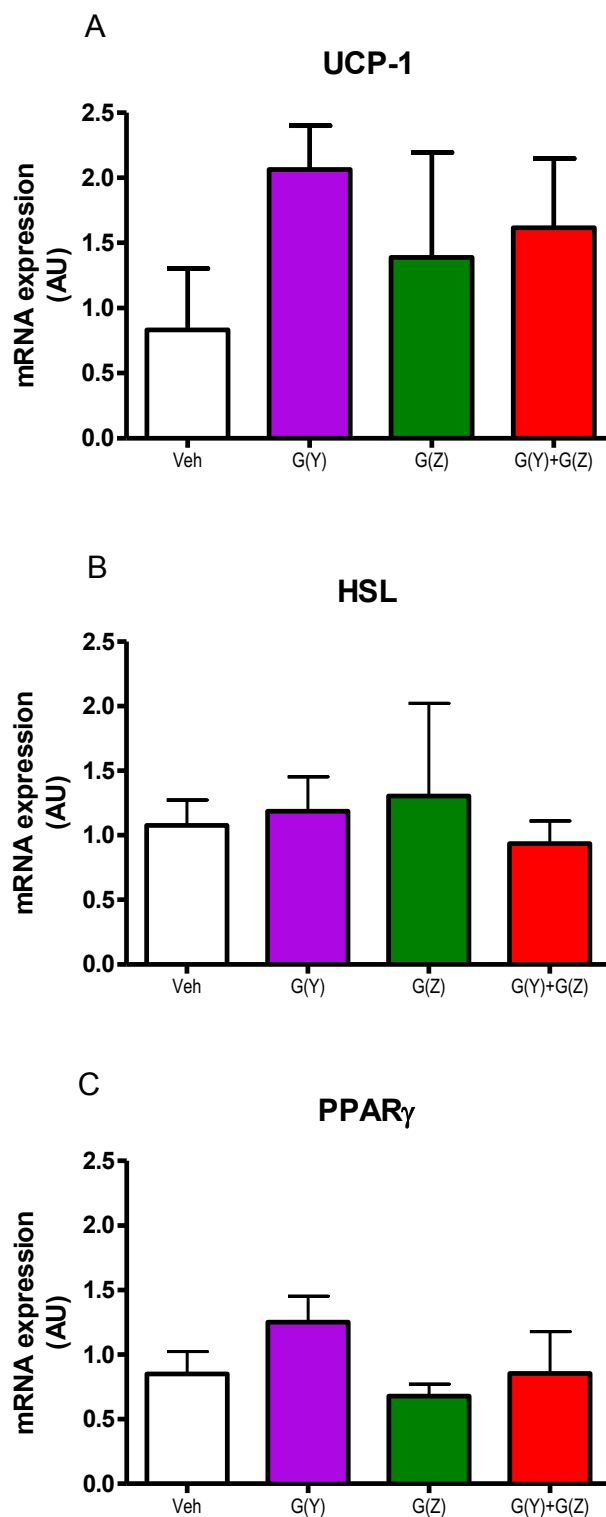


Figure 3.15 mRNA expression in white adipose tissue following chronic administration of G(Y) and G(Z) alone and in combination in DIO mice. Relative mRNA expression of uncoupling protein-1 (UCP-1) (A), hormone sensitive lipase (HSL) (B), peroxisome proliferative activated receptor gamma (PPAR γ) (C) in white adipose tissue. Data were analysed using one-way ANOVA with Tukey's post test. n=2-6/group.

3.4.2.3.3 The effects of chronic administration of long acting glucagon and GLP-1 analogues on circulating leptin, free tri-iodothyronine, triglycerides and β -hydroxybutyrate.

Chronic administration of G(Z) alone and in combination with G(Y) significantly decreased circulating leptin concentration (mean leptin concentration: 34.6 ± 0.4 [vehicle] vs. 29.2 ± 2.1 ng/ml [G(Z)], $p < 0.05$ vs. vehicle; vs. 28.4 ± 1.0 ng/ml [G(Y)+G(Z)], $p < 0.01$ vs. vehicle; $n = 10-11$ /group, fig. 3.16A).

There were no significant differences in free tri-iodothyronine (fT3) (fig. 3.16B), triglycerides (fig. 3.16C) or β -hydroxybutyrate (fig. 3.16D) between any of the groups. Although there was a trend for reduced triglyceride concentration in response to G(Y) administration, this did not reach statistical significance (mean triglyceride concentration: 2.2 ± 0.2 [vehicle] vs. 1.7 ± 0.3 [G(Y)], $p = \text{ns}$ vs. vehicle, $n = 10-11$ /group, fig. 3.16C).

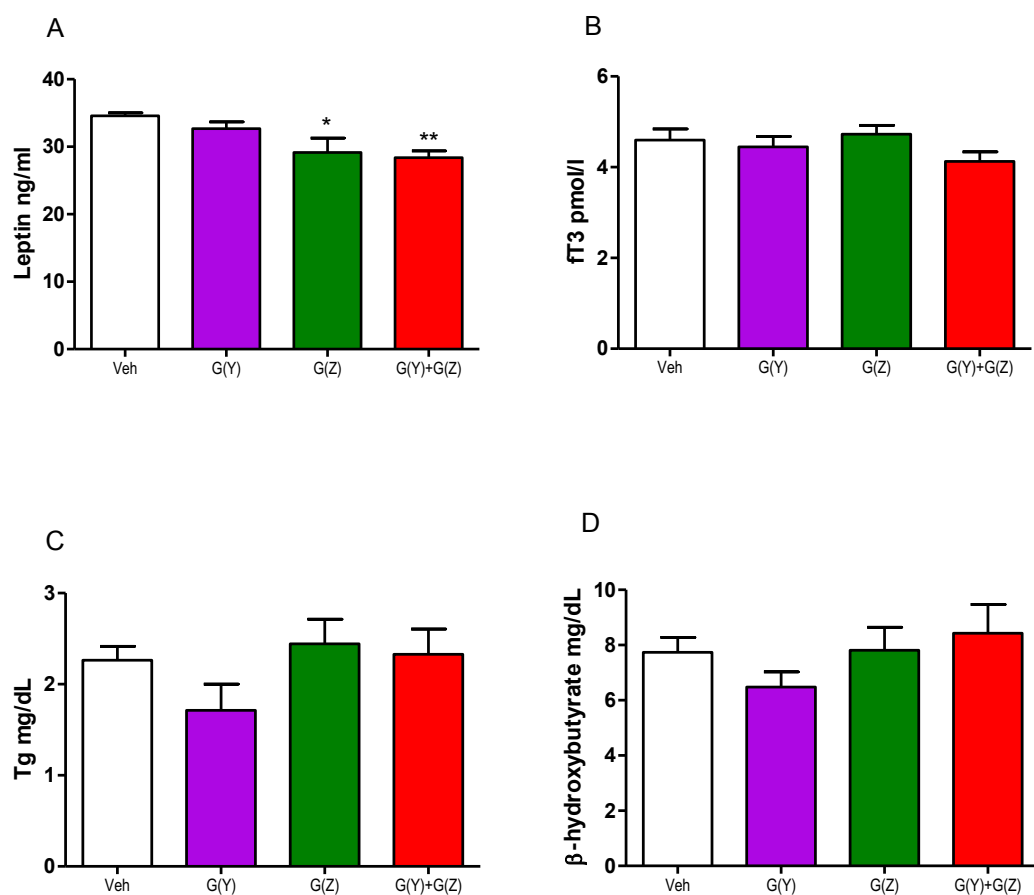


Figure 3.16 The effects of chronic administration of G(Y) and G(Z) alone and in combination on circulating concentration of leptin (A), free tri-iodothyronine (fT3) (B), triglycerides (Tg) (C) and β -hydroxybutyrate (D). Markers of energy homeostasis were measured from plasma samples in DIO mice following chronic administration of G(Y) and G(Z) alone or in combination. Data were analysed using one-way ANOVA with Tukey's post test. * $p < 0.05$, ** $p < 0.01$ vs. vehicle. $n = 10-11$ /group.

3.5 Discussion

These studies demonstrate that a dual glucagon/GLP-1 analogue, G(X), reduces food intake for up to eight hours with no evidence of major adverse behaviour. Despite this, no significant increase in c-fos-ir was detected in the brainstem. The mechanism through which G(X) decreases food intake is unclear. Despite similar amino acid sequences to native glucagon and GLP-1, previous studies showed that it displayed poor binding and activation at both the GCGR and GLP-1R. The anorectic effect may be mediated via a different receptor, although the ability of exendin-4₍₉₋₃₉₎ to almost completely inhibit the anorectic effect suggests action of G(X) at the GLP-1R. Alternatively, G(X) may act as an allosteric modulator at the GLP-1R. Recent evidence shows that allosteric modulation at the GLP-1R can have profound effects on downstream signalling. Small molecule GLP-1R modulators have been identified which enhance the insulinotropic effect of OXM at the GLP-1R⁴³⁰. It is perhaps surprising that G(X) did not cause significant c-fos activation in the brainstem despite a potent anorectic effect. Hypothalamic c-fos activation in response to G(X) administration was not studied since glucagon and GLP-1 alone did not increase c-fos-ir in any hypothalamic nuclei studied. However, activation of an alternative pathway by G(X) to reduce food intake cannot be excluded and may involve hypothalamic appetite circuits.

Due to the discrepancy between receptor binding and activation and anorectic effect following G(X) administration, and the finding that G(X) was less potent than exendin-4 at reducing food intake, two further analogues, G(Y) and G(Z) were designed with greater binding affinity and activation at the GLP-1R and GCGR respectively. These analogues were administered to DIO mice for 70 days, followed by an ipGTT to assess glucose homeostasis. In combination, G(Y) and G(Z) significantly decreased body weight without reducing food intake compared to controls, suggesting increased EE. In addition, the combination of G(Y) and G(Z) improves glucose homeostasis, although this may reflect the weight loss achieved. To further investigate the effects on EE and glucose homeostasis, tissues were harvested from the DIO mice and rtPCR undertaken to look at specific gene markers of EE.

There were no significant differences in markers of EE between any of the groups studied. Unexpectedly, BAT mass was reduced following chronic administration of G(Z) alone and in combination with G(Y). This is in contrast to acute administration of glucagon in rats which has been shown to increase BAT mass²¹⁸. These findings suggest that chronic G(Z) administration does not increase EE through BAT thermogenesis, but

through an alternative mechanism. My findings also do not support a role for WAT 'browning' as a potential mechanism for increased EE in response to G(Z), since WAT UCP-1 expression was not increased. However, interpretation of mRNA expression in WAT in the current study is limited due to small sample numbers. A large number of cycles during rtPCR were required in order to reach the threshold of detection of WAT mRNA for any of the genes studied, suggesting inadequate mRNA in WAT despite quantification during the mRNA extraction process. It is possible that mRNA loss occurred during ethanol precipitation or during reverse transcription into cDNA.

Interestingly, the glucagon analogue G(Z), ameliorated rather than worsened glucose control compared to controls and was similar to the GLP-1 analogue G(Y). This was despite no significant change in body weight or food intake compared to controls or G(Y) treated group suggesting an alternative mechanism for improved glucose homeostasis. In addition, G(Z)-treated mice had lower plasma insulin levels following a glucose load compared to controls or G(Y) treated mice, suggesting improved insulin sensitivity. This is in contrast to findings by other groups, in which antagonism at the GCGR has been targeted to ameliorate glucose control. Administration of glucagon-neutralising antibodies to leptin deficient *ob/ob* mice acutely decreases blood glucose²⁰². Reduction in GCGR expression using antisense oligonucleotides improved glucose profiles in leptin receptor deficient *db/db* mice and Zucker diabetic rats^{203,204}. Furthermore, small molecule GCGR antagonists reduce glucagon-induced hyperglycaemia in both rodents and humans^{431,432}. Finally, long term (18 weeks) inhibition of the GCGR using a monoclonal antibody normalises blood glucose in DIO mice^{205,433}. Interestingly, this effect appears to be dependent on a functional GLP-1 receptor, since the improved glucose tolerance following ipGTT, is diminished by exendin₍₉₋₃₉₎ and in GLP-1R knockout mice⁴³³.

It is possible that chronic glucagon analogue administration, at the dose used in the current studies, either upregulates GLP-1 secretion or binds to the GLP-1R. However, previous studies have shown that GCGR antagonism results in a compensatory rise of circulating glucagon and GLP-1^{205,434}. In mice, the compensatory rise in glucagon and GLP-1 is thought to be due to pancreatic α -cell hyperplasia, upregulating preproglucagon expression and hence increasing active GLP-1 secretion^{204,205}. Similarly, GCGR knockout mice have increased circulating glucagon, increased pancreatic α cell hyperplasia and improved glucose tolerance⁴³⁵. Therefore, antagonism at the GCGR causes a paradoxical rise in circulating glucagon and GLP-1 and improved glucose control.

In contrast, chronically elevated circulating glucagon may cause GCGR receptor downregulation, with a similar compensatory rise in preproglucagon expression. Hepatic glucagon resistance has been shown to occur in rats fed a high fat diet^{436,437}. DIO reduces the number of hepatic GCGRs, and results in a diminished response to glucagon during a hyperglucagonaemic clamp⁴³⁷. Furthermore, *in vitro* studies using hepatocytes have shown that chronic administration of glucagon decreases the adenylyl cyclase response over time suggesting desensitisation⁴³⁸. These findings are in contrast to the current studies which demonstrate lower circulating glucose levels and improved insulin sensitivity in mice following 70 days of G(Z) administration with similar body weight and food intake to controls. Despite these findings, there were no significant changes in PEPCCK or G6Pase mRNA expression in the liver in response to G(Z). Glucagon upregulates G6Pase and PEPCCK transcription in the liver both of which are key mediators of gluconeogenesis⁴¹⁰. This suggests that the improvement in glucose homeostasis shown in the current study is not due to downregulation of PEPCCK or G6Pase.

Administration of G(Z) alone reduced circulating leptin concentration compared to vehicle, despite no significant reduction in body weight. This implies that glucagon reduces adiposity, thereby increasing proportional lean body mass. Although, not investigated in the current study, this could be assessed using whole body MRI and quantification of adiposity⁴³⁹. The combination of G(Y) and G(Z) significantly decreased body weight and circulating leptin concentration. This weight loss occurred despite similar food intake to vehicle, G(Y) or G(Z) alone, suggesting an increase in EE. It is therefore surprising that G(Z) alone acting predominantly through the GCGR did not significantly reduce body weight through increased EE. G(Z) may have been administered at an insufficient dose to achieve weight loss, however in combination with G(Y), a synergistic effect may have occurred. Synergism between two drugs implies a potentiated effect that is significantly greater than the added effects of the two compounds alone⁴⁴⁰. Synergy usually implies multiple sites of action of a drug and is dependent on receptor occupancy⁴⁴¹. Therefore, the current study suggests that glucagon and GLP-1 analogues act synergistically when co-administered to reduce body weight although the mechanism through which this occurs is unknown.

Finally, the GLP-1 analogue, G(Y) significantly reduced liver weight alone, and in combination with G(Z) compared to vehicle. GLP-1 analogues have been shown to protect against NASH by increasing fatty acid oxidation, decreasing lipogenesis and improving hepatic glucose metabolism⁴¹⁶. In one study, these benefits were apparent

despite a lack of weight loss following liraglutide⁴⁴². In addition, there was a trend towards reduced circulating triglyceride levels following chronic G(Y) administration compared to vehicle, although this did not reach significance. Therefore, reduced liver mass in response to G(Y) administration may be secondary to reduced hepatic triglyceride content. This could be investigated further by direct hepatic triglyceride quantification and other markers of hepatic steatosis such as histological analysis and plasma alanine aminotransferase.

In summary, these experiments demonstrate the ability to design and administer glucagon and GLP-1 analogues that in combination cause significant weight loss and improved glycaemic control in mice. Although the mechanism is unclear, an increase in EE appears to be the cause. Long-acting glucagon and GLP-1 receptor analogues may be useful tools in the investigation of the chronic effects of glucagon and GLP-1 receptor agonism in energy homeostasis and reflect a potential therapy for obesity and type 2 diabetes mellitus.

CHAPTER 4:

THE ACUTE EFFECTS OF CO- ADMINISTRATION OF GLUCAGON AND GLP-1 ON ENERGY EXPENDITURE IN HUMANS

4.1 Introduction

Glucagon and GLP-1 are derived from the same preproglucagon gene. Data from my studies in rodents and from others^{87,181,182,343,345} support similar anorectic roles for these peptides. However, they demonstrate different roles in glucose metabolism and EE^{162,209,405,443}. These effects may be exploited to reduce body weight and improve glucose homeostasis. OXM is also derived from the preproglucagon gene, is released postprandially from the gut and is an agonist at both the GCGR and GLP-1R^{220,444}. In rodent and human studies, OXM has been shown to reduce food intake and body weight, increase EE and improve glucose control^{220,222,223}. It has been proposed that the effects of OXM on food intake occur mainly via the GLP-1R, whereas increased EE is due to GCGR agonism²²⁷. Kosinski et al, have developed an OXM agonist that differs from OXM by only one residue, yet has no significant activity at the GCGR²²⁷. This OXM agonist significantly reduces food intake in DIO mice following administration for 14 days, with a modest reduction in body weight (3%). This is in keeping with activation at the GLP-1R. In contrast, OXM which has retained activity at the GCGR and GLP-1R, significantly decreases food intake and reduces body weight by 14%, suggesting a beneficial weight-lowering effect through GCGR agonism²²⁷. Studies in GLP-1R and GCGR knockout mice have also demonstrated the important contribution of GCGR agonism in EE and weight loss^{228,229}. Repeated administration of a dual glucagon and GLP-1R agonist results in weight loss and reduced food intake in wild-type mice, however this effect is attenuated in GCGR knockout mice²²⁸. Similarly, administration of a dual glucagon and GLP-1R agonist for one month to DIO mice, significantly decreases food intake, body weight and fat mass and increases EE compared to controls²²⁹. These effects are still evident following administration in GLP-1R knockout mice²²⁹. Therefore OXM provides a unique model of dual receptor agonism, causing weight loss through a reduction in food intake via the GLP-1R and increased EE via the GCGR.

The mechanism through which glucagon increases EE is unknown and few studies have investigated its effects in humans. Animal and *in vitro* studies suggest glucagon may increase EE via BAT^{213,214}. Glucagon administration in rodents increases whole body oxygen consumption and body temperature in addition to increased BAT mass, blood flow and temperature^{209,215,216}. It is thought that many of these effects occur via the SNS^{217,218,407,445}. In humans, there have been no studies investigating the effects of glucagon infusion alone and EE. Glucagon increases EE in healthy volunteers by 15% when co-infused with somatostatin, whilst somatostatin alone has no effect on EE²¹⁰. In contrast, several studies have investigated the effects of GLP-1 analogues and EE. In

obese non-diabetic patients, daily injection of exenatide for three months does not alter weight-adjusted EE⁴⁰⁵. Similarly, once daily injection of liraglutide in patients with type 2 diabetes does not significantly increase resting EE (REE)⁴⁰⁶. No studies have investigated the effect of co-administration of glucagon and GLP-1 on EE in humans.

Acute administration of glucagon increases circulating glucose in humans⁴⁴³ through hepatic glycogenolysis and gluconeogenesis. Since GLP-1 reduces glucose through an incretin effect, the combination of glucagon and GLP-1 may offer an attractive therapeutic option for obese patients with type 2 diabetes due to reduced food intake, increased EE and improved glucose control. Consistent with this hypothesis, OXM reduces food intake and increases EE causing considerable weight loss in obese volunteers with no net effect on glucose control^{223,446}. However, the separate and combined effects of glucagon and GLP-1 on EE, food intake and glucose control has not been investigated in humans. In addition, the ratio of GCGR to GLP-1R agonism that is optimal for increased EE and reduced food intake whilst improving glycaemia is unknown.

This study therefore aims to investigate the effects of glucagon and GLP-1 administration in overweight human volunteers, when administered independently and in combination, on EE and substrate oxidation. I hypothesised that glucagon increases REE and that this effect is retained following co-administration with GLP-1. In addition, I hypothesised that the acute hyperglycaemic effects following glucagon administration could be ameliorated by co-administration with GLP-1.

4.2 Hypothesis and aims

4.2.1 Hypothesis

I hypothesised that glucagon administration increases EE and any rise in glucose is ameliorated by co-infusion with GLP-1. To investigate this hypothesis, I measured EE using indirect calorimetry in non-diabetic overweight human volunteers during infusion of vehicle, each hormone separately and in combination. In addition, I hypothesised that glucagon and GLP-1 co-administration improved circulating glucose levels compared to glucagon alone.

4.2.2 Aim

My aim was to investigate the effect on EE and glucose homeostasis following glucagon and GLP-1 co-administration in overweight volunteers.

4.3 Materials and Methods

4.3.1 Investigation of the acute effects of glucagon and GLP-1 co-administration on energy expenditure in humans.

4.3.1.1 Peptides

Glucagon and GLP-1₍₇₋₃₆₎ amide were purchased from Novo Nordisk (Crawley, UK) and Bachem Ltd (Switzerland) respectively. Glucagon was dissolved in the solvent provided by Novo Nordisk (1 ml water for injection, lactose monohydrate, hydrochloric acid and sodium hydroxide for pH adjustment) whilst GLP-1₍₇₋₃₆₎ amide was dissolved in 1 ml sterile 0.9% saline (Bayer, Haywards Heath, UK).

4.3.1.2 Subjects

Ten non-diabetic overweight volunteers, three women and seven men, of mean age 35.8 +/- 3.1 years (range 23-49) and mean BMI 29.3 +/- 1.0 kg/m² (range 25.6-36 kg/m²), were recruited by advertisement. All completed the study with no adverse effects. Inclusion criteria were: age 18 years or over; stable body weight for preceding three months and BMI 25-40 kg/m². Exclusion criteria were: substance abuse, current smoker, significant past or current history of physical or psychiatric illness, regular medication other than contraceptives and pregnancy or breastfeeding. All subjects were screened and determined to be healthy by medical history, physical examination, haematological and biochemical testing and 12-lead electrocardiogram. All female participants were premenopausal and had regular menstrual cycles. Women of child-bearing age were advised to avoid pregnancy during the study and underwent urine tests to exclude pregnancy prior to each infusion.

The study was approved by the West London Research Ethics Committee (reference no.: 09/H0707/76). Written informed consent was obtained for all volunteers and the study was carried out according to the principles of the Declaration of Helsinki.

4.3.1.3 Protocol

The study was designed as a randomised, double-blinded, placebo-controlled crossover study comparing four different pairs of infusion as shown in table 4.1. Volunteers received iv infusions of placebo (gelofusine), glucagon (50 ng/kg/min, Novo Nordisk, Crawley, UK), GLP-1₍₇₋₃₆₎ amide (0.8 pmol/kg/min, Clinalfa Basic, Bachem Ltd, Switzerland) or combined glucagon and GLP-1₍₇₋₃₆₎ amide at the above mentioned doses.

The dose of glucagon was selected after a dose finding study to determine the dose of glucagon that increased EE without causing side-effects. The dose of GLP-1 was based on previous studies investigating the effects of GLP-1 on food intake and glucose homeostasis in humans^{156,447,448}.

A	B
Vehicle	Vehicle
GLP-1 (0.8 pmol/kg/min)	Vehicle
Glucagon (50 ng/kg/min)	Vehicle
GLP-1 (0.8 pmol/kg/min)	Glucagon (50 ng/kg/min)

Table 4.1 Randomised, double-blinded peptide infusion administration. Volunteers received two simultaneous infusions in order to compare the effects of GLP-1 and glucagon alone and in combination on EE.

Volunteers attended for five consecutive visits spaced at least three days apart. The initial visit was an unblinded ‘dummy’ visit where gelofusine (Braun, Crawley, UK) was infused to acclimatise volunteers to study procedures. For avoidance of liver glycogen depletion, volunteers were asked to avoid alcohol and strenuous exercise for 24h prior to each study visit and were given a high energy snack (each containing 3.8g fat, 20.4g carbohydrate and 1.9g protein; Alpen, Weetabix, UK) to consume at 2200h the night before the study. They arrived at the clinical research facility at 0830h having consumed a small, low-fat breakfast of two McVitie’s Rich Tea biscuits (each containing 1.3g fat, 5.9g carbohydrate and 0.6g protein; United Biscuits) at 0700h. Upon arrival, volunteers were asked to empty their bladder and then urine was collected at the end of each study visit to provide an estimate of urinary nitrogen excretion.

The protocol for each visit is shown in figure 4.1. Peripheral venous cannulae were inserted in both forearms, one for infusions and one for blood sampling. A three-way tap (BD Connecta Plus Stopcock, Becton Dickinson & Co., USA) was attached to the infusion cannula, to allow connection of two separate infusion lines.

