A Medical Bypass

Can the manipulation of gut hormone levels replicate the favourable effects on energy and glucose homeostasis seen following Gastric Bypass surgery?

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Dr Rachel Clare Troke

BMedSci, MBBS, MRCP

Section of Investigative Medicine

Division of Diabetes, Endocrinology & Metabolism

Faculty of Medicine

Imperial College London

ABSTRACT

The obesity crisis has reached epidemic proportions. Medical therapies are few and far between, offering only modest benefit, with maximal weight loss at one year in the region of 5-6%. Gastric bypass surgery and specifically Roux-en-Y gastric bypass (RYGB) is the only obesity treatment which has shown a marked and sustained weight loss, with initial remission of diabetes in up to 75% of patients. An increase in the post-prandial secretion of satiety gut hormones glucagon-like peptide-1 (GLP