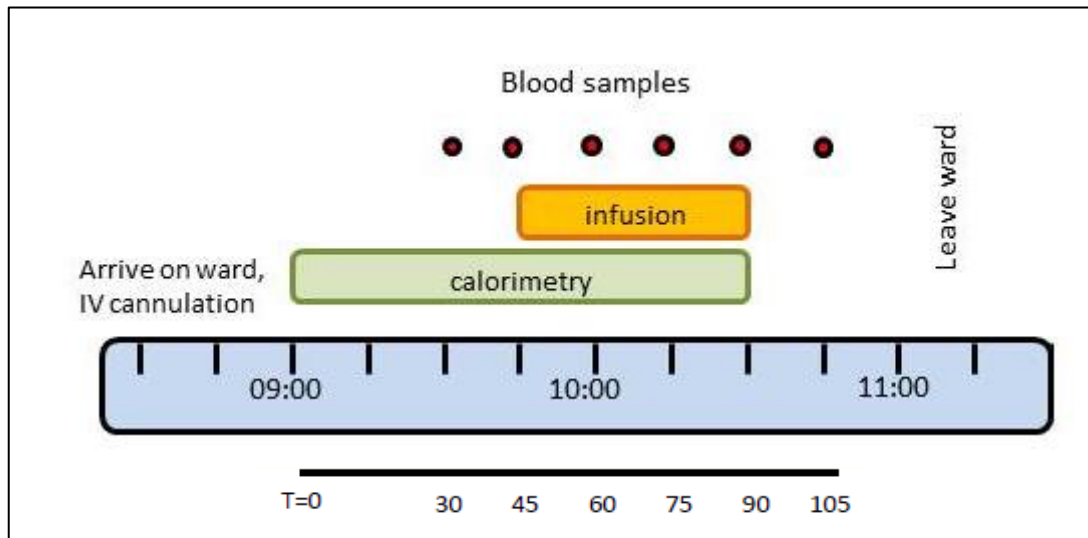


Figure 4.1 Protocol of study. Following acclimatisation, indirect calorimetry readings were commenced at t=0 for a total of 90 minutes. An infusion of vehicle/vehicle, glucagon/vehicle, GLP-1/vehicle or glucagon/GLP-1 was commenced at t=45 minutes and continued for 45 minutes. Blood samples were taken at time points indicated.

At 0 min (0900h), subjects were placed under the indirect calorimetry canopy (Gas Exchange Monitor, GEM Nutrition, Daresbury, UK). Before each measurement, the calorimeter was calibrated with 'zero' (0.00% O₂ and 0.00% CO₂) and 'span' gases (20% O₂ and 1.00% CO₂) (BOC gases, Surrey, UK). Volunteers lay semirecumbent on a bed and were allowed to watch television or listen to music. Calorimeter measurements were allowed to stabilise during the first thirty minutes. REE, respiratory quotient (RQ) and carbohydrate and fat oxidation rates were estimated from measurements of V_{O2} and V_{CO2} recorded each minute, with adjustment made for urinary nitrogen excretion. Protein oxidation rate over the entire study visit was estimated from urinary nitrogen excretion alone^{449,450}. The formulae used to determine EE, carbohydrate, fat and protein oxidation were as follows and are based on the Weir equation⁴⁴⁹:

Energy Expenditure

$$EE \text{ (kcal/min)} = (3.94 \times VO_2 \text{ (l/min)}) + (1.11 \times VCO_2 \text{ (l/min)}) - (2.17 \times \text{nitrogen excretion})$$

Nitrogen excretion

$$\text{Nitrogen excretion (g/min)} = 0.028 \times ((\text{urine urea (mmol/l)} \times \text{urine volume (litres)}) / \text{time elapsed since last passed urine (mins)})$$

Respiratory Quotient

$$RQ = VCO_2 \text{ (l/min)} / VO_2 \text{ (l/min)}$$

CHO oxidation

$$\text{CHO oxidation (g/min)} = (4.55 \times VCO_2 \text{ (l/min)}) - (3.21 \times VO_2 \text{ (l/min)}) - (2.87 \times \text{nitrogen excretion})$$

Fat oxidation

$$\text{Fat oxidation (g/min)} = 1.67 \times (VO_2 \text{ (l/min)} - VCO_2 \text{ (l/min)}) - (1.92 \times \text{nitrogen excretion})$$

Protein oxidation

$$\text{Protein oxidation (g/min)} = \text{nitrogen excretion} \times 6.25$$

Baseline REE was defined as the mean of measurements taken during the final 15 minutes of the 45 minute baseline phase. REE following infusion was determined from measurements taken during the final 15 minutes of the infusion phase.

At 45 minutes, a hormone infusion was started, which lasted for 45 minutes. Each volunteer received two infusions, A and B, simultaneously, at each visit. Infusion allocation was determined in a four-way randomised design. Gelofusine was used as the vehicle for hormone infusions in order to minimise adsorption of peptides to infusion line and syringes⁴⁵¹. In addition, gelofusine was drawn up into the syringes and giving sets and left for 30 minutes prior to expelling. Each infusion was drawn up and delivered in a separate 50 ml syringe (Graseby 3100, SIMS Graseby Ltd, Watford, UK) to allow the use of two different infusion rates. The infusion rate was 'ramped' to establish stable plasma hormone levels rapidly, with an infusion rate four times the nominal rate for 5 minutes reduced to twice the nominal rate for the next 5 minutes and then to the nominal rate for the remaining 35 minutes.

Calorimetry continued during the 45 minute infusion. As with the baseline phase, calorimetry measurements were allowed to stabilise for 30 minutes after the infusion was started. Measurements from the final 15 minutes were used to calculate the infusion-phase REE, RQ and substrate oxidation rates. At 90 minutes, the infusion and calorimetry were stopped and the study terminated at 105 minutes. Blood samples were collected at 30, 45, 60, 75, 90 and 105 minutes, into lithium heparin-coated BD Vacutainer tubes (International Scientific Supplies Ltd, Bradford, UK) containing 1000 kallikrein inhibitor units (0.1ml) (Trasylol, Bayer Scherin Pharma, Berlin, Germany) for gut hormone analysis. In addition, blood samples were collected into plain serum Vacutainer tubes containing clot activators and fluoride oxalate tubes for insulin and glucose assay respectively. Cryogenic vials (Alpha Laboratories, Eastleigh, UK) containing 20 µl of 0.8M HCl were used for ghrelin quantification and lithium heparin-coated tubes for non-esterified fatty acids (NEFAs). Samples were stored on ice until centrifugation (4°C, 4000 rpm, 10 minutes) after which, plasma was separated immediately and stored at -20°C until analysis. The pulse and blood pressure of each subject was measured at t=0, 45 and 90 minutes.

4.3.1.4 Plasma hormone assays

Glucagon and GLP-1 radioimmunoassay (RIA)

Glucagon and total GLP-1 were measured using established in-house radioimmunoassays (RIAs)^{132,162}. All samples were assayed in duplicate. Glucagon and GLP-1 were purchased from Bachem Ltd (Switzerland). All other reagents and materials were supplied by Sigma (Poole, Dorset, UK). The glucagon and GLP-1 labels were prepared by Professor M. Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) who iodinated the peptide using the iodogen method⁴⁵² and was purified by reverse-phase HPLC.

Assays were performed in veronal buffer (1l distilled water containing 10.3g sodium barbitone, 0.3g sodium azide), at pH 8 with 0.3% BSA (and 0.02% tween for the GLP-1 assay) (VWR, UK). Standard curves were prepared in assay buffer at 0.25 and 0.5 pmol/ml for GLP-1 and glucagon respectively, added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 µl. The glucagon antibody (RCS5) was raised in rabbits against the C-terminal of glucagon and is therefore specific for pancreatic glucagon. In this assay the antibody was used at a dilution of 1:50000. The GLP-1 antibody was

raised in rabbits against the N terminal of GLP-1₍₇₋₃₆₎ and binds to all amidated forms of GLP-1 (1-36, 7-36, 9-36). Experimental samples of 50 µl, 100 µl glucagon or GLP-1 antibody solution and 100 µl of glucagon or GLP-1 label solution were used and all tubes were buffered to a total volume of 700 µl with assay buffer. The assays were incubated for 96 hours at 4°C. Free peptide was separated from bound using charcoal adsorption. To each tube, 4 mg of charcoal, suspended in 0.06 M phosphate buffer with gelatine was added immediately prior to centrifugation. The samples were then centrifuged at 1500 rpm, 4°C, for 20 minutes. Bound and free label were separated and both the pellet and supernatant counted for 180 seconds in a γ-counter (model NE1600, Thermo Electron Corporation). Plasma glucagon and GLP-1 concentrations in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard.

Ghrelin ELISA

Ghrelin was measured using total ghrelin (intact and des-octanoyl forms) and active (acylated) ghrelin ELISA assay (Millipore, Watford, UK). In both cases, the manufacturer's protocol was followed. The kits use a competitive inhibition enzyme immunoassay technique, in which monoclonal anti-ghrelin antibodies were immobilised on the surface wells. After washing with buffer, these antibodies bound to ghrelin in the sample (20 µl added), standard or control solutions during a 2 hour incubation. After washing, to remove unbound material, a second biotinylated, monoclonal anti-ghrelin antibody was added to the wells which bound to the already captured ghrelin. Further washing preceded the addition of streptavidin-horseradish peroxidase, which bound to biotin. After excess streptavidin-enzyme conjugate was washed away, a substrate for horseradish peroxidase was added (3,3', 5, 5'-tetramethylbenzidine). The peroxidase reaction was stopped by the addition of 0.3M HCl. Enzyme activity was measured spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) by the increased absorbance at 450 nm and 620 nm. Absorbance at 450nm was subtracted from that at 620 nm. A standard curve was constructed and sample concentration derived from this (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

Other blood samples

NEFAs were measured by the Department of Chemical Pathology, Great Ormond Street Hospital National Health Service Trust using a Wako NEFA assay kit (Alpha laboratories, UK) and the ILab 650 analyser (Instrumentation Laboratory, Massachusetts, US). Insulin, glucose, thyroid hormones and cortisol samples were measured by the Department of Chemical Pathology, Imperial College Healthcare National Health Service Trust. Insulin was measured using a fully automated enzyme immunoassay and an Abbott AxSYM analyser.

4.3.1.5 Statistical analysis

Change from baseline in EE and substrate oxidation rates, were analysed using one way ANOVA with Tukey post test. Two-way repeated measures ANOVA with Bonferroni post test was used to compare differences in glucose, insulin, ghrelin, NEFA, thyroid hormones and cortisol concentration at different time points. Change in pulse and blood pressure from baseline was analysed using one way ANOVA with Tukey post test. Results are presented as mean $\pm$ SEM and statistical significance defined as $p < 0.05$.

4.4 Results

4.4.1 Dose finding study investigating the acute effects of co-administration of glucagon and GLP-1 on energy expenditure and glucose homeostasis in humans.

4.4.1.1 Investigation of the acute effects of glucagon and GLP-1 co-administration on energy expenditure.

Non-diabetic overweight volunteers were recruited as described in section 4.3.1.2 and the protocol followed as described in section 4.3.1.3. The initial dose of glucagon was based on a previous study in which glucagon and somatostatin co-administration increased REE by 15%²¹⁰. In the current dose finding study, the dose was gradually increased until the lowest dose of glucagon that increased REE was determined. As shown in figure 4.2, the lowest effective dose of glucagon was 50 ng/kg/min and increased REE by 22 kcal/day from baseline (n=1). Subsequent infusions for the remainder of the study contained glucagon (50 ng/kg/min) or GLP-1 (0.8 pmol/kg/min) administered independently and in combination.

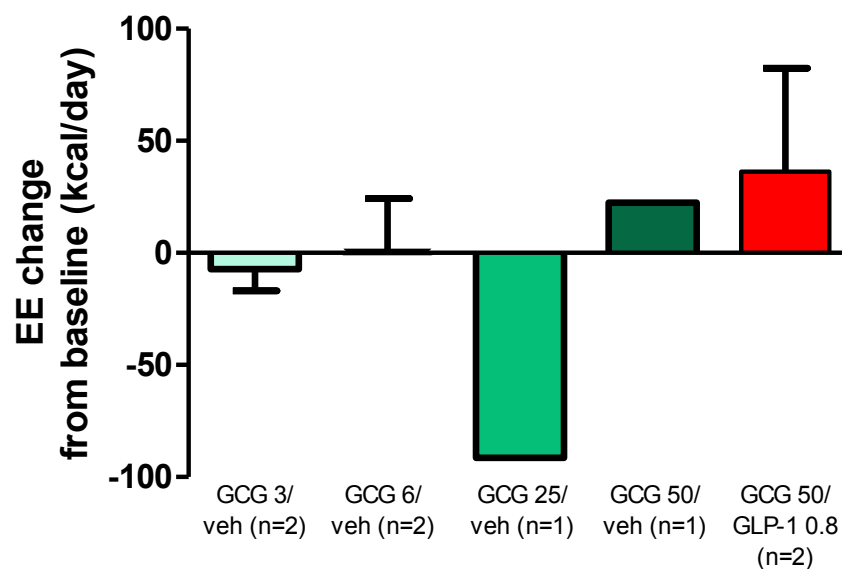


Figure 4.2. Dose finding study to determine energy expenditure following the administration of glucagon and GLP-1 independently and in combination. Energy expenditure (EE) was measured prior to infusion of glucagon (ng/kg/min) and GLP-1 (pmol/kg/min) alone or in combination. The number of volunteers receiving each dose is shown in brackets. Abbreviations: Energy expenditure (EE), glucagon (GCG), vehicle (veh), glucagon-like peptide-1 (GLP-1).

4.4.1.2 Investigation of the acute effects of glucagon and GLP-1 co-administration on plasma insulin and glucose concentrations.

During the dose finding study, glucose and insulin samples were taken at the time points shown in figure 4.3. At the lowest effective glucagon dose that increased EE (50 ng/kg/min), circulating glucose levels did not significantly rise compared to the lowest dose tested (3 ng/kg/min) (figure 4.3A). In contrast, there was a dose-dependent increase in insulin levels following glucagon infusion with the highest levels observed following glucagon/GLP-1 co-administration (figure 4.3B).

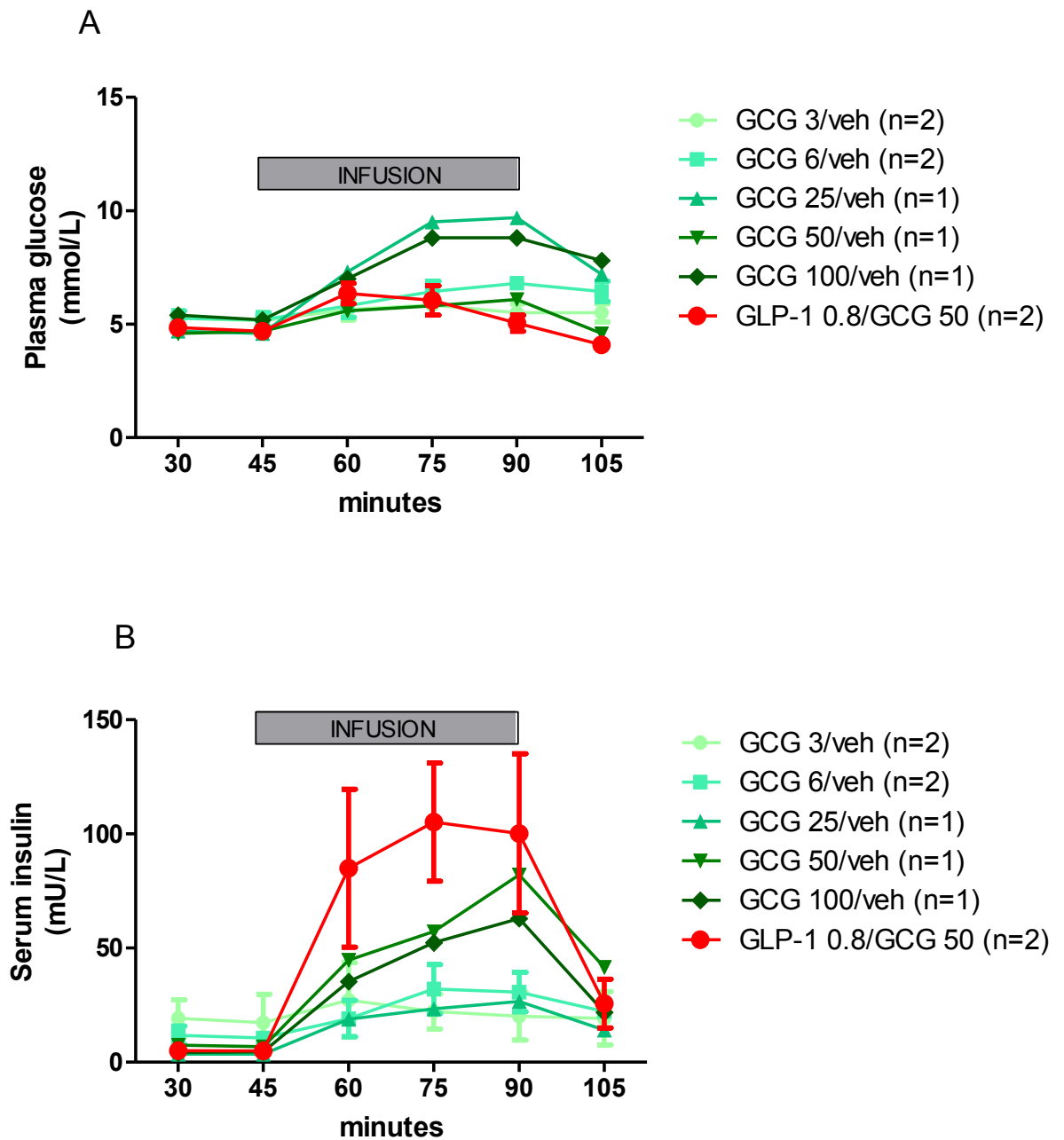


Figure 4.3. Circulating glucose and insulin levels following the administration of glucagon and GLP-1 alone and in combination. Volunteers received an infusion for 45 minutes (denoted by grey bar) of glucagon alone or in combination with GLP-1. Glucose and insulin samples were collected at the time points indicated. The number of volunteers receiving each dose is shown in brackets. Abbreviations: glucagon (GCG), vehicle (veh), glucagon-like peptide-1 (GLP-1).

4.4.2 Investigation of the acute effects of glucagon and GLP-1 co-administration on energy expenditure in humans.

4.4.2.1 Investigation of plasma glucagon and GLP-1 concentrations achieved following co-administration of glucagon and GLP-1

Mean baseline plasma concentration of GLP-1 in all volunteers, prior to the start of the infusion was 18 ± 1.9 pmol/l (range: 5.1-48.8 pmol/l). Infusion of GLP-1 at 0.8 pmol/kg/min, increased circulating GLP-1 levels to 103.1 ± 26.0 pmol/l, 15 minutes after the infusion was started (Fig 4.4A). In the GLP-1/glucagon combination group, GLP-1 levels rose to 89.7 ± 17.2 pmol/l, 15 minutes after the infusion was started. Glucagon alone or vehicle infusion caused no rise in circulating GLP-1.

Mean baseline plasma concentration of glucagon in all volunteers, prior to the start of infusion was 9.6 ± 1.1 pmol/l (range 2.0-31.6 pmol/l). This rose to a peak of 260.9 ± 43.8 pmol/l, 15 minutes after starting the glucagon infusion, and 239.3 ± 42.1 pmol/l following glucagon and GLP-1 co-administration (Fig 4.4B). GLP-1 alone or vehicle infusion did not elevate circulating glucagon. After the infusions were discontinued at 90 minutes, glucagon and GLP-1 levels decreased to baseline within 15 minutes.

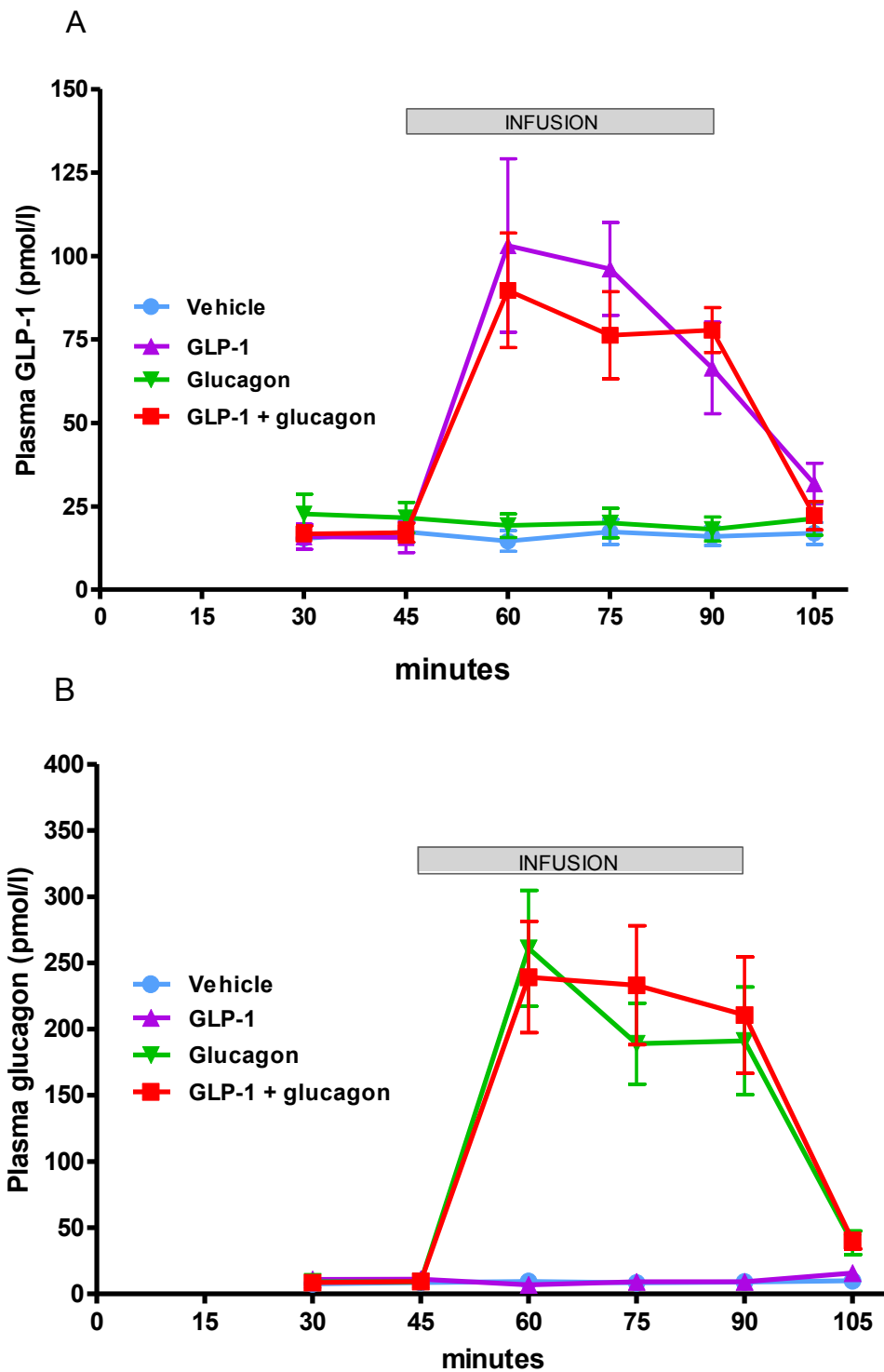


Figure 4.4 Plasma GLP-1 (A) and glucagon (B) following peptide infusion. Volunteers received an infusion for 45 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1 (0.8 pmol/kg/min)/vehicle, glucagon (50 ng/kg/min)/vehicle or GLP-1 (0.8 pmol/kg/min)/glucagon (50 ng/kg/min). n=10.

4.4.2.2 Investigation of the acute effects of glucagon and GLP-1 co-administration on energy expenditure

REE was measured at baseline and following infusion of vehicle, GLP-1, glucagon or combined GLP-1/glucagon. Glucagon significantly increased REE compared to vehicle or GLP-1 alone (mean REE: -7.4 ± 22.0 [vehicle] vs. 140.9 ± 18.4 kcal/day [glucagon], $p < 0.001$) and (mean REE: -10.6 ± 22.3 [GLP-1] vs. 140.9 ± 18.4 kcal/day [glucagon], $p < 0.001$, $n=10$, fig. 4.5). A further increase in REE was seen following the co-administration of GLP-1/glucagon compared to vehicle or GLP-1 alone (mean REE: -7.4 ± 22.0 [vehicle] vs. 161.2 ± 30.9 kcal/day [GLP-1/glucagon co-administration], $p < 0.001$ vs. vehicle, $n=10$, fig 4.5) and (mean REE: -10.6 ± 22.3 [GLP-1] vs. 161.2 ± 30.9 kcal/day [GLP-1/glucagon co-administration], $p < 0.001$, $n=10$, fig. 4.5). There was no significant difference in REE between glucagon and GLP-1/glucagon co-administration.

Substrate oxidation was calculated from RQ measurements during calorimetry at baseline and at the end of infusion. Co-administration of GLP-1/glucagon caused a significant increase in carbohydrate oxidation (mean carbohydrate oxidation: -0.005 ± 0.009 [vehicle] vs. 0.074 ± 0.016 g/min [GLP-1/glucagon co-administration], $p < 0.001$, $n=10$, fig. 4.6A) and a significant decrease in fat oxidation (mean fat oxidation: 0.002 ± 0.004 [vehicle] vs. 0.017 ± 0.006 g/min [GLP-1/glucagon co-administration], $p < 0.05$, $n=10$, fig. 4.6B). Calculation of protein oxidation requires estimation of urinary nitrogen excretion over the entire study period. Therefore comparison between baseline and following infusion could not be done. There were no significant changes in protein oxidation between any of the infusions (fig. 4.6C).

Due to previous evidence suggesting a lipolytic effect of glucagon on adipose tissue⁴¹⁷, plasma NEFAs were measured. Glucagon and GLP-1/glucagon co-administration significantly reduced plasma NEFA during the infusion (mean NEFA: 0.46 ± 0.07 [pre glucagon infusion] vs. 0.18 ± 0.03 mmol/l [post glucagon infusion], $p < 0.001$; 0.38 ± 0.06 mmol/l [pre GLP-1/glucagon infusion] vs. 0.13 ± 0.02 mmol/l [post GLP-1/glucagon infusion], $p < 0.001$, $n=10$, fig. 4.7).

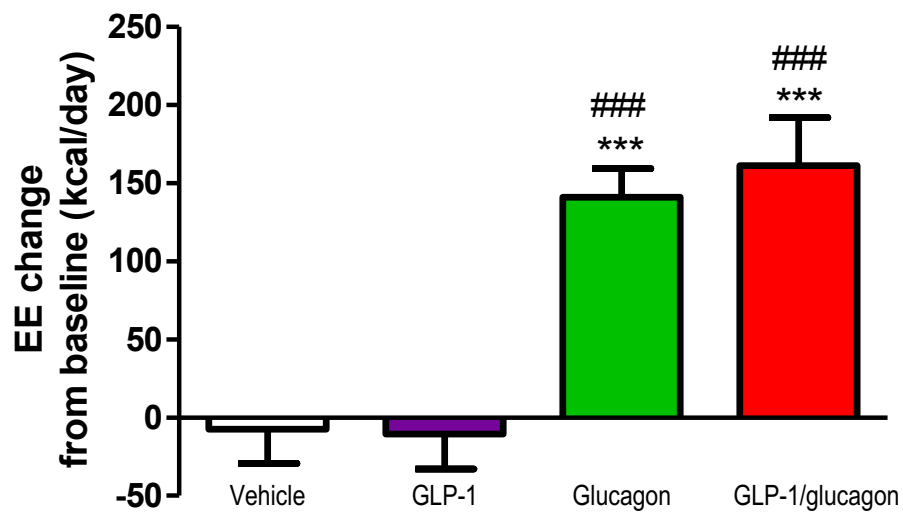


Figure 4.5. Change in energy expenditure (EE) from baseline following administration of vehicle, GLP-1, glucagon or combination of GLP-1 and glucagon. Volunteers received an infusion of vehicle/vehicle, GLP-1 (0.8 pmol/kg/min)/vehicle, glucagon (50 ng/kg/min)/vehicle or GLP-1 (0.8 pmol/kg/min)/glucagon (50 ng/kg/min). EE was measured at baseline, prior to the start of infusion and continued throughout the infusion. Data were analysed using one-way ANOVA with Tukey post test, *** $p < 0.001$ vs. vehicle, ### $p < 0.001$ vs. GLP-1. $n = 10$.

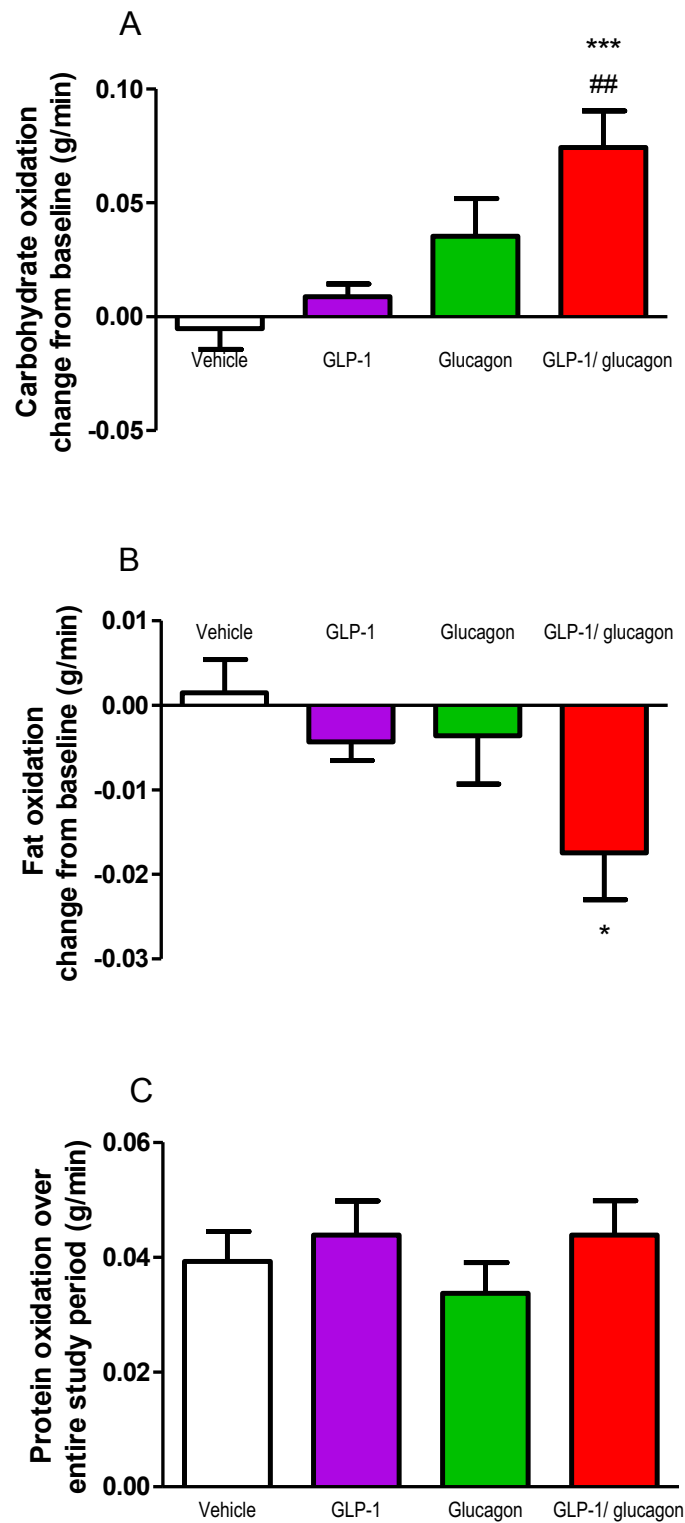


Figure 4.6 Substrate oxidation rates following infusion. Carbohydrate and fat oxidation values were calculated during calorimetry from RQ readings at baseline and following infusion. Protein oxidation was calculated at the end of the study only. Data were analysed using one-way ANOVA with Tukey post test. * $p < 0.05$, *** $p < 0.001$ vs. vehicle, ## $p < 0.01$ vs. GLP-1. $n = 10$.

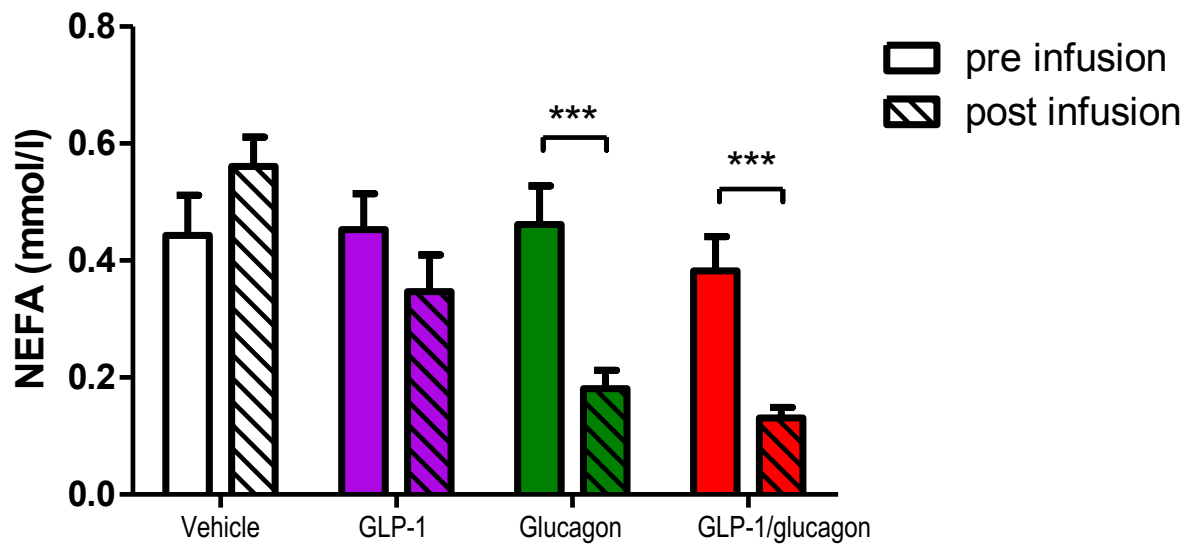


Figure 4.7. Non-esterified fatty acid (NEFA) levels following infusion of GLP-1, glucagon or GLP-1/glucagon in combination. Blood samples were collected for NEFAs prior to infusion (t=30 mins) and at the end of the infusion (t=90 mins). Data were analysed using two-way repeated measures ANOVA with Bonferroni post test. ***p<0.001 vs. pre-infusion. n=10.

4.4.2.3 Investigation of the acute effects of glucagon and GLP-1 co-administration on plasma insulin and glucose concentrations.

Mean baseline glucose values in all volunteers were 5.1 ± 0.1 mmol/l (range: 3.9-6.3 mmol/l) prior to peptide administration. A significant rise in glucose was seen following glucagon infusion, reaching a peak at $t=75$ (30 minutes after the start of infusion) (mean glucose: 5.1 ± 0.1 [vehicle] vs. 8.3 ± 0.4 mmol/l [glucagon], $p<0.001$, $n=10$, fig. 4.8A). At the end of the study ($t=105$), glucose levels remained elevated following glucagon administration despite the infusion having been discontinued 15 minutes prior (mean glucose: 5.0 ± 0.1 [vehicle] vs. 6.7 ± 0.5 mmol/l [glucagon], $p<0.001$, $n=10$, fig. 4.8A).

The increase in glucose following glucagon administration was significantly attenuated by the co-administration of GLP-1/glucagon at $t=75$ and $t=90$ (mean glucose at $t=90$: 6.0 ± 0.7 [GLP-1/glucagon co-administration] vs. 5.1 ± 0.1 mmol/l [vehicle], $p=ns$; vs. 7.9 ± 0.4 mmol/l [glucagon], $p<0.001$, $n=10$, fig. 4.8A). In addition, the glucose level in response to co-administration returned to baseline 15 minutes after the infusion was stopped.

Both glucagon and GLP-1/glucagon co-administration significantly increased insulin concentration compared to vehicle or GLP-1 alone. The amelioration of glucose control in response to GLP-1/glucagon co-administration described above, was accompanied by a greater rise in insulin in response to GLP-1/glucagon co-administration compared to glucagon alone (mean AUC: 5605 ± 1037 [GLP-1/glucagon co-administration] vs. 2919 ± 536 [glucagon] $\text{mU/l}^{-1}\text{min}$, $p<0.01$, $n=10$, fig. 4.9B). Administration of GLP-1 alone did not significantly increase glucose or insulin.

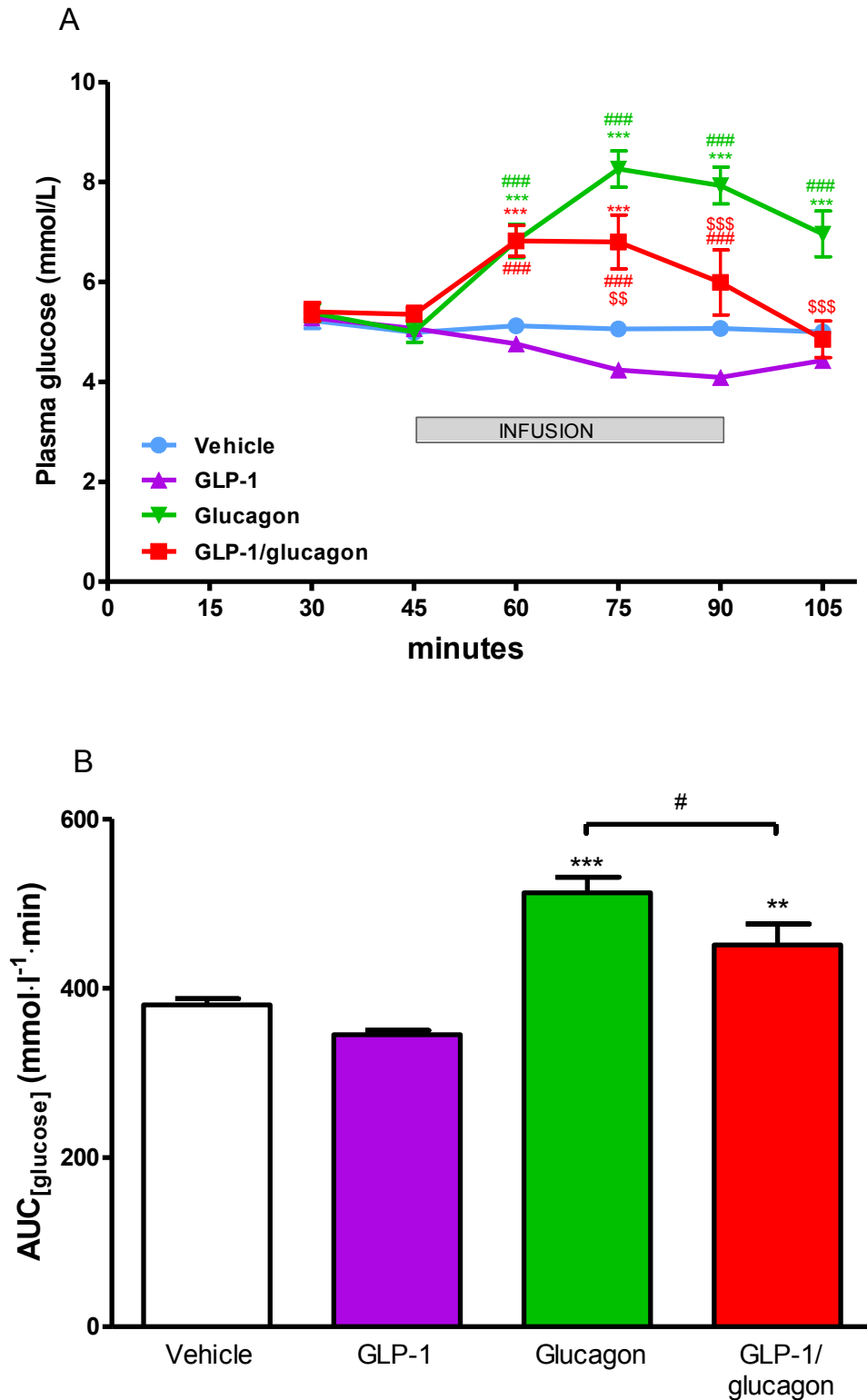


Figure 4.8. Effects of glucagon, GLP-1 or GLP-1/glucagon in combination on plasma glucose concentration. Volunteers received an infusion for 45 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1 (0.8 pmol/kg/min)/vehicle, glucagon (50 ng/kg/min)/vehicle or GLP-1 (0.8 pmol/kg/min)/glucagon (50 ng/kg/min). Data were analysed using (A) two-way repeated measures ANOVA with Bonferroni post test and (B) one way ANOVA with Tukey post test. **p<0.01, *** p<0.001 vs. vehicle; ###p<0.001 vs. GLP-1; \$\$ p<0.01, \$\$\$ p<0.001 vs. glucagon. n=10.

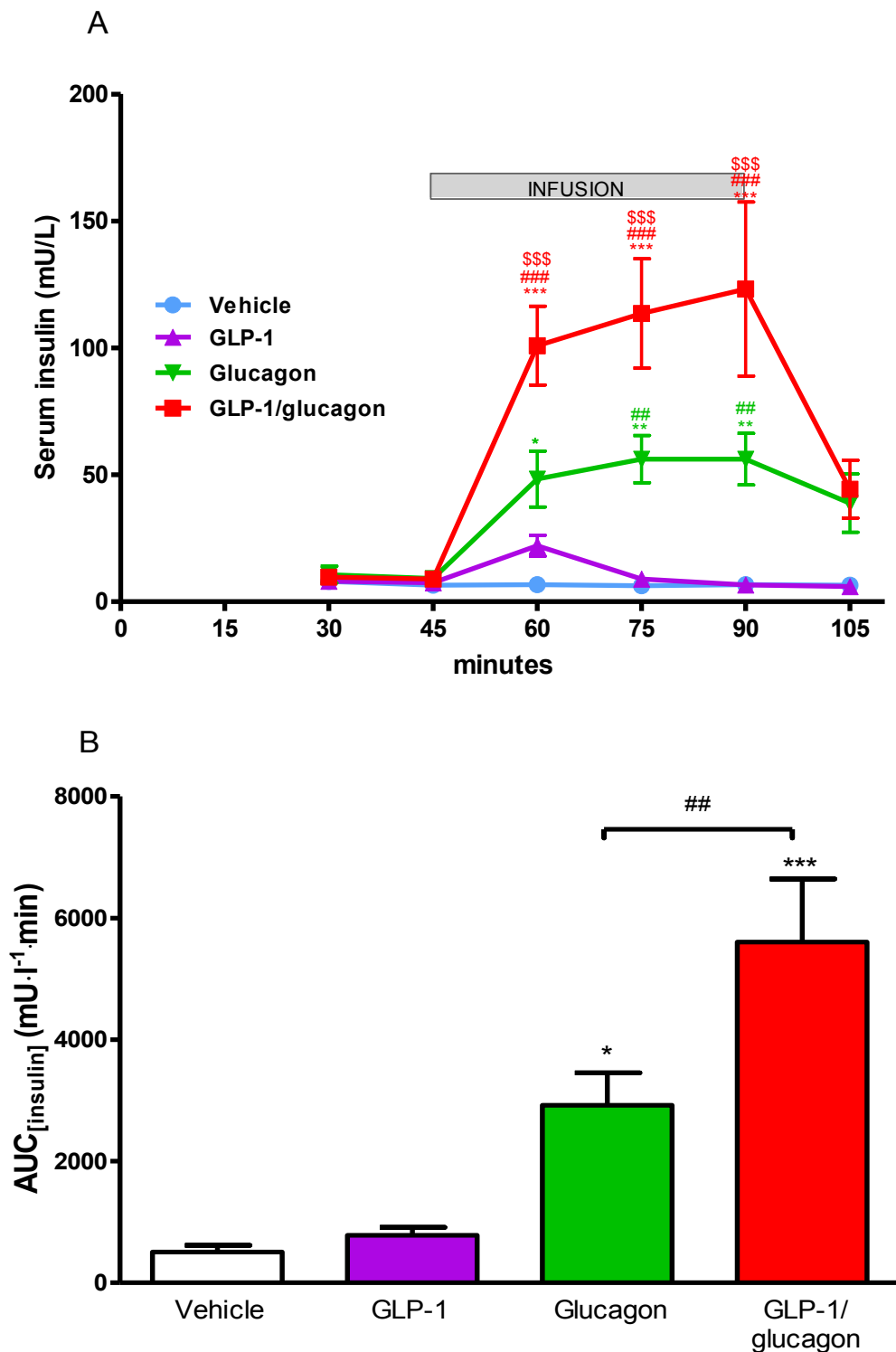


Figure 4.9 Effects of glucagon, GLP-1 and GLP-1/glucagon in combination on serum insulin concentrations. Volunteers received an infusion for 45 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1 (0.8 pmol/kg/min)/vehicle, glucagon (50 ng/kg/min)/vehicle or GLP-1 (0.8 pmol/kg/min)/glucagon (50 ng/kg/min). Data were analysed using (A) two-way repeated measures ANOVA with Bonferroni post test and (B) one way ANOVA with Tukey post. *p<0.05, **p<0.01, *** p<0.001 vs. vehicle; ##p<0.01, ###p<0.001 vs. GLP-1; \$\$\$ p<0.001 vs. glucagon. n=10.

4.4.2.4 Investigation of the acute effects of glucagon and GLP-1 co-administration on cardiovascular parameters

Following infusion of glucagon or GLP-1/glucagon co-administration, there was a rise in systolic blood pressure (fig. 4.10A) and pulse (fig. 4.10C) although this did not reach significance. There was no significant change in diastolic blood pressure in response to any of the infusions administered (fig. 4.10B).

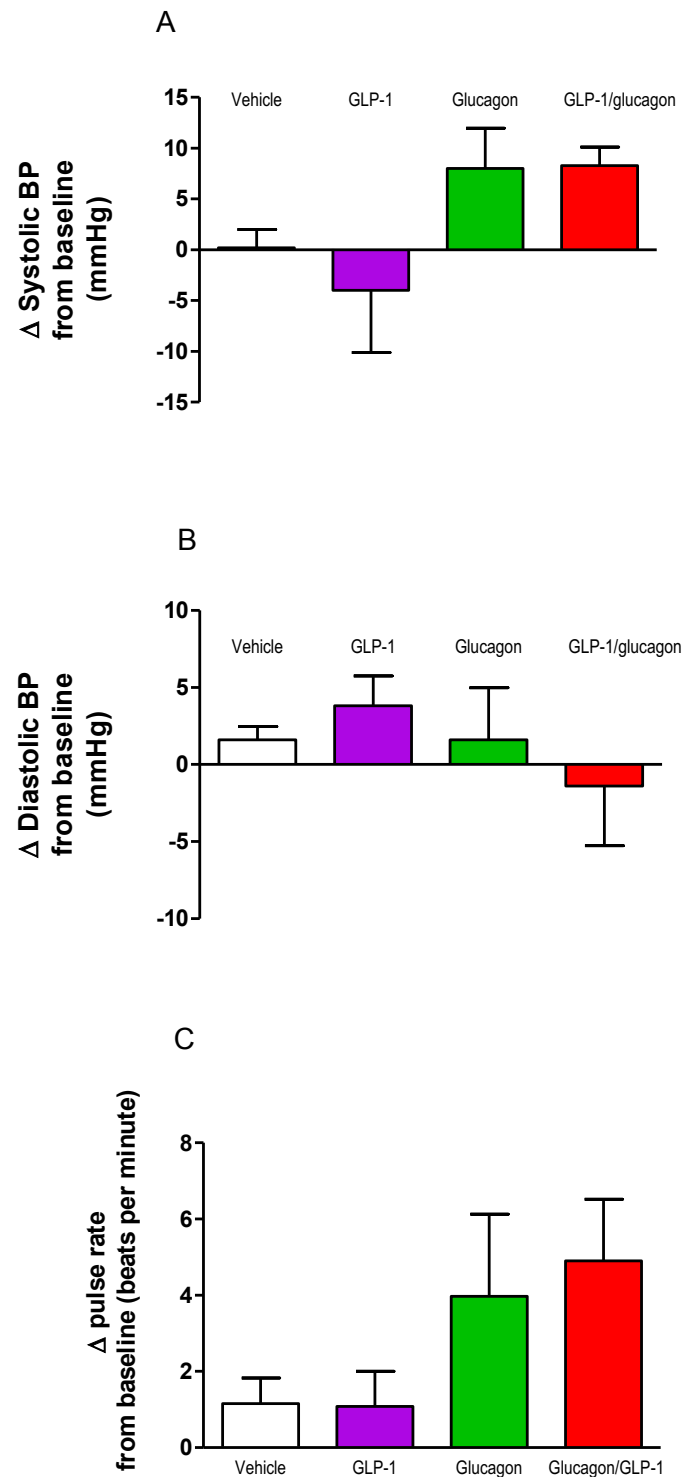


Figure 4.10. Cardiovascular parameters following infusion of GLP-1, glucagon and GLP-1/glucagon in combination. Systolic (A), diastolic (B) blood pressure and pulse rate (C) change from baseline (t=45 mins) following infusion (t=90 minutes) of GLP-1 (0.8 pmol/kg/min), glucagon (50 ng/kg/min) and combination of GLP-1 (0.8 pmol/kg/min)/glucagon (50 ng/kg/min). Data were analysed using one way ANOVA with Tukey post test. n=10. Abbreviations: blood pressure (BP), millimetres of mercury (mmHg).

4.4.2.5 Investigation of the acute effects of glucagon and GLP-1 co-administration on plasma concentrations of ghrelin, cortisol and thyroid hormones.

There were no significant changes in cortisol, fT4 and fT3 (fig. 4.11 and 4.12A and B), however there was a significant reduction in TSH following glucagon infusion (mean TSH levels: 1.24 ± 0.25 [TSH pre infusion] vs. 1.05 ± 0.22 mU/l [TSH post infusion], $p < 0.01$, $n=10$, fig. 4.12C).

Previous studies have demonstrated a reduction in circulating ghrelin in response to glucagon and GLP-1 alone⁴⁵³⁻⁴⁵⁵. Ghrelin samples were collected in this study to assess the effect on ghrelin levels in response to GLP-1/glucagon co-administration. Glucagon alone reduced total and acylated ghrelin levels although this did not reach significance. No effect on total or acylated ghrelin was seen in response to GLP-1 infusion. In contrast co-administration of GLP-1/glucagon significantly reduced total and acylated ghrelin (mean acylated ghrelin level: 390.5 ± 129.7 [pre GLP-1/glucagon infusion] vs. 191.0 ± 57.9 pg/ml [post GLP-1/glucagon infusion], $p < 0.05$, $n=10$, fig. 4.13B).

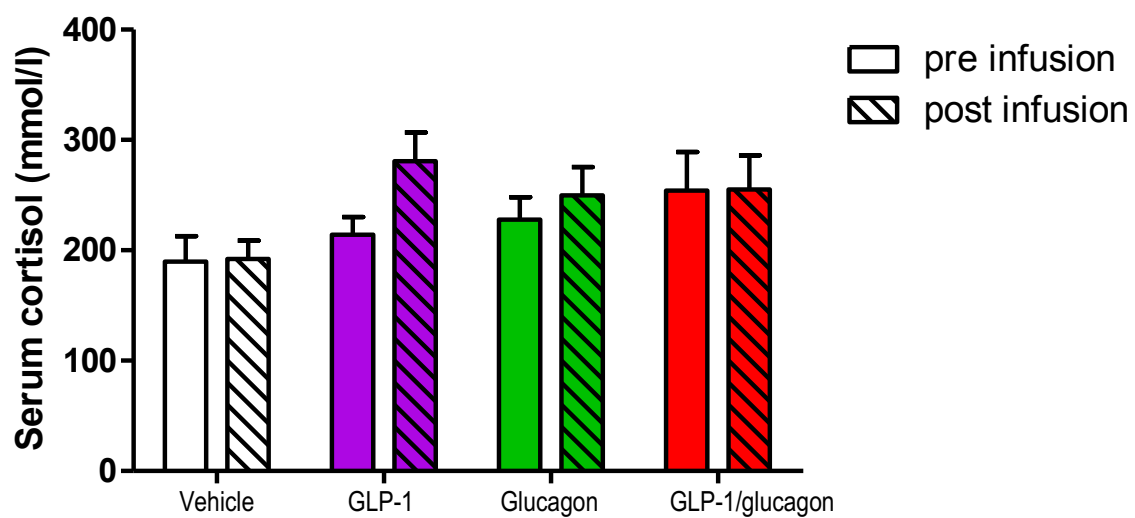


Figure 4.11 Serum cortisol following infusion of GLP-1, glucagon or GLP-1/glucagon in combination. Blood samples were collected prior to infusion (t=30 mins) and at the end of the infusion (t=90 mins). Data were analysed using two-way repeated measures ANOVA with Bonferroni post test. n=10.

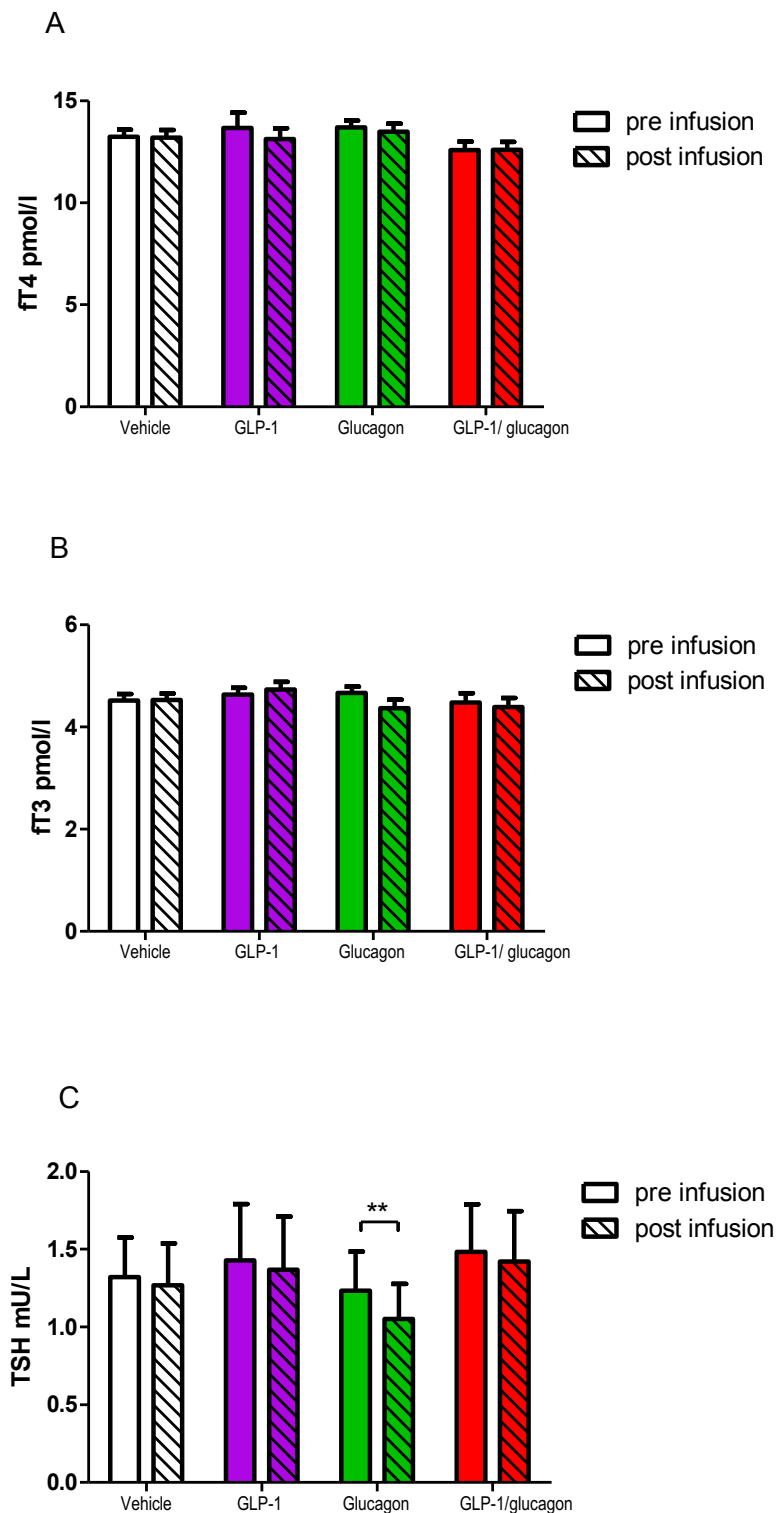


Figure 4.12. fT4 (A), fT3 (B) and TSH (C) levels following infusion of GLP-1, glucagon or GLP-1/glucagon in combination. Blood samples were collected for thyroid hormones prior to infusion (t=30 mins) and at the end of the infusion (t=90 mins). Data were analysed using two-way repeated measures ANOVA with Bonferroni post test. **p<0.01 vs. pre-infusion. n=10. Abbreviations: free thyroxine (fT4), free tri-iodothyronine (fT3), thyroid stimulating hormone (TSH).

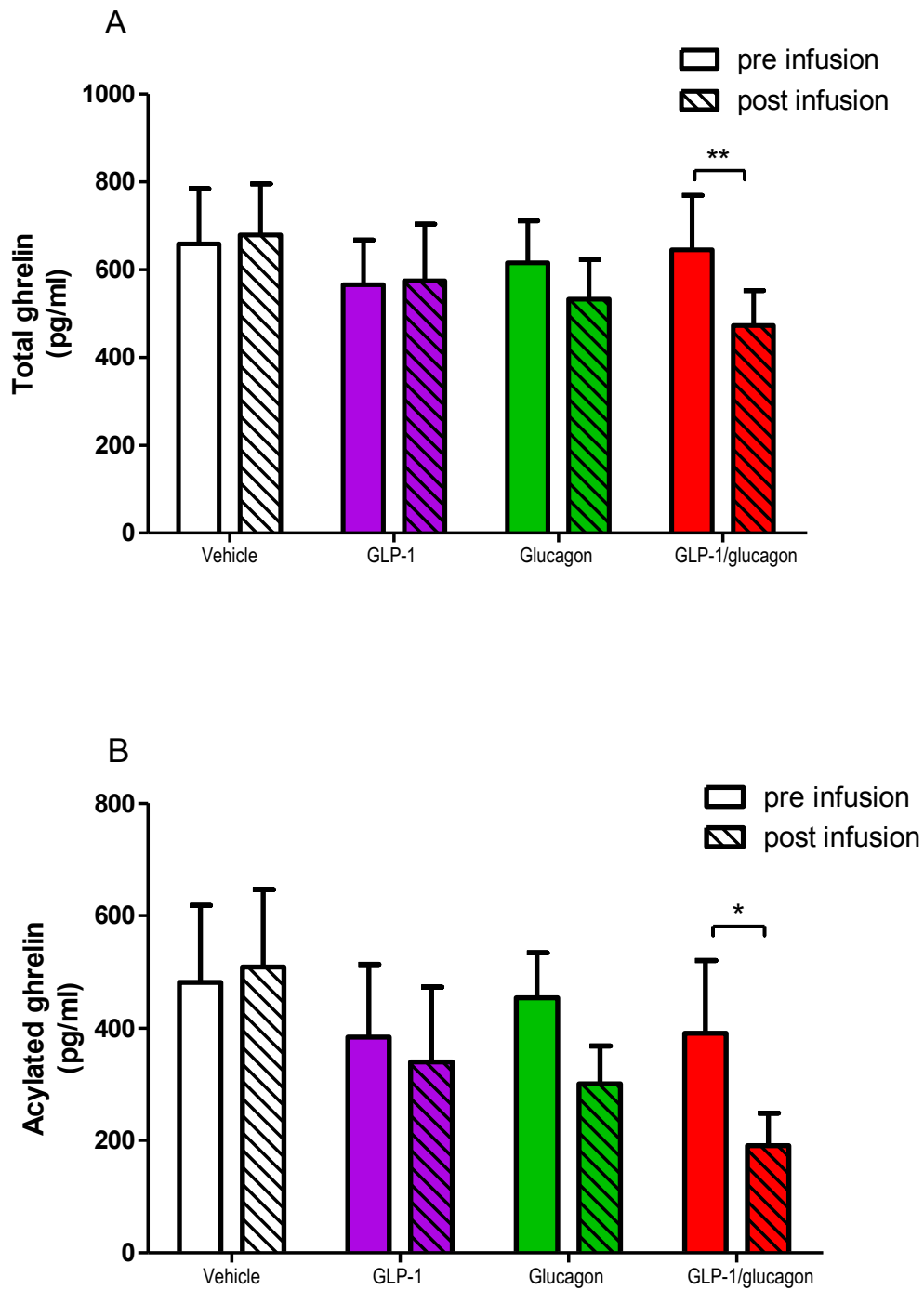


Figure 4.13. Total (A) and acylated (B) ghrelin following infusion of GLP-1, glucagon or GLP-1/glucagon in combination. Plasma samples were collected for ghrelin prior to infusion (t=30 mins) and at the end of the infusion (t=90 mins). Data were analysed using two-way repeated measures ANOVA with Bonferroni post test. *p<0.05, **p<0.01 vs. pre-infusion. n=10.

4.4 Discussion

This study demonstrates that glucagon alone and in combination with GLP-1, increases REE in humans by approximately 150 kcal/day. In addition, co-administration of GLP-1/glucagon ameliorates the rise in glucose in response to glucagon infusion alone. The effects on EE in response to glucagon may be triggered by glucagon-induced sympathetic nervous activation of BAT or increased blood flow to BAT increasing thermogenesis. Alternatively, the rise in REE may be due to glucagon-induced release of catecholamines^{456,457}. However a significant rise in pulse and blood pressure would be expected⁴⁵⁸. In this study, whilst although there was a trend towards increased pulse and systolic blood pressure, this did not reach statistical significance. Circulating catecholamines were not measured in the current study.

Interestingly, some studies suggest that glucagon stimulates catecholamine release indirectly via insulin^{377,459}. This is in keeping with my findings, in which glucagon increased insulin levels and REE, whereas GLP-1 had no effect on either outcome. Hyperinsulinaemia produced using a euglycaemic insulin clamp technique in healthy volunteers, results in a dose dependent increase in REE⁴⁶⁰. However, the glucose requirements to maintain euglycaemia are increased during the insulin clamp and it has been suggested that the increased EE is largely dependent on glucose-induced thermogenesis⁴⁶⁰. Interestingly, the rise in EE during a euglycaemic insulin clamp study is blunted in obese patients and abolished in patients with type 1 diabetes and obese type 2 diabetics^{461,462}. Furthermore, the change in REE is significantly correlated to the rate of glucose uptake⁴⁶¹. These findings suggest that the rise in EE is dependent on intact insulin function, facilitating glucose transport into cells. In contrast, somatostatin (which inhibits insulin release) and glucagon co-infusion in healthy volunteers increases REE despite suppressed insulin levels²¹⁰. However, during somatostatin and glucagon co-infusion, circulating levels of glucose are elevated, supporting the view that increased EE may be secondary to glucose-induced thermogenesis alone. Therefore, in the current study, the rise in REE in response to glucagon and GLP-1/glucagon co-administration could be secondary to an insulinotropic effect, increasing glucose utilisation in thermogenic tissues. This mechanism requires further investigation.

The current study shows increased REE in response to glucagon administration. OXM increases activity-related EE in humans but has no significant effects on REE²²³. Similarly, a long-acting glucagon agonist increases EE in mice but is associated with increased locomotor activity⁴⁶³. The difference in findings may be related to the doses

used and the degree of GCGR agonism in each study. Although not measured in this study, the effect of glucagon on activity-related EE could be measured using portable monitoring devices that calculate activity-related EE.

Both glucagon and GLP-1 can cause nausea at high doses^{464,465}. Subjective feelings of nausea at the doses of glucagon and GLP-1 administered was not investigated in the current study. Participants were placed under the indirect calorimetry hood for the duration of the infusion with minimal movement allowed. This prevented completion of VAS to assess nausea and satiety. Before further development of a glucagon/GLP-1 agonist as a potential treatment for obesity, further investigation of the incidence of nausea and vomiting would be needed.

In the current study, increased EE in response to GLP-1/glucagon co-administration was accompanied by an increase in carbohydrate oxidation and reduced fat oxidation. In addition, glucagon alone and in combination with GLP-1 significantly reduced plasma NEFAs. This is in contrast to the traditional understanding of glucagon in the stimulation of lipolysis, increasing fat oxidation and NEFA production⁴⁶⁶. However, my findings are consistent with the insulinotropic effect observed following GLP-1/glucagon co-administration, whereby the resultant rise in insulin causes inhibition of HSL^{467,468}. Similarly, a recent study has shown a significant decrease in NEFA concentration in healthy overweight human volunteers, during concomitant hyperinsulinaemia in response to 1mg im glucagon⁴⁶⁹. These findings are in contrast to Day *et al*²²⁹, in which chronic treatment with a GLP-1/glucagon co-agonist in DIO mice causes phosphorylation and activation of HSL. However, in the same study, a significant reduction in circulating insulin was shown which could account for the discrepant results.

These results show that at the dose administered, glucagon increased plasma glucose levels, most likely through glycogen breakdown. Co-administration of GLP-1 and glucagon ameliorated this hyperglycaemic effect although glucose remained elevated compared to levels in the vehicle control group. This was due to an apparent synergistic insulinotropic effect of glucagon and GLP-1 co-administration. Both hormones are known to stimulate insulin release from pancreatic β cells *in vivo* and *in vitro* in response to glucose⁴⁷⁰⁻⁴⁷². An additive insulinotropic effect is also seen following co-infusion of gastric inhibitory peptide (also a member of the secretin family) and GLP-1 in healthy volunteers⁴⁷⁰. This study shows that GLP-1 alone did not increase insulin levels compared to vehicle. However, GLP-1 co-administered with glucagon significantly

increased insulin levels to above those achieved with glucagon alone. It is likely that the lack of GLP-1 induced insulin release is secondary to low glucose levels, since the incretin effect of GLP-1 is glucose-dependent. Nevertheless, the apparent synergistic effect in response to GLP-1 and glucagon co-administration was not completely glucose dependent since combination therapy caused the largest rise in insulin, yet only a modest rise in glucose was seen. This is in keeping with previous studies that demonstrate glucagon-induced insulin release from human pancreatic β cells is independent of hepatic glucose production^{443,467}. The apparent synergistic effect in response to GLP-1 and glucagon co-administration has not been investigated. However, a reduction in GLP-1-induced insulin release in human pancreatic islets is seen in response to administration of a glucagon antagonist⁴⁷³. This suggests that glucagon and GLP-1 may act synergistically at the pancreatic β cell to regulate glucose metabolism.

In the work outlined in this chapter, the enhanced insulinotropic effect and increased EE in response to GLP-1/glucagon co-administration was demonstrated in non-diabetic overweight volunteers. Whether these effects are still evident in patients with insulin resistance or type 2 diabetes remains unknown. The sensitivity of the pancreatic islets to the incretin action of physiological levels of GLP-1 is reduced in patients with type 2 diabetes⁴⁷⁴, however pharmacological doses are still able to stimulate significant insulin release⁴⁷⁴. This could be investigated using a similar protocol as described here, however comparing weight-matched non-diabetic and type 2 diabetic volunteers.

Finally, the current study shows that GLP-1/glucagon co-administration reduces total and acylated ghrelin levels. There was no effect on ghrelin secretion following GLP-1 administration alone. This is in contrast to a previous study which shows that GLP-1 suppresses ghrelin secretion possibly through increased insulin secretion⁴⁵³. In my study, there was no significant increase in insulin in response to GLP-1 administration and may explain the different results. In contrast, there was a trend towards suppression of ghrelin levels in response to glucagon administration alone. This is in keeping with previous studies in which suppression of ghrelin secretion was seen following 1 mg im glucagon^{454,455}. These findings are in contrast with recent work by Gagnon *et al*, in which glucagon administration *in vitro* showed a dose-dependent increase in ghrelin secretion⁴⁷⁵. However the *in vitro* findings may simply reflect the absence of another modulator of ghrelin secretion through which glucagon acts indirectly to reduce ghrelin secretion.

In conclusion, my work demonstrates that glucagon increases EE when administered alone and in combination with GLP-1. However, the mechanism through which glucagon increases EE remains to be investigated. Activation of the SNS or increased circulating catecholamines may be involved. Alternatively, the increased EE may be due to an insulintropic effect of glucagon with subsequent increased glucose uptake in thermogenic tissues. A secondary finding from this study demonstrates that co-administration of GLP-1 and glucagon ameliorates the hyperglycaemia observed in response to glucagon alone. This is associated with a significant insulintropic effect above that seen with either hormone alone. Despite this rise in insulin secretion, the doses used in combination did not significantly improve glucose control compared to vehicle over the 45 minute infusion period. Whether these potentially beneficial effects on EE and glucose control in humans are sustained over a longer period requires further investigation. Nausea, a frequently described side-effect of both glucagon and GLP-1 administration, was not assessed in this study and requires further investigation should glucagon/GLP-1 co-administration be of clinical benefit as a treatment for obesity.

CHAPTER 5:

THE ACUTE EFFECTS OF CO- ADMINISTRATION OF LOW DOSE GLUCAGON AND GLP-1 ON ENERGY BALANCE AND NAUSEA IN HUMANS

5.1 Introduction

GLP-1R agonists are used to treat patients with type 2 diabetes, improving glucose control through their insulinotropic effects and resulting in weight loss due to reduced food intake¹⁵⁷. However, the magnitude of weight loss is restricted by dose-limiting nausea and vomiting⁴⁷⁶. Glucagon reduces food intake in rodents^{181,182,343,351} and humans^{180,186} and increases EE^{209,210}. Similarly, its use is limited by nausea and vomiting⁴⁷⁷.

Data from my earlier rodent studies demonstrate that co-administration of sub-anorectic doses of glucagon and GLP-1 significantly reduces food intake compared to vehicle whilst maintaining euglycaemia (chapter 2). Rodents do not vomit and there is considerable discussion as to whether studies, including CTA protocols, can reliably and accurately assess nausea in rodents⁴²⁴. Using manganese-enhanced magnetic resonance imaging (MEMRI) in mice, it has been shown that signal intensity in the VMN following GLP-1 administration closely resembles that generated by peripheral injection of LiCl, an agent used to study adverse behaviour such as nausea in animal studies³⁶⁶. This suggests that GLP-1 and LiCl may cause adverse symptoms through a common hypothalamic pathway in rodents. It has been postulated that the brainstem NTS plays a major role in integrating the emetic response in humans and is a target for anti-emetic agents⁴⁷⁸. Rodent studies suggest that both glucagon and GLP-1 reduce food intake via vagal mediated pathways^{87,351} and data from my earlier c-fos studies (chapter 2) demonstrate that peripheral administration of glucagon and GLP-1 increases c-fos expression in the NTS. This suggests that glucagon and GLP-1 may cause nausea through a common pathway via the NTS in the brainstem. Recently, functional magnetic resonance imaging has been used in humans to assess signals generated within the CNS in response to anorectic doses of gut hormones⁴⁴⁸. Unfortunately, the imaging techniques at present are not sensitive enough to detect changes in individual hypothalamic or brainstem nuclei. Therefore animal data demonstrating hypothalamic and brainstem pathways involved in nausea in response to anorectic gut hormones have still not been confirmed in humans.

Previous studies in humans have shown that GLP-1/PYY co-administration^{345,448} and OXM/PYY co-administration⁴⁷⁹ reduce food intake additively. However, following co-infusion of anorectic doses of OXM/PYY, one-third of participants experienced nausea despite the low doses used⁴⁷⁹. In contrast, no nausea was reported following GLP-1/PYY co-infusion³⁴⁵. In addition, the duration of peptide infusion appears to have a significant

effect on the incidence of nausea. Nausea has been reported following 110 minutes iv OXM infusion in healthy volunteers⁴⁷⁹. In contrast, OXM infusion at the same dose for 90 minutes demonstrates no side effects²²². Similarly, reported nausea following iv PYY₍₃₋₃₆₎ infusion in healthy human participants, increases with the duration of infusion⁴⁸⁰. There is currently no data investigating the duration of GLP-1 or glucagon infusion and the incidence of nausea. A previous study administered iv glucagon at 3ng/kg/min which was shown to reduce food intake with no reported nausea compared to saline controls¹⁸⁰. Although plasma glucagon levels were not measured, previous studies have shown that the same dose of glucagon administered iv increases plasma glucagon from 30 to 75-150 pmol/l⁴⁸¹⁻⁴⁸³. Iv administration of GLP-1 at 0.83 pmol/kg/min reduced food intake by 12% when infused over 2 hours with peak levels reaching 60-90 pmol/l¹⁵⁷. Although GLP-1 infusion reduced satiety and feelings of hunger, nausea was not specifically measured. In a separate study, 0.4 pmol/kg/min GLP-1 similarly reduced food intake compared to vehicle (although this did not reach significance) and circulating GLP-1 levels rose to 61.5 pmol/l³⁴⁵ following 90 minutes infusion. Throughout the study, there were no reported symptoms of nausea³⁴⁵.

Data from my earlier work in chapter 4 shows increased EE following glucagon and glucagon/GLP-1 co-administration during a 45 minute infusion. However, subjective feelings of nausea and satiety were not measured. Therefore, in the current study the protocol was adjusted to compare low doses of glucagon and GLP-1 in combination over a longer duration, to investigate whether these beneficial effects on energy homeostasis are sustained with minimal nausea. In addition to food intake, the effects of low dose infusion of glucagon and GLP-1 on glucose homeostasis and EE were investigated.

5.2 Hypothesis and aims

5.2.1 Hypothesis

I hypothesised that low doses of glucagon and GLP-1 in combination have an additive anorectic effect and increase REE without causing nausea. I hypothesised that co-administration of glucagon and GLP-1 at these doses ameliorates the rise in glucose levels in response to glucagon alone.

5.2.2 Aim

My aim was to investigate the effect on food intake, REE and glucose homeostasis following co-administration of glucagon and GLP-1 in non-diabetic, overweight human volunteers. I chose sub-anorectic doses of glucagon and GLP-1 to aim to minimise nausea and vomiting.

5.3 Materials and Methods

5.3.1 Peptides

Glucagon and GLP-1 were obtained as outlined in section 4.3.1.1.

5.3.2 Subjects

Sixteen non-diabetic, overweight volunteers were recruited for the study. Three were excluded due to abnormal eating behaviour at the first acclimatisation visit. The remaining thirteen completed the study. Four women and nine men, of mean age 31.6 $\pm$ 2.0 years (range: 21-41 years) and mean BMI 27.3 $\pm$ 0.7 kg/m² (range 25-33.3 kg/m²), were recruited by advertisement. Inclusion criteria were: age 18 years or over; stable body weight for preceding three months and BMI 25-40 kg/m². Exclusion criteria were: substance abuse, current smoker, significant past or current history of physical or psychiatric illness, regular medication other than contraceptives and pregnancy or breastfeeding. All subjects were screened and determined to be healthy by medical history, physical examination, haematological and biochemical testing and 12-lead electrocardiogram. Potential subjects were screened to exclude those with abnormal eating habits, as assessed by the SCOFF questionnaire⁴⁸⁴ and those with a high level of restrained eating as assessed by the Dutch Eating Behaviour Questionnaire⁴⁸⁵. All female participants were premenopausal and had regular menstrual cycles. Women of child-bearing age were advised to avoid pregnancy during the study and underwent urine tests to exclude pregnancy prior to each infusion.

The study was approved by the West London Research Ethics Committee (reference no.: 10/H0707/80). All subjects gave written informed consent and the study was carried out according to the principles of the Declaration of Helsinki.

5.3.3 Protocol

The study was designed as a randomised, double-blinded, placebo-controlled crossover study comparing four different pairs of infusion as shown in table 5.1.

A	B
Vehicle	Vehicle
GLP-1 (0.4 pmol/kg/min)	Vehicle
Glucagon (10 ng/kg/min)	Vehicle
GLP-1 (0.4 pmol/kg/min)	Glucagon (10 ng/kg/min)

Table 5.1. Randomised, double-blinded peptide infusion administration. Volunteers received two simultaneous infusions in order to compare the effects of GLP-1 and glucagon alone and in combination on food intake, EE and glucose homeostasis.

In my previous study investigating the effects of glucagon and GLP-1 on REE in man (chapter 4), the dose of GLP-1 used was 0.8 pmol/kg/min. In the current study, I chose to investigate lower doses as the duration of infusion was almost three times longer and my aim was to achieve positive effects on energy balance but with minimal nausea. Doses were selected based on published work investigating the effects of glucagon and GLP-1 on food intake in humans^{156,180,447,448}. During the dose-finding study, low doses of glucagon and GLP-1 were administered alone and in combination. The initial protocol for the dose finding study is shown in figure 5.1. A peptide infusion was commenced at t=0 during which a range of glucagon (1.5-100 ng/kg/min) and GLP-1 (0.4-0.8 pmol/kg/min) doses were administered alone and in combination. A meal was served at 90 minutes. Blood samples and visual analogue scale (VAS) scores (appendix C) were taken at the time points indicated.

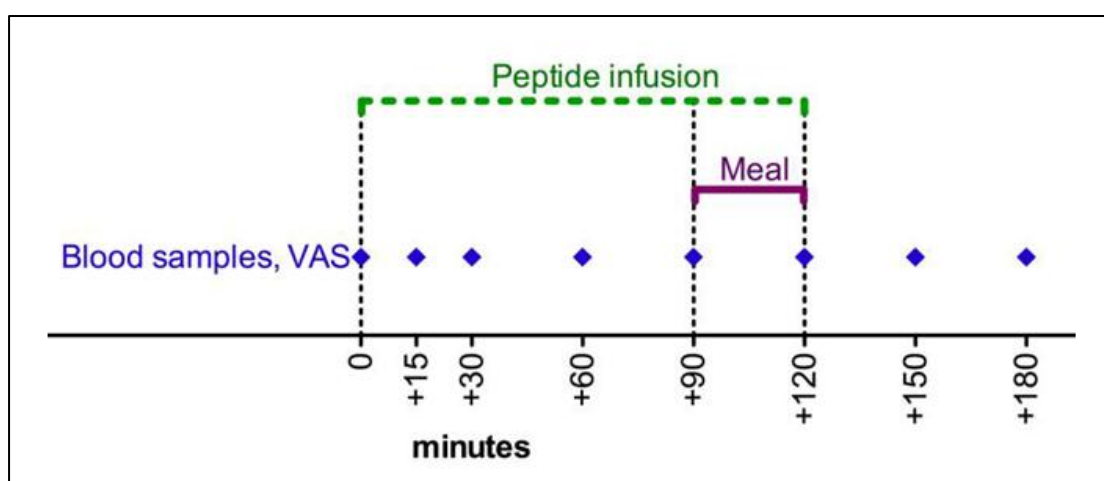


Figure 5.1 Protocol of dose finding study. Volunteers were cannulated and an infusion of vehicle/vehicle, glucagon/vehicle, GLP-1/vehicle or GLP-1/glucagon infused for 120 minutes. A meal was served at 90 minutes and blood samples and visual analogue scale (VAS) scores taken as outlined.

Following the dose finding study, the protocol was amended to include indirect calorimetry and alteration of sampling time points as illustrated in figure 5.2. Blood samples were collected at $t=-60$ and -30 to ascertain fasting blood levels of glucagon, GLP-1, insulin and glucose prior to the start of infusion and indirect calorimetry. Therefore, volunteers received iv infusions of placebo (gelofusine), glucagon (10 ng/kg/min, Novo Nordisk, Crawley, UK), GLP-1₍₇₋₃₆₎ amide (0.4 pmol/kg/min, Clinalfa Basic, Bachem, Switzerland) or combined glucagon and GLP-1₍₇₋₃₆₎ amide at the above mentioned doses. Volunteers attended for five consecutive visits spaced at least three days apart. The initial visit was an unblinded 'dummy' visit where gelofusine (Braun, Crawley, UK) was infused to acclimatise volunteers to study procedures. Since the primary outcome was food intake, subjects were asked to fast overnight from 2200h onwards.

Upon arrival, volunteers were asked to empty their bladder and then urine was collected at the end of each study visit to provide an estimate of urinary nitrogen excretion. Peripheral venous cannulae were inserted in both forearms, one for infusions and one for blood sampling. A three-way tap (BD Connecta Plus Stopcock, Becton Dickinson & Company, USA) was attached to the infusion cannula, to allow connection of two separate infusion lines.

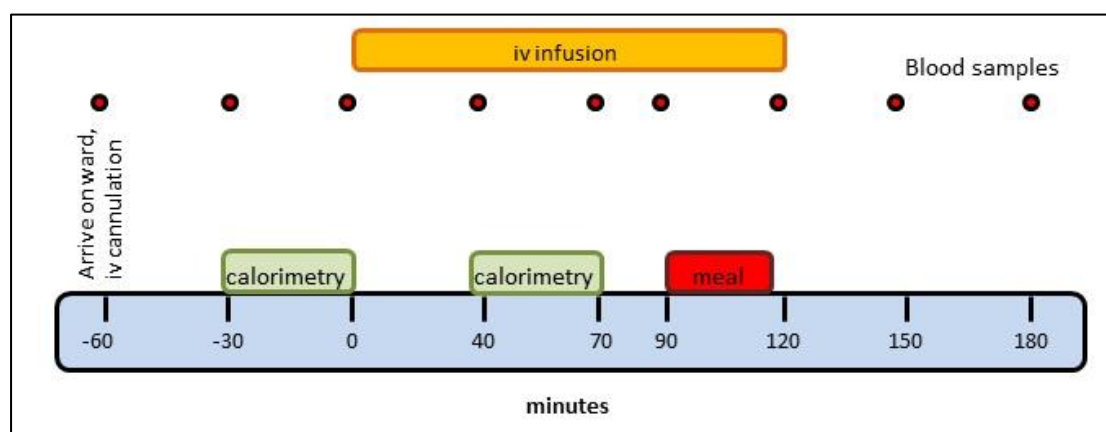


Figure 5.2 Protocol of study. Following acclimatisation, baseline indirect calorimetry readings were taken 30 minutes prior to the start of the infusion. An infusion of vehicle/vehicle, glucagon (10 ng/kg/min)/vehicle, GLP-1 (0.4 pmol/kg/min)/vehicle or glucagon (10 ng/kg/min)/GLP-1 (0.4 pmol/kg/min) was commenced at $t=0$ and continued for 120 minutes. Further calorimetry readings were taken between 40 and 70 minutes after the start of the infusion. A test meal was given at 90 minutes and the infusion stopped following this. Blood samples were taken at the time points indicated.

At -30 minutes (0900h), subjects were placed under the indirect calorimetry canopy (Gas Exchange Monitor, GEM Nutrition, Daresbury, UK) for 30 minutes to assess baseline REE. Calibration and setting up of the calorimeter is described in section 4.3.1.3. The iv infusion was started at t=0 (1100h) and lasted for 120 minutes. The preparation of peptide infusions and the ramping protocol was identical to that described in section 4.3.1.3. Indirect calorimetry was repeated from 40 to 70 minutes to assess REE during the infusion. Blood samples were taken at -60, -30, 0, 40, 70, 90, 120, 150 and 180 minutes for plasma glucose, glucagon, GLP-1 and serum insulin as described in section 4.3.1.3. Pulse and blood pressure were taken at each time point and volunteers asked to complete a VAS sheet (appendix C). An *ad libitum* meal, provided to excess, was served at 90 minutes (1130h) and volunteers allowed 20 minutes to eat until they felt comfortably full. Pre-prepared and standardised ready meals (Sainsbury's®) of known macronutrient and calorific content were used (chicken tikka masala (178 kcal/100g), macaroni cheese (189 kcal/100g), tomato and mozzarella pasta bake (127 kcal/100g), spaghetti bolognaise (188 kcal/100g)). Meals were weighed immediately before and after eating. Calorie intake was calculated according to weight of food consumed. The infusion was discontinued at 120 minutes (1200h). The duration of infusion in the current study was prolonged compared to that used in chapter 4, to investigate whether any beneficial effects on energy homeostasis and glucose control is sustained in the absence of nausea. Blood sampling and VAS measurements continued at 30 minute intervals until 180 minutes (1300h). Volunteers were then allowed to go home.

5.3.4 Plasma hormone assays

Glucagon and GLP-1 were analysed using in-house RIAs as described in section 4.3.1.4. Plasma glucose and serum insulin samples were analysed in the Department of Chemical Pathology, Imperial College Healthcare National Health Service Trust.

5.3.5 Statistical analysis

Food intake, change from baseline in REE and substrate oxidation rates, pulse and blood pressure and palatability VAS score, were analysed using one way ANOVA with Tukey multiple comparison post test. Two-way repeated measures ANOVA with Bonferroni post test was used to compare differences in glucose, insulin, pulse, blood pressure and

VAS scores at each time point. Results are presented as mean $\pm$ SEM and statistical significance defined as $p < 0.05$.

5.4 Results

5.4.1 Dose finding study investigating the acute effects of co-administration of low dose glucagon and GLP-1 on energy balance and nausea in humans.

5.4.1.1 Investigation of the acute affects of low dose glucagon and GLP-1 co-administration on food intake.

A dose finding study was undertaken to determine the lowest effective dose of glucagon and GLP-1 in reducing food intake compared to vehicle. The lowest effective anorectic dose of GLP-1 and glucagon alone was 0.8 pmol/kg/min and 15 ng/kg/min respectively (Fig. 5.3).

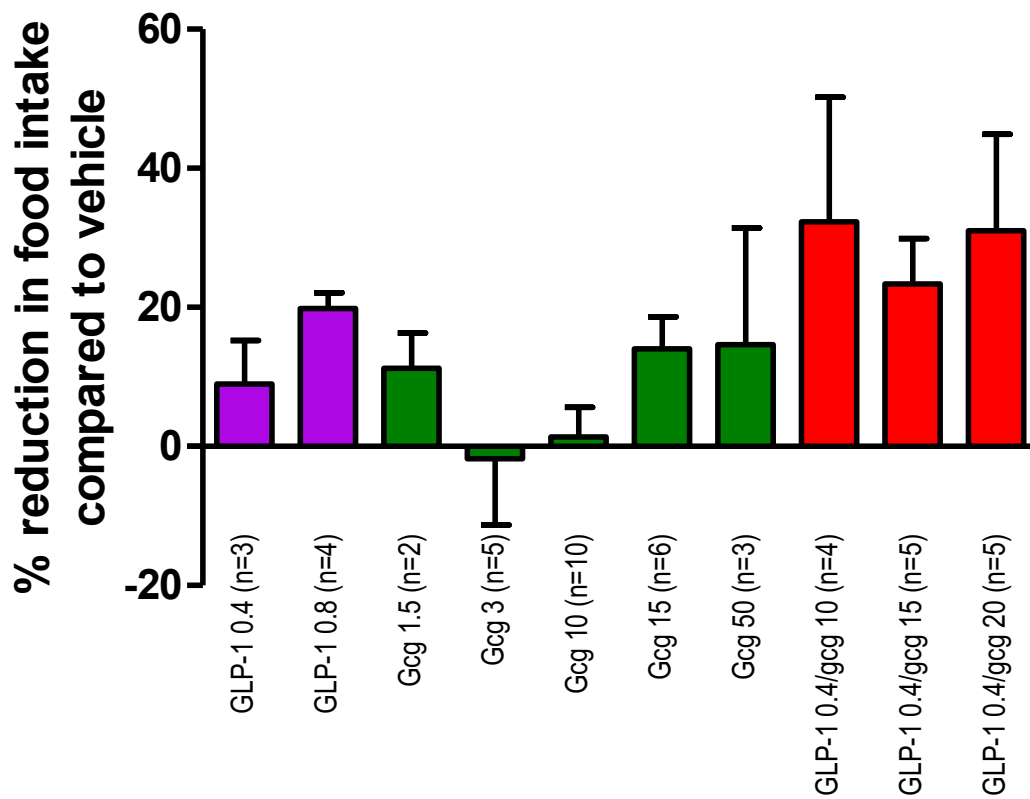


Figure 5.3. Percentage reduction in food intake compared to vehicle following the administration of glucagon and GLP-1 alone and in combination. Volunteers received an infusion of vehicle/vehicle, glucagon/vehicle, GLP-1/vehicle or GLP-1/glucagon for 120 minutes. A meal was served at 90 minutes and the percentage reduction in food intake compared to the vehicle visit calculated. The number of volunteers receiving each dose is shown in brackets. Data were analysed using one-way ANOVA with Tukey post test. Abbreviations: Glucagon-like peptide-1 (GLP-1), glucagon (GCG).

5.4.1.2. Investigation of the acute effects of low dose glucagon and GLP-1 co-administration on the incidence of nausea.

VAS scores were measured to assess the incidence of nausea. There was a dose-dependent increase in the incidence of nausea and one person vomited following glucagon administration (mean VAS nausea score at t=120: 0.9 ± 0.5 [vehicle] vs. 41.7 ± 21.1 mm [glucagon 50 ng/kg/min], $p < 0.01$ vs. vehicle, $n = 3-7$, fig. 5.4). Similarly, a dose-dependent increase in nausea was shown in response to GLP-1 0.4 pmol/kg/min and escalating doses of glucagon (mean VAS nausea score at t=150: 0.6 ± 0.3 [vehicle] vs. 26.8 ± 14.0 mm [GLP-1 0.4 pmol/kg/min + glucagon 20 ng/kg/min], $p < 0.001$ vs. vehicle, $n = 5-7$, fig. 5.4).

In contrast, there was no significant increase in VAS nausea score in response to GLP-1 administration at 0.4 or 0.8 pmol/kg/min compared to vehicle. However, there was a trend towards an increase in VAS nausea score following 0.8 pmol/kg/min GLP-1 compared to 0.4 pmol/kg/min (mean VAS nausea score at t=120: 0.3 ± 0.3 [GLP-1 0.4 pmol/kg/min] vs. 11.0 ± 10.5 mm [GLP-1 0.8 pmol/kg/min], $p = \text{ns}$, $n = 3-4$, fig. 5.4).

Although 0.8 pmol/kg/min GLP-1 was more effective at reducing food intake, the aim of this study was to investigate the effects of glucagon and GLP-1 co-administration on energy homeostasis without causing nausea. Since there was a trend for increased nausea following GLP-1 0.8 pmol/kg/min, doses of 0.4 pmol/kg/min GLP-1 and glucagon 10 ng/kg/min doses were chosen for further investigation with the aim to avoid nausea whilst demonstrating an additive anorectic affect.

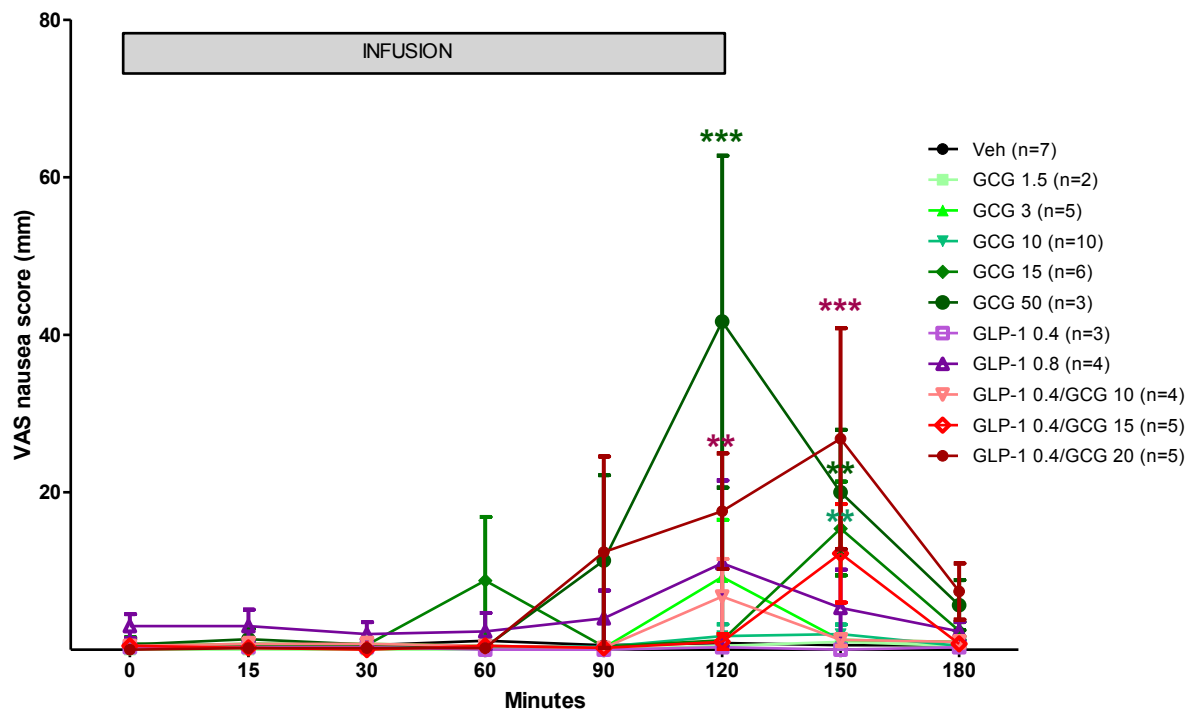


Figure 5.4. Incidence of nausea and vomiting following the administration of glucagon and GLP-1 alone and in combination. Volunteers received an infusion of vehicle/vehicle, glucagon/vehicle, GLP-1/vehicle or GLP-1/glucagon for 120 minutes. A meal was served at 90 minutes. The incidence of nausea and vomiting was documented throughout the infusion at various doses of glucagon and GLP-1, alone and in combination. Data were analysed using two-way ANOVA with Bonferroni post test. ** $p < 0.01$, *** $p < 0.001$ vs. vehicle. Abbreviations: Veh (vehicle), glucagon-like peptide-1 (GLP-1), glucagon (GCG).

5.4.2 Investigation of the acute effects of co-administration of low dose glucagon and GLP-1 on energy balance and nausea in humans.

5.4.2.1 Investigation of plasma glucagon and GLP-1 concentrations achieved following co-administration of low dose glucagon and GLP-1.

Mean baseline plasma concentration of GLP-1 in all volunteers prior to the start of the infusion was 21.7 ± 1.6 pmol/l (range: 6.1 - 60.2 pmol/l). Infusion of GLP-1 at 0.4 pmol/kg/min, increased circulating GLP-1 concentration to a peak of 117.2 ± 17.3 pmol/l, 70 minutes after the infusion was started (Fig 5.5A). In the GLP-1/glucagon combination group, GLP-1 levels rose to 111.5 ± 12.1 pmol/l, 70 minutes after the infusion was started. In all four infusion arms, there was a small rise in circulating GLP-1 levels following the meal.

Mean baseline plasma concentration of glucagon in all volunteers, prior to the start of infusion was 16.0 ± 1.9 pmol/l (range 4.0-59.4 pmol/l). At forty minutes, glucagon levels rose to 169.1 ± 35.1 pmol/l following glucagon infusion, and 159.0 ± 18.0 pmol/l following glucagon and GLP-1 co-administration (Fig 5.5B). GLP-1 alone or vehicle infusion did not elevate circulating glucagon. In addition, there was no change in circulating glucagon concentration following the meal. After the infusions were discontinued at 120 minutes, circulating GLP-1 decreased to post-prandial vehicle levels although this took 60 minutes. In contrast, circulating glucagon returned to baseline within 30 minutes of stopping the glucagon infusion and 60 minutes after glucagon and GLP-1 co-administration.

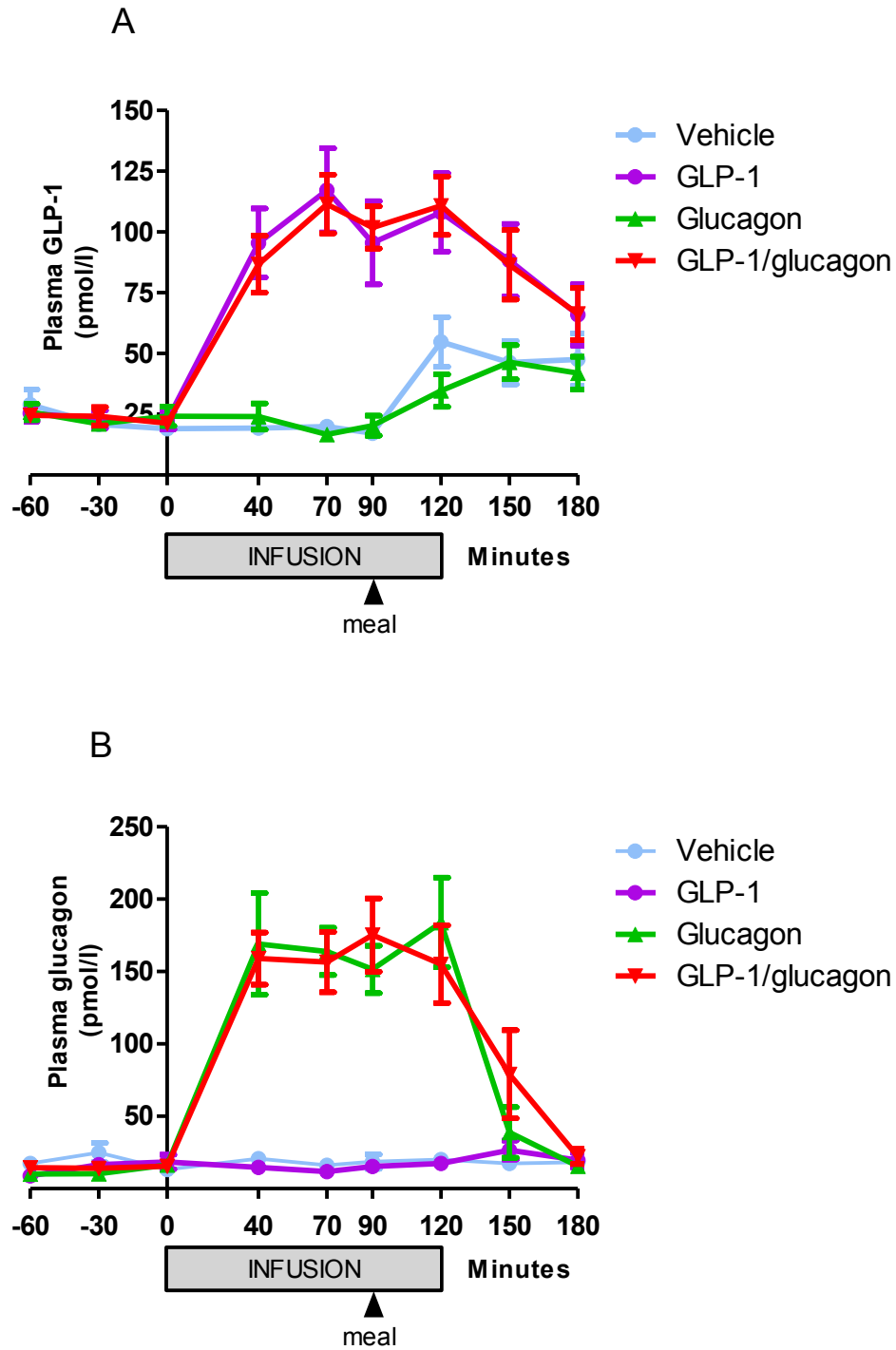


Figure 5.5 Plasma GLP-1 (A) and glucagon (B) following peptide infusion. Volunteers received an infusion for 120 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1 (0.4pmol/kg/min) / vehicle, glucagon (10ng/kg/min) / vehicle or GLP-1 (0.4 pmol/kg/min) / glucagon (10ng/kg/min). n=13.

5.4.2.2 Investigation of the acute effects of low dose glucagon and GLP-1 co-administration on food intake

Glucagon and GLP-1 alone did not significantly alter food intake at the doses administered. In contrast, glucagon and GLP-1 co-administration significantly reduced food intake compared to vehicle (mean energy intake: 1086 ± 94.2 [vehicle] vs. 878.5 ± 94.2 kcal/day [GLP-1/glucagon co-administration], $p < 0.05$ vs. vehicle, fig. 5.6, $n=13$).

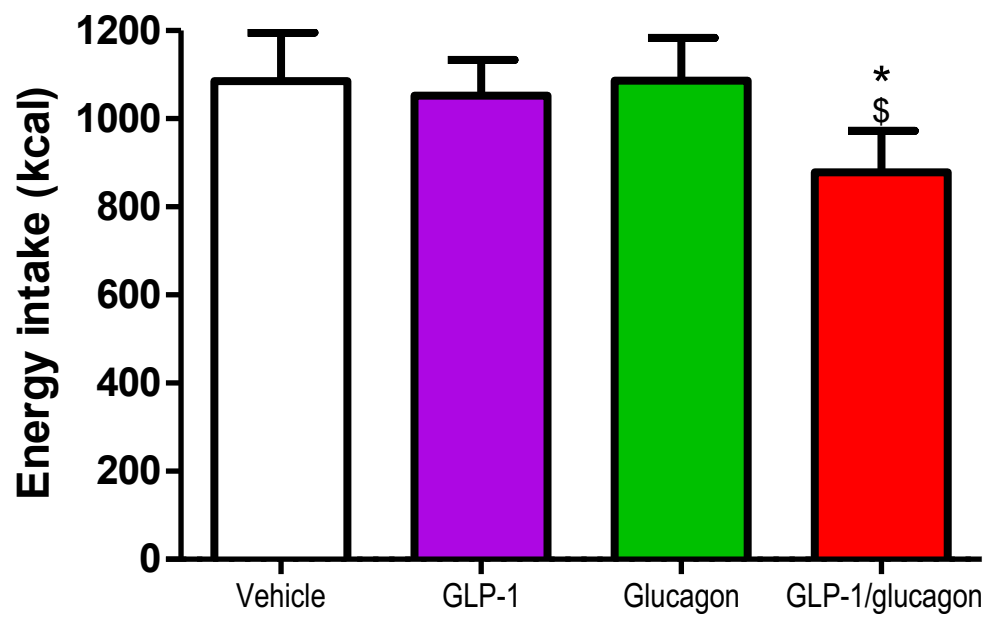


Figure 5.6. Energy intake following buffet meal in human subjects. Participants were given a meal of known quantity and calorie content during an infusion of vehicle/vehicle, GLP-1 (0.4pmol/kg/min) / vehicle, glucagon (10ng/kg/min) / vehicle or GLP-1 (0.4 pmol/kg/min) / glucagon (10ng/kg/min). Data were analysed using one-way ANOVA with Tukey multiple comparison post test. * $p < 0.05$ vs. vehicle, \$ $p < 0.05$ vs. glucagon. $n = 13$.

5.4.2.3 Investigation of the acute effects of low dose glucagon and GLP-1 co-administration on subjective nausea and satiety

Following the meal (t=120), there was a significant increase from baseline in the VAS score related to nausea whilst receiving GLP-1/glucagon co-administration compared to vehicle (mean VAS score (change from baseline): 0.8 ± 0.8 [vehicle] vs. 9.7 ± 8.2 mm [GLP-1/glucagon co-administration], $p < 0.01$ vs. vehicle, fig. 5.7B, n=13). There were no significant differences in VAS scores between any volunteers in response to other questions asked (fig. 5.7). In response to the question 'How tasty was the meal?', GLP-1/glucagon co-administration scored the lowest out of all infusion arms however again, this did not reach significance (mean VAS score: 82.3 ± 4.2 [vehicle] vs. 66.9 ± 8.0 mm [GLP-1/glucagon co-administration], n=13, fig. 5.7F).

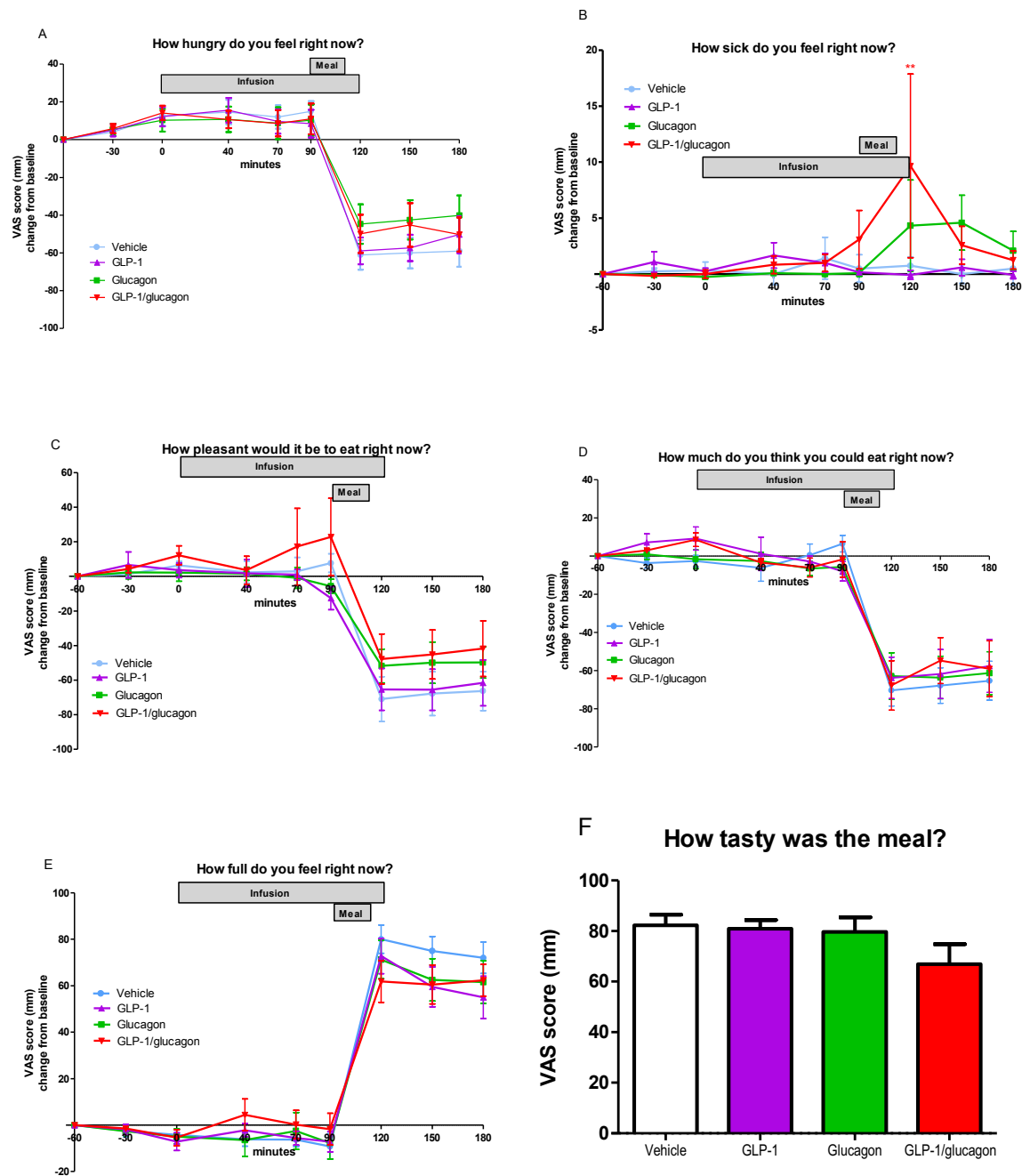


Figure 5.7 Change from baseline in visual analogue scale (VAS) scores in human participants. VAS scores were measured at each time point in response to the following questions: (A) 'How hungry do you feel right now?', (B) 'How sick do you feel right now?', (C) 'How pleasant would it be to eat right now?', (D) 'How much do you think you could eat right now?', (E) 'How full do you feel right now?' and (F) 'How tasty was the meal?'. Volunteers received an infusion for 120 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1 (0.4pmol/kg/min)/vehicle, glucagon (10ng/kg/min)/vehicle or GLP-1 (0.4 pmol/kg/min)/glucagon (10ng/kg/min). Data were analysed with two-way repeated measures ANOVA with Bonferroni post test (A-E) and one way ANOVA with Tukey post test (F). ** $p < 0.01$ vs. vehicle. $n = 13$.

5.4.2.4 Investigation of the acute effects of low dose glucagon and GLP-1 co-administration on energy expenditure

An increase in REE was demonstrated following administration of glucagon although this failed to reach significance (mean change from baseline in REE: -8.6 ± 12.6 [vehicle] vs. 66.8 ± 29.2 kcal/day [glucagon], $n=13$, fig. 5.8A). Similarly, GLP-1/glucagon co-administration increased EE but did not reach significance (mean change from baseline in REE: -8.6 ± 12.6 [vehicle] vs. 52.5 ± 27.1 kcal/day [GLP-1/glucagon co-administration], $n=13$, fig. 5.8A).

Glucagon alone significantly increased carbohydrate oxidation (mean change in carbohydrate oxidation: -0.005 ± 0.012 [vehicle] vs. 0.052 ± 0.013 g/min [glucagon], $p<0.01$ vs. vehicle, $n=13$, fig. 5.8B). Co-administration of glucagon and GLP-1 increased carbohydrate oxidation compared to vehicle to a greater extent than glucagon alone (mean change in carbohydrate oxidation: -0.005 ± 0.012 [vehicle] vs. 0.099 ± 0.012 g/min [GLP-1/glucagon co-administration], $p<0.001$, $n=13$, fig. 5.8B). In contrast, glucagon alone significantly decreased fat oxidation (mean change in fat oxidation: 0.001 ± 0.005 [vehicle] vs. -0.015 ± 0.005 g/min [glucagon], $p<0.05$ vs. vehicle, $n=13$, fig. 5.8C). Glucagon and GLP-1 co-administration caused a greater reduction in fat oxidation compared to vehicle than glucagon alone (mean change in fat oxidation: 0.001 ± 0.005 [vehicle] vs. -0.035 ± 0.005 g/min [GLP-1/glucagon co-administration], $p<0.001$ vs. vehicle, $n=13$, fig. 5.8C). There were no significant differences in protein oxidation over the entire study compared to vehicle. Glucagon alone significantly increased protein oxidation compared to GLP-1 alone (mean protein oxidation: 0.06 ± 0.007 [glucagon] vs. 0.044 ± 0.004 g/min [GLP-1], $p<0.05$ vs. GLP-1, $n=13$, fig. 5.8 D).

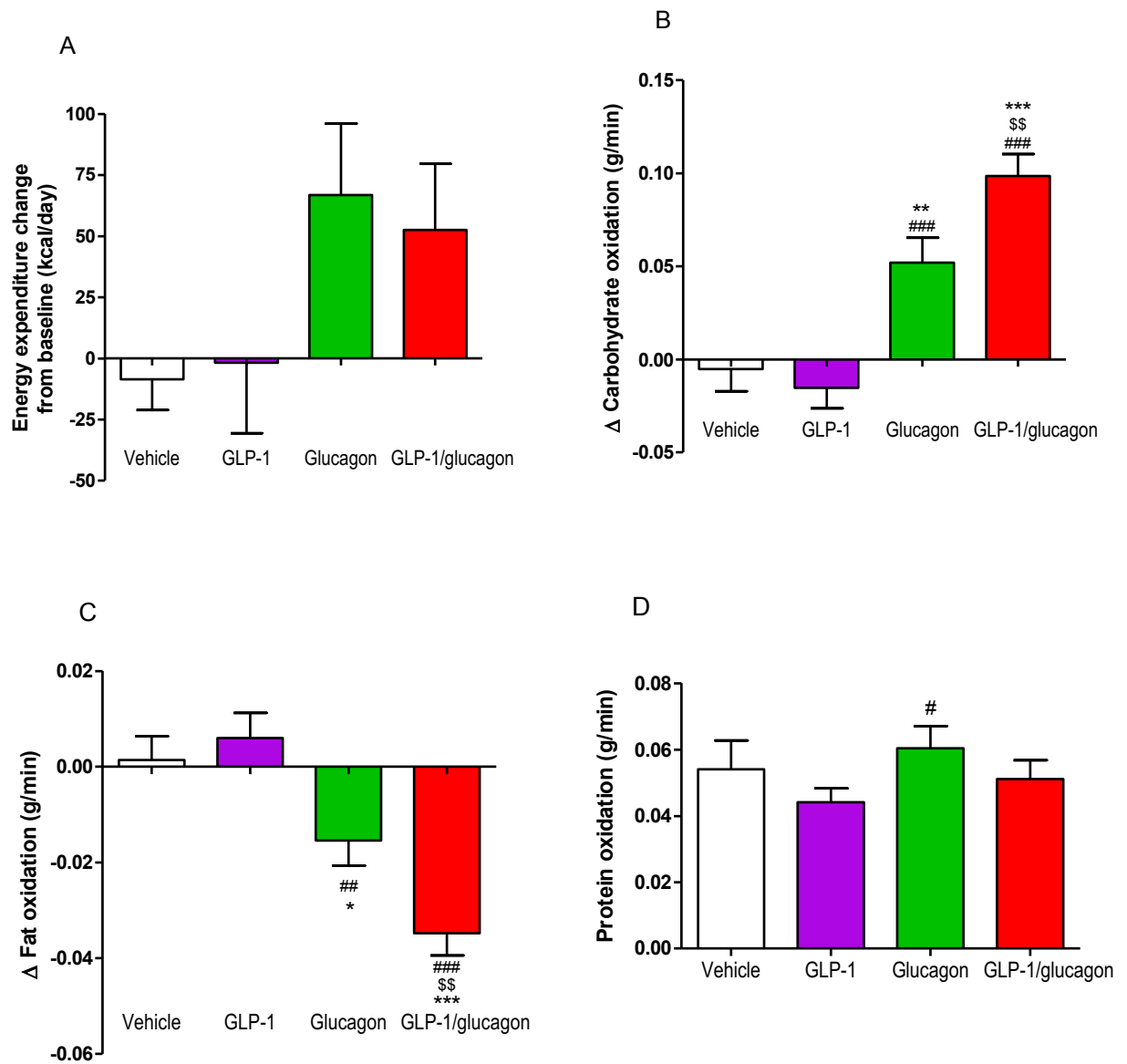


Figure 5.8 Change from baseline in resting energy expenditure (A), carbohydrate (B), fat (C) and protein (D) oxidation rates in human subjects. Indirect calorimetry was used to measure energy expenditure (A) before and at the end of an infusion of vehicle/vehicle, GLP-1 (0.4pmol/kg/min) / vehicle, glucagon (10ng/kg/min) / vehicle or GLP-1 (0.4 pmol/kg/min) / glucagon (10ng/kg/min). In addition, substrate oxidation rates were calculated for carbohydrate (B), fat (C) and protein (D). Data were analysed using one way ANOVA with Tukey multiple comparison post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. GLP-1; \$\$ $p < 0.01$ vs. glucagon. $n = 13$.

5.4.2.5 Investigation of the acute effects of low dose glucagon and GLP-1 co-administration on plasma insulin and glucose concentrations.

Mean baseline glucose concentration in all volunteers was 4.9 ± 0.1 mmol/l (range: 2.9-6.0 mmol/l) immediately prior to infusion. A significant rise in glucose was seen in response to glucagon administration, reaching a peak 40 minutes after the start of infusion (mean glucose: 4.6 ± 0.2 [vehicle] vs. 6.5 ± 0.3 mmol/l [glucagon], $p < 0.001$ vs. vehicle, $n=13$, fig. 5.9A). Glucose returned to baseline 60 minutes after the infusion was terminated. The increase in glucose following glucagon administration was significantly attenuated by the co-administration of GLP-1/glucagon. Forty minutes after the infusion started, there was a trend towards an increase in glucose concentration following GLP-1/glucagon co-administration (mean glucose: 4.6 ± 0.2 [vehicle] vs. 5.6 ± 0.4 mmol/l [GLP-1/glucagon co-administration], $n=13$, fig. 5.9A). At 70 minutes, glucose levels following GLP-1/glucagon co-administration were indistinguishable from those during the vehicle infusion.

Following a meal at $t=90$ minutes, glucose concentration increased during vehicle, GLP-1 and combined GLP-1/glucagon infusion. The glucose rise in response to the meal was lower in the GLP-1/glucagon co-administration arm compared to vehicle although this was not significant (120-180 minutes). Following the meal, glucose concentration did not fall during infusion of vehicle, GLP-1 or GLP-1/glucagon despite the infusion stopping at $t=120$ minutes. In contrast, during the glucagon infusion, glucose levels remained the same after the meal with a subsequent decline to baseline levels following discontinuation of the infusion (figure 5.9A). The differences in circulating glucose levels were also accompanied by changes in insulin secretion. All groups demonstrated a rise in insulin in response to the meal, with the highest insulin levels seen following glucagon infusion.

Prior to the meal, GLP-1/glucagon co-administration significantly increased insulin compared to vehicle (mean insulin: 6.7 ± 1.5 [vehicle] vs. 51.6 ± 6.8 mU/l [GLP-1/glucagon co-administration], $p < 0.01$ vs. vehicle, $n=13$, fig. 5.9B). Glucagon also increased insulin release compared to vehicle however this did not reach significance. There was no change in insulin secretion during GLP-1 infusion (prior to the meal). Despite this, there appeared to be a reduction in glucose levels compared to vehicle although this was not significant (fig. 5.9A).

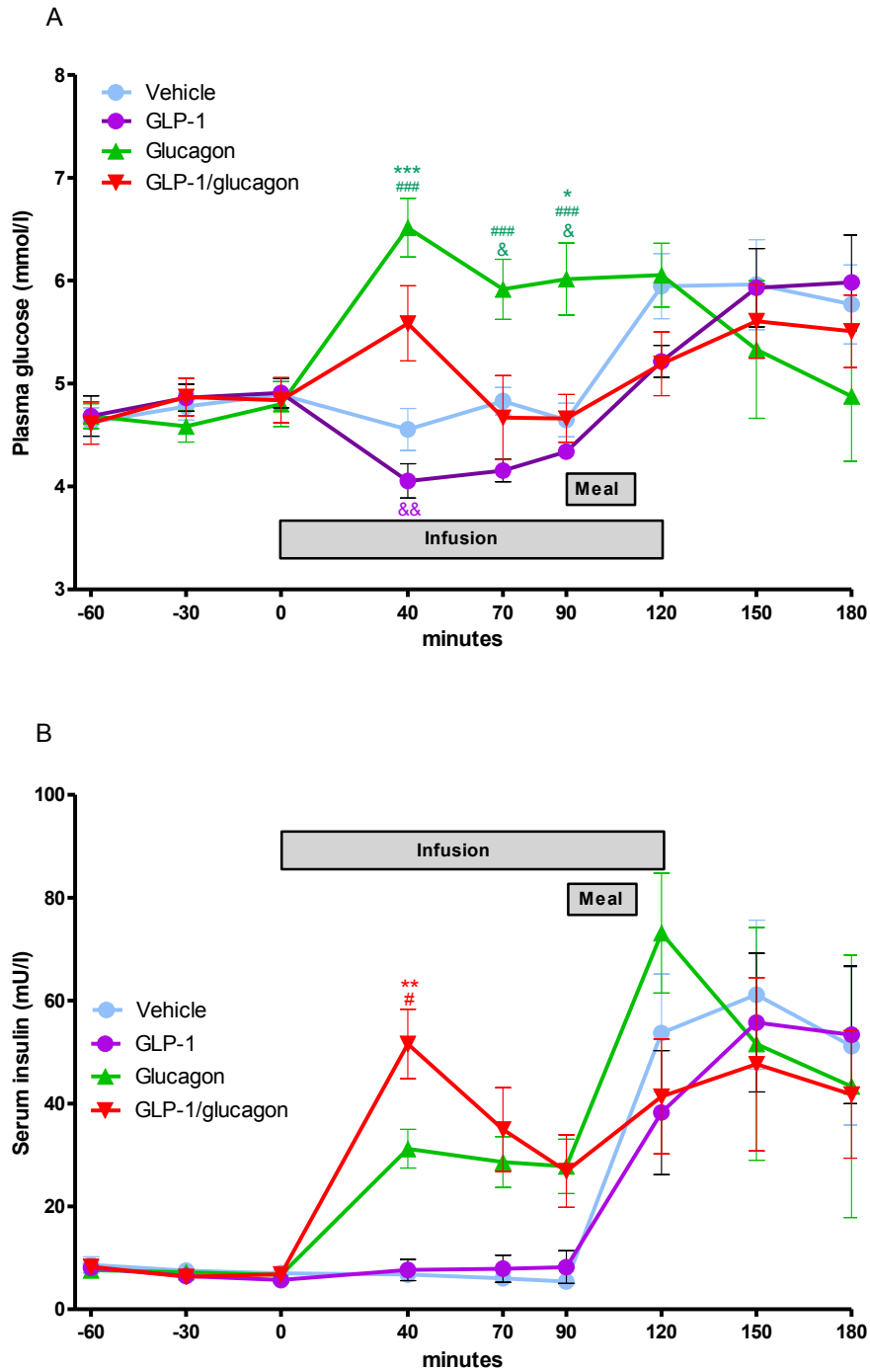


Figure 5.9 Glucose (A) and insulin (B) levels in human subjects during the study. Volunteers received an infusion for 120 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1 (0.4pmol/kg/min) / vehicle, glucagon (10ng/kg/min) / vehicle or GLP-1 (0.4 pmol/kg/min) / glucagon (10ng/kg/min). Glucose and insulin samples were collected at the time points indicated and a meal was served at 90 minutes. Data were analysed using two way ANOVA with Bonferroni multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle; # $p < 0.05$, ### $p < 0.001$ vs. GLP-1; & $p < 0.05$, && $p < 0.01$ vs. GLP-1/glucagon. $n = 13$.

5.4.2.6 Investigation of the acute effects of low dose glucagon and GLP-1 co-administration on cardiovascular parameters

There were no significant differences in pulse, systolic or diastolic pressure between the infusion arms (figure 5.10). A rise in pulse was seen in response to the meal at t=120 minutes in all volunteers. Change between baseline and 90 minutes during infusion showed no significant changes in response to any of the infusion arms, however there was a trend for glucagon alone and in combination with GLP-1 to increase pulse rate and diastolic blood pressure (figure 5.10B and 5.10F).

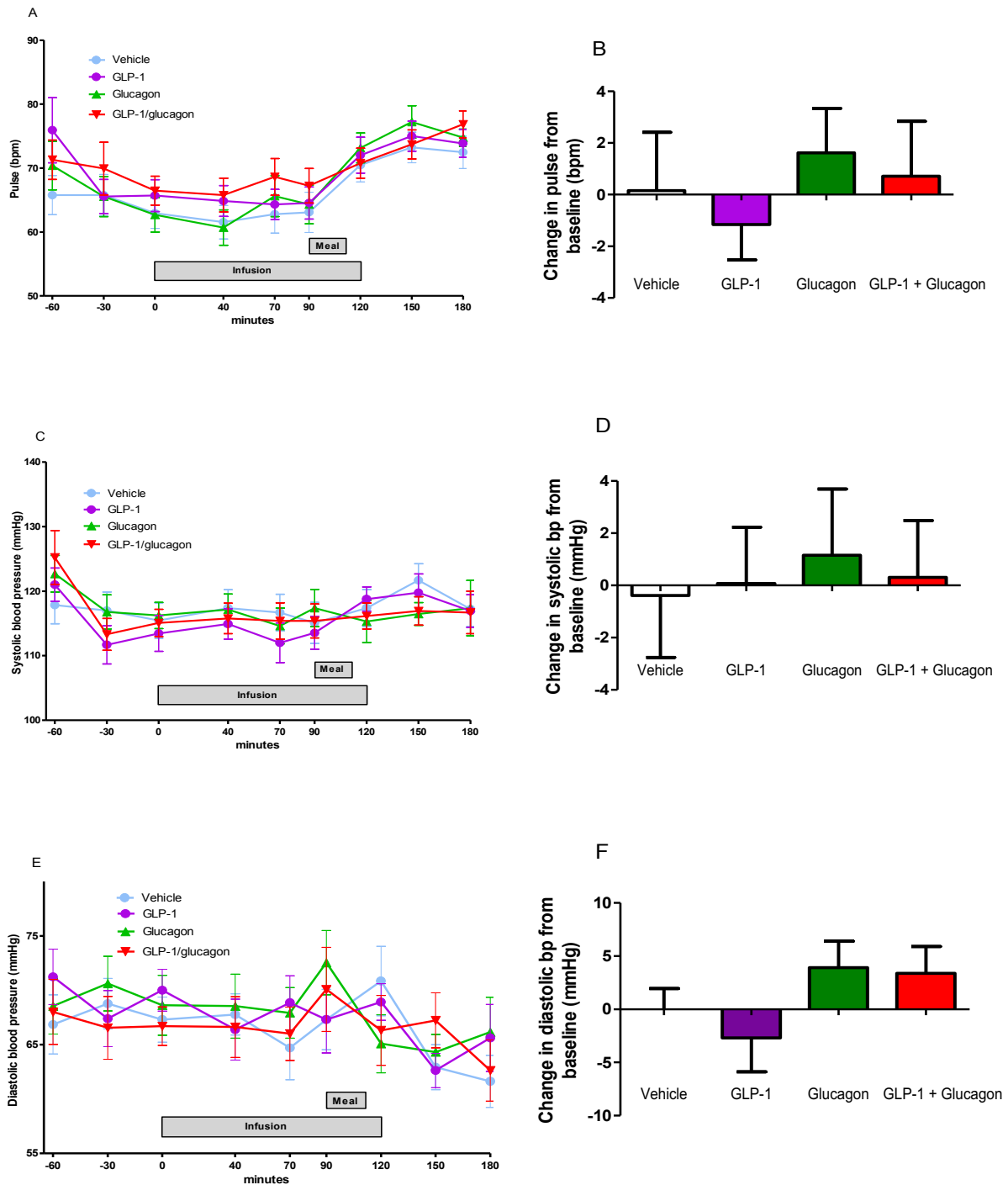


Figure 5.10 Pulse (A+B), systolic (C+D) and diastolic blood pressure (E+F) in human subjects during the study. Volunteers received an infusion for 120 minutes (denoted by grey bar) of vehicle/vehicle, GLP-1(0.4pmol/kg/min)/vehicle, glucagon (10ng/kg/min)/vehicle or GLP-1 (0.4 pmol/kg/min)/glucagon (10ng/kg/min). Pulse and blood pressure was measured at the time points indicated and a meal was served at 90 minutes. Comparison is also made between change in pulse and blood pressure from baseline (t=0) and prior to the meal (t=90). Data were analysed using two way ANOVA with Bonferroni multiple comparison test (A,C+E) and one-way ANOVA with Tukey post test (B,D+F). n=13. Abbreviations: beats per minute (bpm), millimetres of mercury (mmHg).

5.5 Discussion

This study demonstrates that sub-anorectic doses of GLP-1 and glucagon significantly reduce food intake when co-administered. This additive anorectic effect supports a role for glucagon in appetite control in humans. The first study to investigate the effects of iv glucagon infusion on food intake in humans was published by Geary *et al* in 1992¹⁸⁰. Prior to this, glucagon had been administered im at 1-2mg doses prior to a test meal with reports of nausea^{186,187}. Geary *et al* demonstrated that glucagon infusion (3ng/kg/min) for 10 minutes reduced food intake compared to saline infusion in healthy normal weight male volunteers. In the current study, the infusion lasted two hours and a dose of 10ng/kg/min was given. The discrepancy in effective doses in reducing food intake is likely to be due to the different protocols followed and subjects recruited. In the current study, subjects were overweight (mean BMI 27.3) and fasted from 10pm the night before the study. In contrast, subjects studied by Geary *et al* were of normal weight (mean BMI 21.8) and had been given a standardised breakfast consisting of 300 kcal two and a half hours prior to the start of the study, followed by a small meal (190 kcal) five minutes prior to the start of the infusion. Furthermore, the infusion only lasted 10 minutes and participants were given the test meal during this time period. Therefore in addition to the length of infusion and subjects studied, the protocols differ in that the studies are comparing fasted versus fed states.

The current study demonstrates for the first time that glucagon and GLP-1 co-administration reduces food intake in an additive manner in humans. It has been suggested that peptides from discrete families, which act through entirely independent receptor pathways, are more likely to inhibit feeding additively⁴⁸⁶. Co-administration of glucagon and other anorectic gut hormones such as PYY or PP has not been studied but would test this hypothesis. GLP-1/PYY and OXM/PYY co-administration at sub-anorectic doses have additive effects on food intake reduction^{345,448,479}. In contrast, PYY and PP co-administration do not reduce food intake compared to controls in rodents or humans⁴⁸⁶. Although PYY and PP reduce food intake via different receptors (Y2 and Y4 receptors respectively), both hormones can also activate the Y5 receptor leading to an increase in food intake ^{239,487}.

Similarly, glucagon and CCK alone decrease food intake although co-administration has no effect on food intake compared to controls¹⁸⁰. The reason for this is unknown. CCK-1Rs are found on pancreatic α cells^{488,489}. However, *in vivo* studies have found no effect of CCK-8 infusion on plasma glucagon levels in humans⁴⁹⁰ or sheep⁴⁹¹. Similarly, infusion

of a CCK antagonist, does not alter plasma glucagon levels in humans⁴⁹². It is possible that CCK and glucagon interact elsewhere in the body to prevent a reduction in food intake when administered in combination. Vagotomy studies suggest that both hormones act via the vagus nerve to reduce food intake^{87,351}. Therefore antagonism at the level of the brainstem or higher centres within the brain could account for the results.

The aim of the current study was to investigate whether beneficial effects of glucagon and GLP-1 on energy homeostasis and glucose control was sustained using lower doses over a longer infusion period, without causing nausea. Post-prandial nausea score was significantly increased following the co-administration of GLP-1 and glucagon. GLP-1R agonists used in the treatment of type 2 diabetes are known to cause nausea and vomiting especially upon initiation of treatment, however the incidence of such side effects generally diminish over time⁴⁶⁴. Continued glucagon administration in humans has not been studied and hence, the effect on nausea is unknown. Most clinical indications for glucagon use suggest im injection for short-term use only and include reversal of hypoglycaemia⁴⁹³, relaxation of smooth muscle in the gut for radiological studies⁴⁶⁵, β blocker overdose⁴⁹⁴ and cardiac failure⁴⁹⁵. Although many of these studies describe nausea as a side effect, the effect appears to be transient which is in keeping with the rapid glucagon clearance following im administration.

The mechanism through which nausea and vomiting is largely unknown. Evolutionarily, these symptoms served as a defense against the consumption of harmful substances⁴⁹⁶. Activation of the autonomic system results in sweating, salivation and vasoconstriction which frequently accompanies symptoms of nausea and vomiting. Nausea can be difficult to study in animal models such as rodents that lack an emetic response. Although CTA studies have been used as a marker of nausea, it has been argued that it does not reliably assess nausea since some drugs of addiction also produce the same response⁴⁹⁷. In addition, CTA studies rely on complex CNS pathways involved with learning and memory which can confound results related to nausea and appetite. Although a final common neural pathway or 'vomiting centre' has not been defined, animal studies (including those in animals with a vomiting response) have suggested some key areas within the brain important in the emetic response. Gut vagal afferents converge on the NTS in the brainstem and it is possible that toxic substances in the bloodstream act on the AP, which lacks a complete BBB⁴⁹⁶. In addition, afferent inputs from the cerebral cortex and vestibular system converge in the NTS supporting the concept that the NTS is a key integrator for nausea signals⁴⁹⁶. Data from my rodent

studies in chapter 2, showed that glucagon and GLP-1 co-administration increased c-fos expression in the cNTS. This would support the hypothesis that co-administration of both hormones reduces food intake but at the expense of nausea. It would be interesting to investigate whether co-administration of glucagon and GLP-1 modulates fMRI signal in the NTS of humans. Furthermore, there is a lack of data concerning why some individuals are more prone to nausea than others. Factors such as adiposity, renal and hepatic clearance, hormonal interactions including higher oestrogen levels in women and sex hormone binding globulin, and perhaps genetic predisposition may all influence the pharmacokinetics of a drug and hence the degree of nausea⁴⁹⁸.

In the current study, administration of glucagon alone and in combination with GLP-1 increased REE although this did not reach statistical significance. Interestingly there was a significant rise in carbohydrate oxidation and reduction in fat oxidation following glucagon alone and in combination with GLP-1 which supports findings from my previous study investigating EE in man (chapter 4). The results also suggest that substrate oxidation is not the sole determinant of increased EE in response to glucagon. In this study, there was a trend towards increased pulse and systolic blood pressure in response to glucagon alone and in combination with GLP-1, although this did not reach statistical significance.

The current study also shows that glucagon and GLP-1 co-administration has a greater insulinotropic effect than either hormone alone. Furthermore, although an initial rise in glucose was seen in the co-administration arm, this returned to control values within 70 minutes. Following a meal, there was a smaller rise in glucose following GLP-1/glucagon co-administration compared to other infusion arms and may reflect the reduced food intake observed following GLP-1/glucagon co-administration. In addition, both GLP-1 and glucagon delay gastric emptying^{164,398} which would delay a rise in glucose seen and may explain the results. Paracetamol absorption studies or other radiolabelled tracer studies could be performed to investigate this.

Interestingly, administration of GLP-1 did not increase insulin levels yet there was a trend for reduced glucose levels. There is increasing evidence that GLP-1 and GLP-1R agonists reduce hyperglycaemia independently of insulin. Incubation of human hepatocytes with exendin-4 activates cAMP⁴⁹⁹ and glycogen synthase activity⁵⁰⁰. In humans, GLP-1 (9-36) amide infusion in fasting and glucose clamp studies decreases hepatic glucose output by up to 50% in obese and insulin resistant subjects⁵⁰¹. Within the liver, glucose output can also decrease in response to increased glucose utilisation.

Glucokinase is an important enzyme which converts glucose into glucose-6-phosphate and glucokinase activators are being developed as an alternative treatment in type 2 diabetes⁵⁰². Recently, a study has shown that acute and chronic administration of exendin-4 dose dependently increases hepatic glucokinase activity in *db/db* mice⁵⁰³. This effect is abolished by the prior administration of exendin-4₍₉₋₃₉₎ (a GLP-1R antagonist)⁵⁰³. Furthermore, chronic treatment of exendin-4 in streptozotocin-treated mice, a model of diabetes, restores hepatic glycogen with no significant changes in serum insulin levels⁵⁰³. Similarly, GLP-1R knockout mice demonstrate impaired ability to suppress endogenous glucose production and hepatic glycogen accumulation during insulin clamps⁵⁰⁴. This implies that the GLP-1R is required to control hepatic glucose output despite the presence of insulin. Therefore, GLP-1 may improve glycaemic control through at least two mechanisms, firstly stimulation of insulin secretion by pancreatic β cells and secondly, a direct effect to reduce hepatic glucose output. Results from the current study support a role for GLP-1 in reducing hepatic glucose output, since there was a trend for reduced glucose concentration following GLP-1 infusion, yet insulin concentration remained low.

In conclusion, this study shows that at low doses of GLP-1 and glucagon, co-administration reduces food intake additively in non-diabetic, overweight human volunteers. However, the increase in nausea is likely to limit the dose at which glucagon/GLP-1 co-agonists can be administered. At the doses given, there is a non-significant increase in REE and no significant deterioration in glucose control over the infusion period. These findings suggest that drug therapy aimed at glucagon and GLP-1 receptor co-agonism could potentially result in weight loss through the dual mechanism of reduced food intake and increased EE, without detrimental effects on glucose homeostasis. Longer term studies however are required to investigate this and whether symptoms of nausea diminish with the duration of administration.

CHAPTER 6:

GENERAL DISCUSSION AND FUTURE WORK

6.1 General discussion

The worldwide incidence of obesity is increasing^{1,505}. 65% of men and 58% of women in England are overweight or obese⁵⁰⁶. Between 2001 and 2011, the number of hospital admissions with a primary diagnosis of obesity increased eleven-fold⁵⁰⁶. Furthermore, obesity is increasing amongst children with 10% of 4-5 year-olds and 20% of 10-11 year-olds classified as obese⁵⁰⁶. Although frequently regarded as a cosmetic problem, obesity increases the risk of type 2 diabetes, heart attacks, strokes, cancer and hypertension⁵⁰⁷. In addition, obesity reduces life expectancy by approximately nine years and accounts for almost six hundred deaths per week in England alone⁵. Therefore, obesity has significant health and socioeconomic implications.

There are currently no effective drug treatments for obesity that result in sustained weight loss. Gastric bypass surgery remains the most successful approach in targeting obesity⁵⁰⁸. The rapid weight loss achieved is associated with a rise in gut hormones, including PYY⁵⁰⁹ and GLP-1⁵⁰⁹. However certain criteria must be fulfilled before eligibility for surgery is considered. These criteria include BMI > 40 kg/m² or BMI 35-40 kg/m² and other co-morbidities, such as diabetes, that would be improved by weight loss⁵¹⁰. Many patients do not meet these criteria or are unsuitable for surgery which itself is associated with significant complications⁵¹¹. Therefore there is a clinical need for the development of effective drug treatments to combat obesity. Orlistat® is currently the only medication available for the treatment of obesity in the UK. Weight loss is achieved primarily through reduced intestinal fat absorption with significant gastrointestinal side-effects as a result⁵¹². Weight loss following orlistat treatment is disappointing, ranging from no significant benefit⁵¹³ to 3-4% over two years⁵¹⁴. Recently, rimonabant and sibutramine were withdrawn by the Medicines and Healthcare product Regulatory Agency (MHRA) due to psychiatric and cardiovascular side-effects respectively^{510,515}. A second class of drugs, the GLP-1 analogues are licensed for the treatment of type 2 diabetes, and result in approximately 3% weight loss at 6 months^{516,517}. Recently the Food and Drug Administration (FDA) approved two new anti-obesity agents, Qysmia®, (combination of phentermine and topiramate) and Belviq® (lorcaserin hydrochloride), a serotonin receptor agonist. Ongoing trials are also underway for several other drugs including Contrave® (bupropion and naltrexone combination)⁵¹⁸, Tesofensine®⁵¹⁹, a noradrenaline, dopamine and serotonin reuptake inhibitor and a combination of bupropion and zonisamine (an anti-epileptic drug)⁵²⁰. Several gut hormones are also undergoing clinical trials as potential anti-obesity agents

and include ghrelin antagonists⁵²¹ and analogues of PYY⁵²², PP⁵²³ and OXM⁵²⁴. In addition, glucagon/GLP-1 receptor co-agonists are currently in phase I clinical development^{525,526}.

To date, all treatments for obesity have focused on reducing food intake or fat absorption. However, it has recently become evident that increasing EE may provide an alternative approach to weight loss^{228,229}. OXM, a GCGR and GLP-1R agonist, exemplifies this dual approach to weight loss. Animal and human studies have demonstrated the efficacy of OXM in modulating energy homeostasis and decreasing body weight^{219,220,222-225,227,446,527,528} and phase I trials in man are ongoing⁵²⁴. Although OXM binds to the GLP-1R and GCGR, it does so with lower affinity than the cognate hormones. Furthermore, the mechanisms through which OXM exerts its beneficial effects on body weight are not fully understood. Therefore, this thesis investigates glucagon and GLP-1 co-administration and energy homeostasis, focusing on food intake and EE as a dual approach to weight loss.

Glucagon and GLP-1 co-administration and the effect on food intake

In chapter 2, I demonstrated that glucagon and GLP-1 reduced food intake in rodents and activated similar areas within the brainstem and amygdala. The anorectic effect following glucagon/GLP-1 co-administration was subsequently confirmed for the first time in humans as described in chapter 5. Although central mechanisms underlying the anorectic effect of glucagon are unknown, diminished anorectic response to glucagon in vagotomised animals and those with AP and NTS lesions³⁵³ suggest that a vagal mediated pathway via the brainstem may be the main site of action. Similarly, vagotomy attenuates the anorectic effect of GLP-1 and hypothalamic c-fos expression in rats⁸⁷. The effects of vagotomy on c-fos expression following peripheral glucagon administration are unknown. Such a future study may identify vagal-dependent pathways involved in the anorectic action of glucagon.

The anorectic effect following GLP-1 administration is also thought to be mediated via the circulation^{360,529}. Previous work using radiolabelled GLP-1 has shown that GLP-1 crosses the BBB through simple diffusion³⁶⁰. Although GCGRs are expressed in the hypothalamus and brainstem, their expression in specific nuclei within these areas is unknown. Central administration of glucagon reduces food intake, suggesting a potential hypothalamic site of action³⁶¹⁻³⁶³. Glucagon may reduce food intake directly via the

circulation in a similar way to GLP-1. Using a radiolabelled technique as described above, together with localisation studies to identify neuronal populations activated, may improve our understanding of the mechanism underlying the anorectic effect of glucagon.

It was previously thought that glucagon reduces food intake indirectly through modulation of glucose³⁵⁴⁻³⁵⁶, insulin^{190,359}, ACTH³⁷⁷ or ghrelin^{454,455}. However, neither hyperglycaemia or infusions of insulin or insulin antibodies affect the anorectic response to glucagon in animals^{356,357,359}. A recent study in chicks suggests that central administration of glucagon suppresses food intake via a CRH-induced anorexigenic pathway³⁶¹. Although glucagon increases ACTH secretion from the anterior pituitary, the c-fos studies presented in this thesis (chapter 2) do not support a role for glucagon and hypothalamic neuronal activation. In addition, iv glucagon administration did not increase circulating cortisol in man (chapter 4). In contrast, glucagon and GLP-1 co-administration significantly reduced circulating ghrelin in man (chapter 4). Similar findings are seen following iv and ICV OXM^{222,530}, although the effect of ICV OXM is attenuated by the prior administration of the GLP-1R antagonist exendin-4₍₉₋₃₉₎⁵³⁰. This suggests that the effect on ghrelin levels is mediated via the GLP-1R. In contrast, previous studies have shown that glucagon alone decreases ghrelin although this effect may be insulin-dependent^{454,455}.

An alternative explanation may be that glucagon reduces food intake through stimulation of the SNS and release of catecholamines. Administration of glucagon increases SNS activation^{379,407} and circulating catecholamines^{456,457}. Future work in animals to test this hypothesis could include attenuation of catecholamine output by adrenal medullectomy, adrenal sympthomimectomy or administration of selective β -adrenoceptor antagonists, prior to studying the effects of glucagon administration on food intake.

In this thesis, I demonstrate that glucagon and GLP-1 co-administration reduces food intake additively in rodents (chapter 2) and humans (chapter 5). In man, this effect was associated with an increase in nausea score at the doses administered (chapter 5). Although the mechanism through which glucagon or GLP-1 induce nausea is unknown, direct activation of brainstem neurones eliciting an emetic response, or reduced gastric emptying, are likely to be involved. Both glucagon and GLP-1 reduce gastric emptying^{164,398}, an effect which may be additive following co-administration of glucagon

and GLP-1. This could be further studied in humans using paracetamol absorption studies⁵³¹, 13-C breath test⁵³¹ or radiolabelled food⁴⁸⁰ and assessing gut transit time.

My earlier rodent studies (chapter 2) show that glucagon and GLP-1 increase c-fos expression in similar areas within the brain, notably the AP, cNTS, PBN and CeA. This suggests that both hormones may act through a common pathway to reduce food intake. Furthermore, these areas are also implicated in the emetic response⁴⁹⁶ and gastric emptying⁴⁹⁶. GLP-1 neurones in the brainstem innervate vagal efferent and catecholamine neurones associated with cardiovascular control⁵³². These findings support a role for GLP-1 and modulation of gastric emptying and cardiovascular function. Immunohistochemistry or another method of characterisation could determine whether glucagon and GLP-1 induce similar patterns of neuronal activation. If both hormones reduce food intake via the same mechanism, this may limit the use of glucagon/GLP-1 co-agonists in the treatment of obesity, particularly since side-effects may also be additive.

Glucagon and GLP-1 co-administration and the effect on energy expenditure (EE)

Administration of glucagon alone, and in combination with GLP-1, was shown for the first time to increase REE in humans (chapter 4). Although the mechanism through which glucagon increases REE is unknown, activation of the SNS and BAT thermogenesis may be involved acutely^{209,214-216,218,407}. The increase in REE in response to glucagon was associated with a trend towards a rise in pulse and systolic blood pressure, suggesting activation of the SNS. ICV administration of glucagon in wild-type mice increases BAT thermogenesis, sympathetic nerve activity and increased expression of PGC1- α and UCP-1⁴⁰⁷. Recently, several imaging modalities including FDG-PET⁵³³, MRI^{534,535} and MIBG^{536,537} have demonstrated BAT activation and the ability to quantify BAT mass in humans. Furthermore, thermal imaging has been used as a non-invasive technique for assessing BAT activation in children⁵³⁸. These imaging techniques may offer an exciting opportunity to investigate the role of peripherally administered glucagon and GLP-1 and BAT activation in humans.

Until recently, the investigation of glucagon and EE has focused on its acute effects. The chronic effects of glucagon administration and EE are largely unknown. Long-term glucagon administration is limited due to enzymatic breakdown within the circulation, resulting in a short half-life. Recent development of a GCGR agonist was shown to

increase EE in wild-type mice following three days of s/c administration⁴⁶³. These effects were abolished in FGF-21 knockout mice⁴⁶³ suggesting a role for glucagon in the modulation of FGF-21 expression and EE. However, the rise in EE was accompanied by increased food intake and subsequently no change in body weight. Therefore, any future GCGR-based treatment for obesity will need to demonstrate increased EE in addition to reduced food intake to achieve successful weight loss.

Previous groups have designed hybrid glucagon/GLP-1 co-agonists that bind and activate the GCGR and GLP-1R^{228,229}. Chronic administration of these co-agonists over 14-28 days in mice increases weight loss through a simultaneous reduction in food intake and increased EE. In chapter 3, I investigated glucagon and GLP-1 analogues designed by the Department of Investigative Medicine, Imperial College, London. Analogues were selected based on their ability to bind and activate the GCGR and GLP-1R *in vitro*, to reduce food intake in rodents and the circulating concentrations achieved following administration. Chronic administration of these analogues showed greater weight loss following glucagon and GLP-1 analogue co-administration compared to either hormone alone. This was most likely due to increased EE through GCGR activation since cumulative food intake was the same as controls. rtPCR was used to quantify mRNA expression of various gene markers known to be involved in the regulation of EE. In contrast to a previous study²²⁹, chronic glucagon analogue administration in DIO mice did not increase UCP-1 mRNA expression in BAT or WAT. Furthermore, there was no significant decrease in body weight or food intake in response to chronic glucagon analogue administration alone. These findings suggest that at the dose administered, long-term glucagon analogue administration has no effect on EE. In contrast, glucagon and GLP-1 analogue co-administration significantly reduced body weight and circulating leptin with no effect on cumulative food intake. This suggests that chronic glucagon/GLP-1 analogue co-administration has a synergistic effect on body weight, possibly through increased EE. Unexpectedly, glucagon/GLP-1 analogue co-administration significantly decreased BAT weight. Although the reason for this remains unknown, GCGR agonism may initially increase BAT thermogenesis and BAT mass, but prolonged administration may result in BAT atrophy. BAT atrophy has been associated with hyperinsulinaemia⁵³⁹, leptin administration⁵⁴⁰, starvation⁵⁴¹ and reduced sympathetic activity to BAT^{542,543}. My results suggest an alternative mechanism for BAT atrophy in response to glucagon/GLP-1 analogue co-administration, since insulin and leptin levels were low, and β -hydroxybutyrate levels did not support evidence of starvation.

Alternatively, glucagon may increase EE through futile substrate cycling whereby energy-consuming cyclical metabolic pathways are stimulated²¹². In chapter 4, acute GLP-1/glucagon co-administration in man increased circulating insulin and carbohydrate oxidation. In DIO mice, chronic GLP-1/glucagon analogue administration improved glucose control compared to vehicle, with lower circulating levels of glucose and insulin, both fasting and in response to ipGTT. These findings may suggest increased glucose utilisation in thermogenic tissues. This could be investigated further by measuring glucose uptake by peripheral tissues⁵⁴⁴⁻⁵⁴⁶ and through the use of imaging modalities such as FDG-PET⁵⁴⁷. The role of glucagon and skeletal muscle-induced thermogenesis is unknown. Skeletal muscle accounts for approximately 20-30% of total resting oxygen uptake³³⁴ and up to 50% of adrenaline-induced thermogenesis in humans has been demonstrated to originate from skeletal muscle³³⁵. The underlying mechanism is unknown however UCP-3 has been implicated in skeletal muscle thermogenesis³⁴¹. In addition, a recently discovered protein, irisin, is secreted by skeletal muscle and stimulates UCP-1 expression and BAT-type differentiation in WAT³⁴². Furthermore, increased levels of irisin are associated with a rise in EE and weight loss³⁴². A role for glucagon and skeletal muscle-induced thermogenesis could be investigated using *ex vivo* muscle samples following chronic administration of glucagon and quantifying mRNA expression of UCP-3 and plasma irisin.

Finally, REE was measured in response to glucagon and GLP-1 co-administration in man using indirect calorimetry (chapter 4). OXM increases activity-related EE in humans²²³. Although it is unknown whether this effect occurs due to GCGR or GLP-1R activation, increased locomotor activity is observed in mice in response to a long-acting glucagon agonist⁴⁶³. Although the focus in this thesis was REE, the effect of glucagon/GLP-1 co-administration on activity-related EE could be measured using a portable monitoring device⁵⁴⁸. Similarly, chronic administration of glucagon/GLP-1 analogues in DIO mice suggested weight loss secondary to increased EE. This could be confirmed using a Comprehensive Laboratory Animal Monitoring System (CLAMS). Furthermore, my studies measured inter-scapular BAT and epididymal WAT weight. MRI would provide a more accurate assessment of adiposity and lean body mass⁴⁶³.

Glucagon and GLP-1 co-administration and the effect on glucose homeostasis

In chapters 2, 4 and 5, I demonstrated that GLP-1 administration in rodents and humans ameliorated the acute rise in glucose following glucagon administration. Furthermore, glucagon and GLP-1 co-administration in humans had an additive insulinotropic effect (chapters 4 and 5). These findings are consistent with glucagon and GLP-1 both stimulating insulin release via their respective receptors on pancreatic β -cells⁵⁴⁹⁻⁵⁵¹.

Subsequent studies in DIO mice (chapter 3), showed that chronic co-administration of glucagon and GLP-1 analogues reduced glucose and insulin levels. These findings may be secondary to improved insulin sensitivity, in part due to the weight loss observed. However, similar findings in glucose homeostasis were demonstrated following chronic glucagon analogue administration in DIO mice, yet these effects were independent of weight loss. This suggests improved insulin sensitivity through a different mechanism. One hypothesis may be that chronically elevated GCGR agonism results in glucagon tachyphylaxis and receptor desensitisation. Tachyphylaxis is the attenuation of an effect observed with repeated administration of a substance and has been shown to occur with the gut hormones CCK⁵⁵², GLP-1⁵⁵³ and PP⁵⁵⁴. Previous *in vitro* studies have shown that cAMP production is attenuated following repeated glucagon administration, suggesting receptor desensitisation⁵⁵⁵. Furthermore, GCGRs undergo internalisation within thirty minutes of glucagon administration⁵⁵⁶. Glucagon administration and tachyphylaxis has been shown during the investigation of hepatic glycogenolysis⁵⁵⁷, myocardial contractility⁵⁵⁸ and smooth muscle relaxation in the gut⁵⁵⁹. Consistent with these findings, obese Zucker rats require higher doses of ip glucagon compared to lean controls to elicit the same anorectic response¹⁸⁹ and repeated ip glucagon reduces the ability of the hormone to reduce food intake in rats¹⁹⁰.

Glucagonomas are tumours of pancreatic α -cells and are characterised by high circulating levels of glucagon, type 2 diabetes and weight loss⁵⁶⁰. It has been suggested that patients with glucagonomas are 'glucagon-resistant'^{561,562}. In one case report of a glucagonoma patient, long-term alterations in energy balance were minimal and the weight loss seen was not attributable to increased EE⁵⁶³. In another case report, the circulating cAMP response to iv glucagon infusion was studied pre- and post-operatively following removal of the glucagonoma⁵⁶¹. Pre-operatively, in the presence of hyperglucagonaemia, there was no rise in plasma cAMP. However, following curative surgery, and restoration of physiological glucagon levels, the cAMP response to glucagon

infusion was normal. This suggests that chronically elevated glucagon levels lead to GCGR desensitisation, which may be ameliorated following restoration of normal circulating glucagon concentrations.

A glucagon resistant state or tachyphylaxis in association with receptor internalisation may explain the marked improvement in glucose regulation and also lack of expected weight loss due to chronic GCGR activation. In chapter 3, chronic co-administration of glucagon and GLP-1 analogues in DIO mice demonstrated an apparent synergistic effect on weight loss despite no reduction in food intake. These findings suggest that GLP-1 may ameliorate tachyphylaxis of glucagon-induced EE. This could be tested initially *in vitro* to see whether GCGR internalisation still occurs in the presence of GLP-1. In addition, quantification of GCGR mRNA in tissue samples following chronic glucagon administration may assist in showing an association between prolonged glucagon administration and yet apparent lack of glucagon effect on weight loss.

GCGR knockout mice are animal models which have been used to investigate the metabolic consequences of glucagon down-regulation. Liver-specific GCGR knockout mice develop hyperglucagonaemia and pancreatic α cell hyperplasia, yet have reduced fasting blood glucose and improved insulin sensitivity⁵⁶⁴. Similar findings are found in other models of glucagon down-regulation, and are associated with increased expression of GLP-1 in pancreatic α -cells and circulating levels of GLP-1^{204,205,433,435}. This suggests that α cell hyperplasia results in up-regulation of the preproglucagon gene. Although not studied in chapter 3, it would be interesting to investigate the effects of chronic glucagon analogue administration in mice and the changes in pancreatic α cell morphology, pancreatic GLP-1 expression and circulating levels of GLP-1.

In summary, chronic glucagon analogue administration may lead to GCGR internalisation with subsequent pancreatic α cell hyperplasia, hyperglucagonaemia and increased pancreatic GLP-1 secretion. Although this initially may lead to increased glucose and insulin levels, up-regulation of glucose transporters and futile substrate cycling would ameliorate hyperglycaemia within the circulation and increase EE via peripheral tissue thermogenesis. This is summarised in figures 6.1 and 6.2. To explore this hypothesis further, it would be interesting to investigate whether the expression of glucose transporters such as glucose transporter 4 (GLUT4), which modulates glucose disposal in peripheral tissues, is up-regulated in response to chronic glucagon receptor agonism. GLUT4 over-expressing mice exhibit increased fasting glucose disposal in

tissues and thus lowered blood glucose⁵⁶⁵, and enhanced peripheral glucose utilisation in skeletal muscle^{566,567}. DPPIV inhibitors, which result in prolonged GLP-1 action, are associated with up-regulation of GLUT4 expression in skeletal muscle in rats⁵⁶⁸. In addition, over-expression of GLUT4 in mice increases UCP3 mRNA expression in skeletal muscle and decreases UCP-1 mRNA expression in BAT ⁵⁶⁹. Therefore, up-regulation of glucose transporters in skeletal muscle may explain the amelioration in circulating glucose observed following chronic glucagon analogue administration in DIO mice (chapter 3). This could be investigated in studies using a similar protocol to those outlined in chapter 3, collecting skeletal muscle samples and quantifying GLUT4 and UCP-3 mRNA expression using rtPCR. Until recently, researchers have focused predominantly on chronic GCGR antagonism in an attempt to improve glycaemia. However, these studies suggest that paradoxically, long-term administration of glucagon analogues may improve glucose control, independently of hepatic glucose output.

Therefore, the actions of glucagon and increased EE may differ according to the duration of treatment. Acute glucagon administration may increase EE through mechanisms involving BAT thermogenesis, futile substrate cycling and SNS activation. In contrast, chronic administration of glucagon may lead to activation of more complex pathways involving up-regulation of glucose transporters, leading to improved glucose control and increased EE via peripheral tissue thermogenesis and substrate cycling. Elucidating the mechanisms through which glucagon and GLP-1 co-administration increases EE and ameliorates hyperglycaemia will assist in the development of dual agonists of the GCGR and GLP-1R.

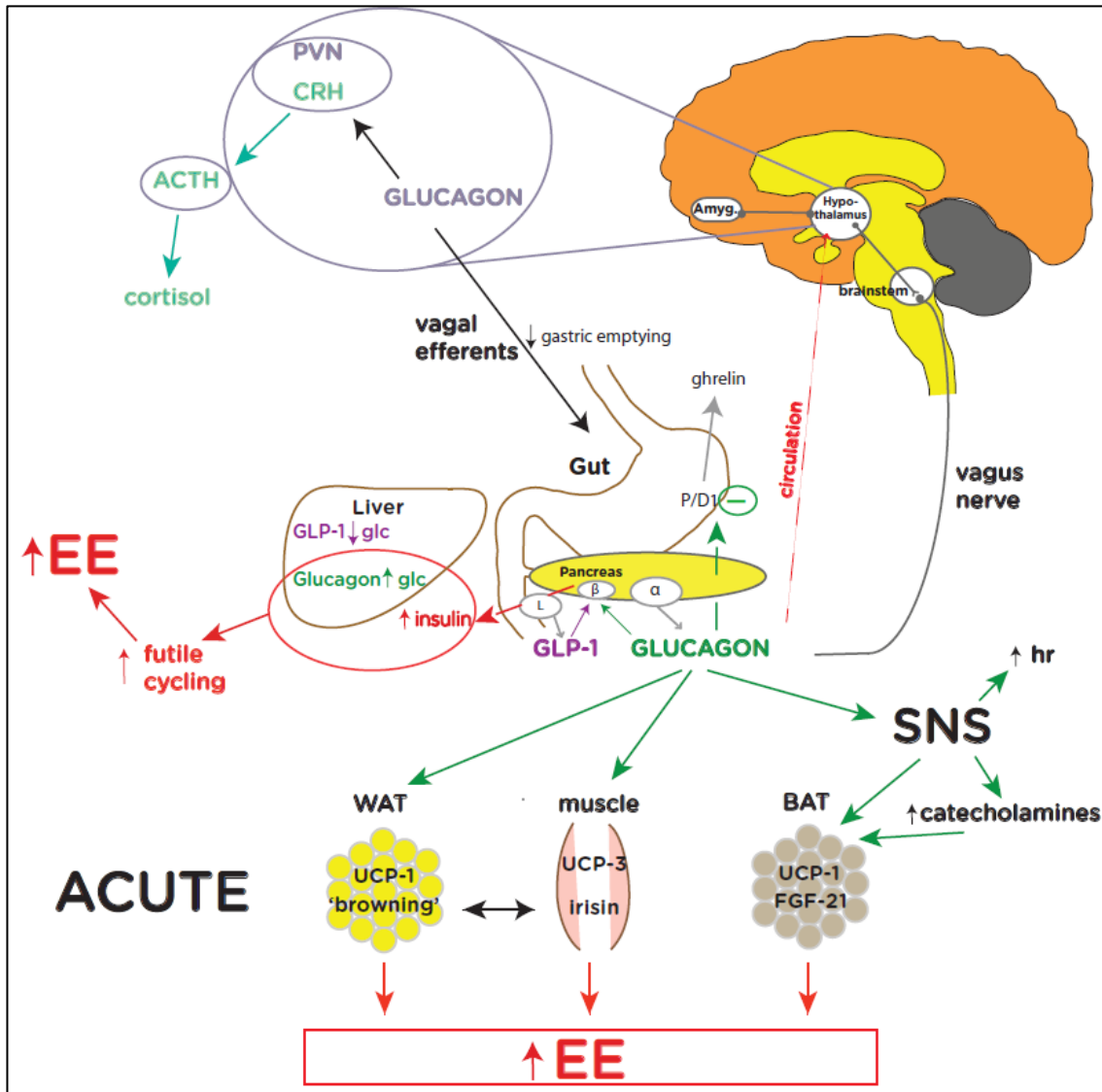


Figure 6.1 Schematic diagram depicting the acute effects of glucagon and GLP-1 administration on food intake, energy expenditure and glucose homeostasis. Glucagon may increase EE acutely via different mechanisms including upregulation of UCP-1 and FGF-21 effect in BAT, UCP-3 and irisin in skeletal muscle and UCP-1 in WAT. Glucagon and GLP-1 both stimulate the release of insulin from pancreatic β cells, which in conjunction with an acute rise in glucose in response to glucagon, increases futile cycling in thermogenic tissues and EE. In addition, glucagon activates the SNS causing release of catecholamines and a rise in heart rate. Glucagon and GLP-1 are thought to reduce food intake via vagal afferent nerves which terminate in the brainstem. In addition, GLP-1 may act directly via the circulation to activate neuronal pathways in the hypothalamus that are involved with the regulation of appetite. Glucagon and GLP-1 have been shown to reduce circulating levels of ghrelin which may also potentiate the anorectic effect. Although central pathways through which glucagon reduces food intake are unknown, it has been suggested that a CRH-dependent pathway may be involved. Furthermore, stimulation of vagal efferents with reduced gastric emptying may reduce food intake but also cause nausea. Abbreviations: Paraventricular nucleus (PVN), corticotrophin hormone (CRH), adrenocorticotrophic hormone (ACTH), amygdala (Amyg.), glucose (glc), glucagon-like peptide-1 (GLP-1), energy expenditure (EE), heart rate (hr), uncoupling protein-1 (UCP-1), uncoupling protein-3 (UCP-3), fibroblast growth factor-21 (FGF-21).

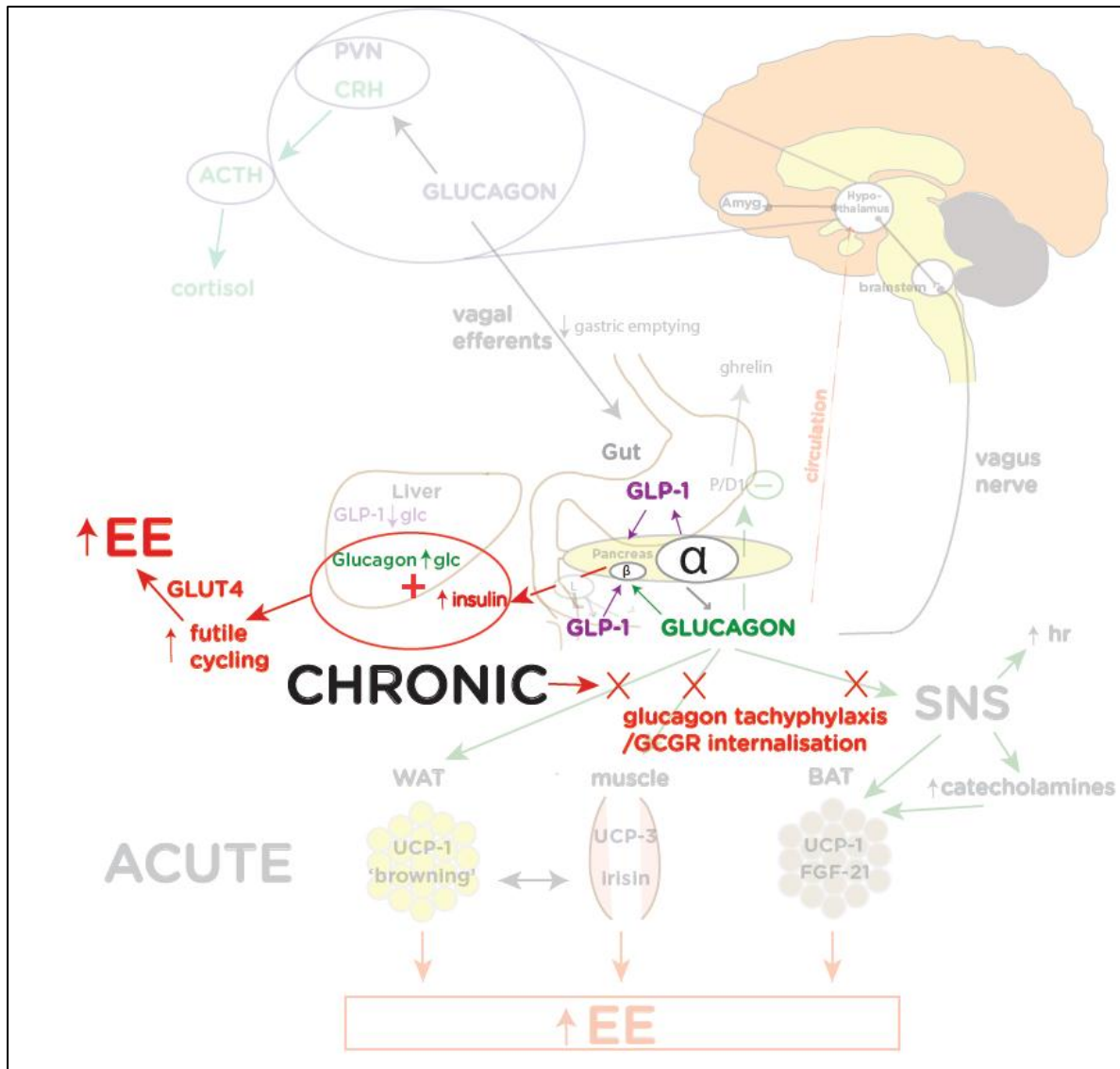


Figure 6.2 Schematic diagram depicting the hypothesised chronic effects of glucagon administration on energy expenditure and glucose homeostasis. Chronic hyperglucagonaemia may result in a state of 'glucagon tachyphylaxis' and GCGR internalisation. As such, direct acute effects of glucagon on BAT, muscle and WAT have minimal effects on EE. However, chronically elevated levels of glucagon and inability to stimulate GCGRs adequately results in pancreatic α cell hyperplasia and elevated expression of pancreatic GLP-1. Initial insulin and glucose levels subsequently rise as a result of elevated glucagon and GLP-1 concentration. However, in the presence of upregulated GLUT4 expression in peripheral thermogenic tissues, rapid glucose disposal increases futile cycling of substrates and hence EE. Abbreviations: glucose (glc), glucagon-like peptide-1 (GLP-1), glucagon receptor (GCGR), energy expenditure (EE), glucose transporter 4 (GLUT4).

Glucagon/GLP-1 co-administration as a potential treatment in obesity

The work presented in this thesis demonstrates that glucagon and GLP-1 co-administration reduces food intake, increases EE and improves glycaemic control. However, the mechanisms through which these effects occur are unknown and further studies are warranted before glucagon/GLP-1 receptor co-agonists are considered as a potential treatment for obesity. The increased incidence of nausea in subjects following glucagon and GLP-1 co-administration requires further investigation. In particular, neuronal pathways involved in glucagon and GLP-1-mediated anorectic effects need further characterisation. If both hormones reduce food intake via the same pathway, then the therapeutic potential of glucagon and GLP-1 in combination may be limited by the presence of nausea and vomiting. OXM has been shown to cause these symptoms in human subjects⁴⁴⁶. Although GLP-1 analogues can also cause nausea and vomiting, these symptoms frequently resolve with the duration of treatment. Further studies are required to see whether similar attenuation of symptoms are seen following chronic glucagon administration.

Recently, there has been concern about the use of GLP-1 analogues and the associated risk of pancreatitis⁵⁷⁰ and thyroid medullary cell cancer^{571,572}. There is some evidence that glucagon administration may be beneficial in the treatment of pancreatitis⁵⁷³. In addition, recent studies suggest that the presence of pancreatic α cells improves β cell function and survival⁵⁷⁴. Prior to any phase I study involving the administration of glucagon/GLP-1 receptor co-agonists, toxicology and safety testing will need to be performed and should include histological analysis of the pancreas and thyroid gland.

In the human studies reported here (chapters 4 and 5), there was a trend towards increased pulse and blood pressure in response to glucagon. This raises concerns about the cardiovascular safety profile of glucagon and GLP-1 in combination. A recent meta-analysis has suggested an association between GLP-1 analogues and a small increase in heart rate⁵⁷⁵. In contrast, there is some evidence that GLP-1 therapies are beneficial in the control of blood pressure^{155,576}. OXM has been shown to increase heart rate in mice but not humans^{223,446,577}. Increased heart rate is positively associated with a higher mortality rate in man⁵⁷⁸. In patients with type 2 diabetes, an increase in resting heart rate of just 10 beats per minute is associated with a 15% increase in mortality⁵⁷⁹. Since both glucagon and GLP-1 can activate the SNS, long-term studies in animal and humans are required to assess cardiovascular safety parameters.

In conclusion, glucagon and GLP-1 co-administration may provide an alternative approach to weight loss through a simultaneous reduction in food intake and increased EE. However, further long-term studies are required, particularly in humans, to assess the incidence of side-effects such as nausea and cardiovascular parameters. In order to replicate the successful weight loss observed following gastric bypass surgery, it is likely that a combination of hormones will be required. The current trends in obesity highlight the urgency with which new treatments are required and the importance of research in this area.

6.2 Future work

- To investigate the effects of chronic glucagon and GLP-1 administration in humans, I aim to undertake a randomised, single-blinded, cross-over study in overweight volunteers during which participants will receive an infusion of vehicle, glucagon alone, GLP-1 alone, or a combination of glucagon and GLP-1. Each infusion will be administered s/c via a portable infusion for one week whilst participants are at home. REE, food intake and carbohydrate tolerance will be measured at the end of each week. Heart rate will be monitored using a non-invasive portable device which also has the ability to measure activity-related EE. Subjective feelings of nausea and satiety will be recorded using VAS scores during each treatment week.
- The Department of Investigative Medicine, Imperial College, London is currently undertaking a programme of dual glucagon/GLP-1 analogue development. These analogues may permit the study of longer-term glucagon and GLP-1 receptor agonism in humans through prolonged action and improved pharmacokinetic profile compared to the native hormones. I aim to undertake a multiple ascending dose finding study in human volunteers using a selected glucagon/GLP-1 analogue. The analogue will be selected based on *in vitro* work demonstrating the ability of this analogue to bind and activate the GCGR and GLP-1R. Prior to studies in man, the analogue will be administered to rodents and dogs to study its effects on food intake, glucose levels and its duration within the circulation. In man, I will measure breakdown products of this glucagon/GLP-1 analogue and study its pharmacokinetic profile following administration. In addition, I will investigate the effects of this analogue on glucose, insulin and food intake in addition to feelings of nausea. The results from this initial dose-finding study in humans will determine the dose of glucagon/GLP-1 analogue that will be administered in a subsequent phase I study.

APPENDICES

APPENDIX A: ABBREVIATIONS FOR AMINO ACIDS

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

APPENDIX B: PEPTIDE STABILITY DATA

B1. Amino acid sequences of glucagon, GLP-1, exendin-4, oxyntomodulin and the glucagon/GLP-1 analogues G(X), G(Y) and G(Z).

Due to the commercial sensitivity of this data, the amino acid sequences of G(X), G(Y) and G(Z) have been removed.

Figure B1. Amino acid sequences of glucagon (GCG), glucagon-like peptide-1 (GLP-1), exendin-4, oxyntomodulin (OXM) and the analogues G(X), G(Y) and G(Z). Fully conserved regions are shown in yellow. Abbreviations: glucagon (GCG), glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM). Amino acids: alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), tyrosine (Tyr). For full amino acid abbreviation list, see Appendix A.

B2. Receptor binding and cAMP activation assays in response to G(X), G(Y) and G(Z).

Receptor Binding Assays (n=1-3)

IC50 (nM)	mGCGR	hGLP-1R
G(X)	6794	309.2
G(Y)	>10000	6.49
G(Z)	0.45	141

cAMP activation (n=1-4)

EC50 (nM)	hGCGR lower GCG) (fold than	hGLP-1R (fold lower than GLP- 1)
G(X)	630	9.63
G(Y)	>10000	0.89
G(Z)	4	36.5

Table B1. Receptor Binding and cAMP assays for G(X), G(Y) and G(Z). HEK 293 cells were transfected with mouse GCGR (mGCGR) for RBA studies and human GCGR (hGCGR) for cAMP activation. Human GLP-1R (hGLP-1) was transfected for both RBA and cAMP studies.

B3. Pharmacokinetic profiles of G(Y) and G(Z) in rats.

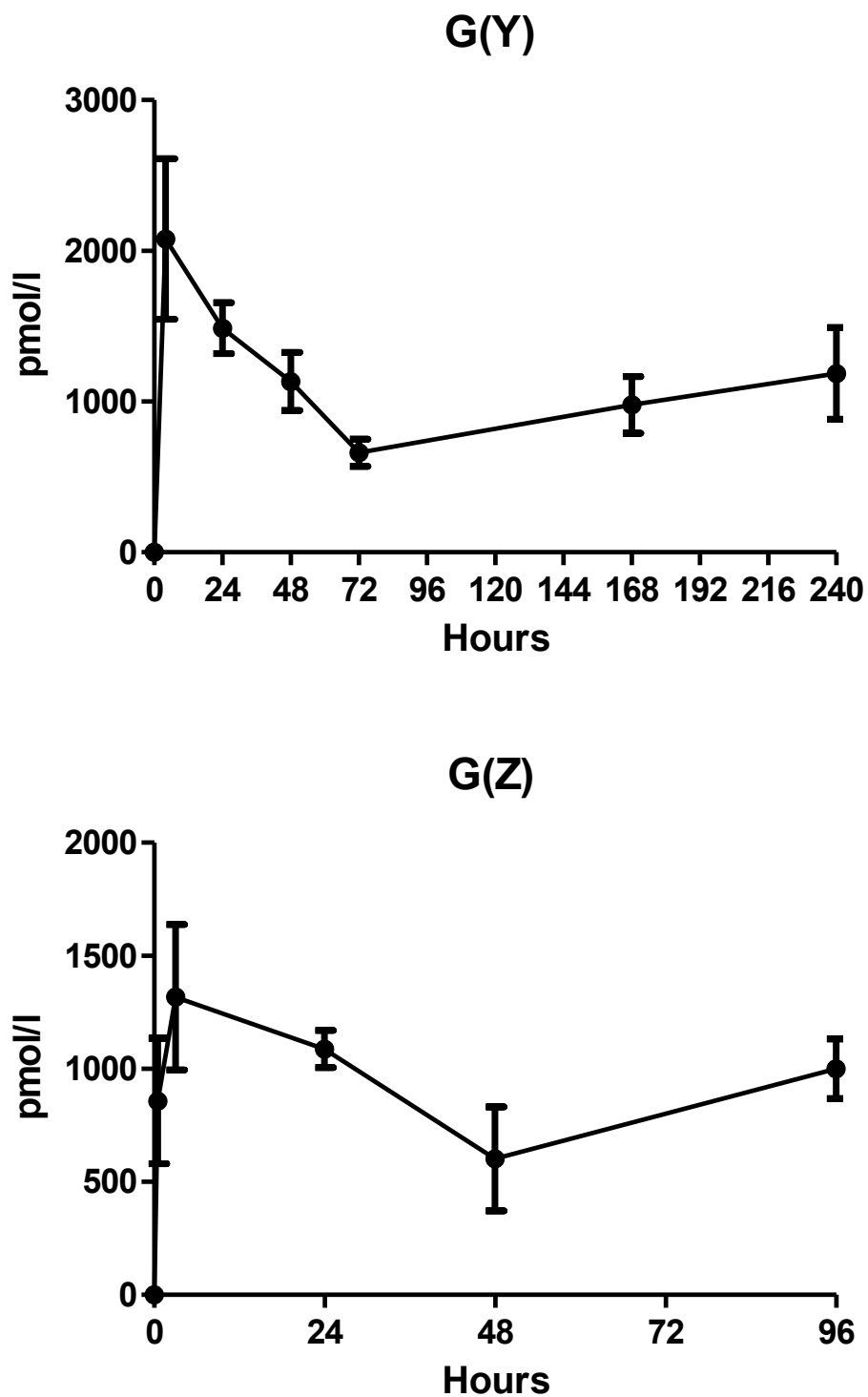


Figure B2. Pharmacokinetic profiles of G(Y) and G(Z) in rats. *Ad libitum* fed rats were injected s/c at the onset of the light phase with G(Y) or G(Z). Tail vein blood samples were taken at the time points indicated. n=3-5.

B4. Acute feeding studies following administration of G(X), G(Y) and G(Z).

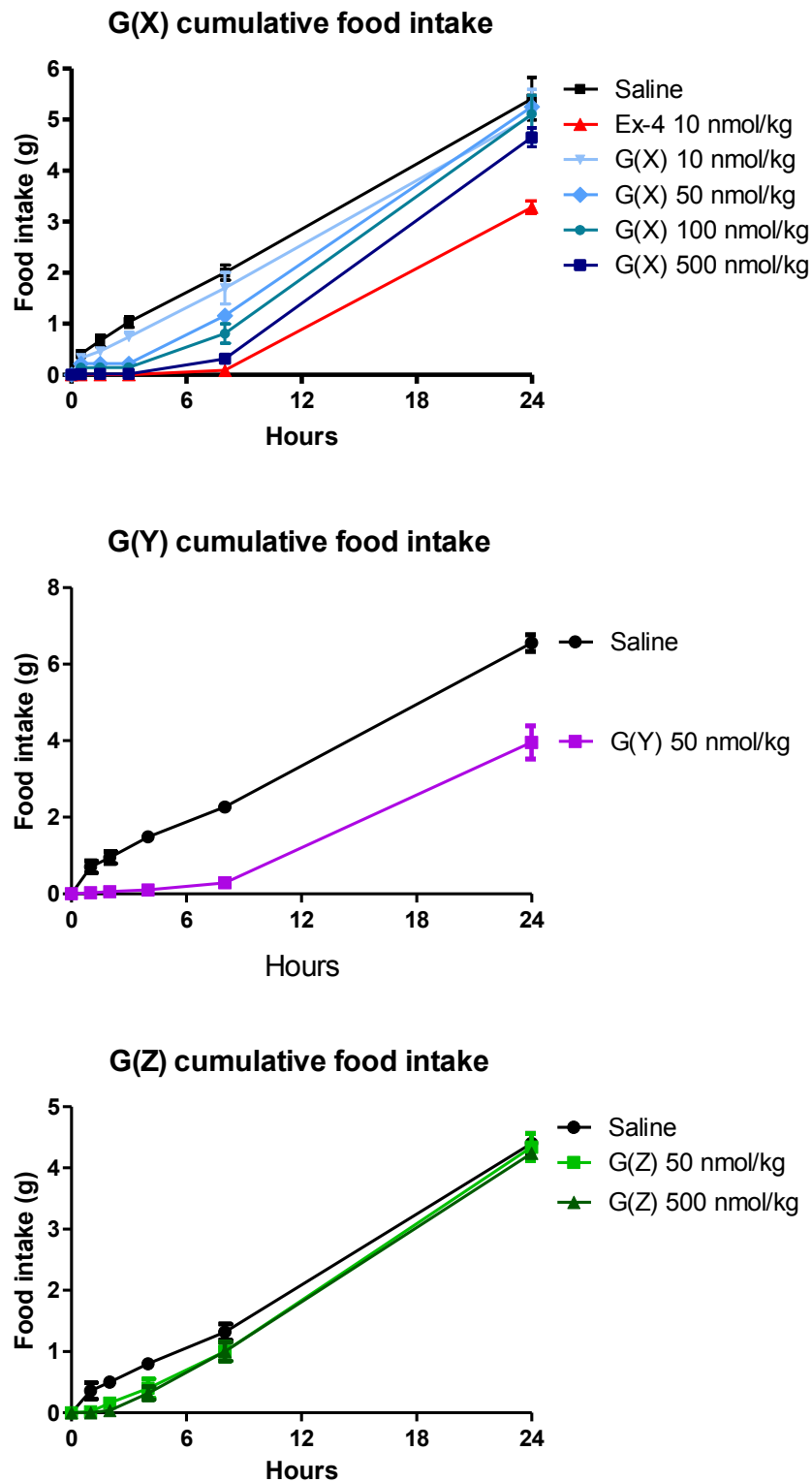


Figure B3. Acute feeding studies in mice following the administration of G(X), G(Y) and G(Z). Mice were fasted from 1600 the preceding day and injected s/c at the onset of the light phase. Mice were then returned to their cages with a known quantity of food. Food weight was measured at time points indicated. n=5. Abbreviations: exendin-4 (Ex-4).

APPENDIX C: EXAMPLE OF VISUAL ANALOGUE SCALE SHEET

How hungry do you feel right now?

NOT AT ALL  EXTREMELY

How sick do you feel right now?

NOT AT ALL  EXTREMELY

How pleasant would it be to eat right now?

NOT AT ALL  EXTREMELY

How much do you think you could eat right now?

NOTHING  A LARGE AMOUNT

How full do you feel right now?

NOT AT ALL  EXTREMELY

How tasty was the meal?

NOT AT ALL  EXTREMELY

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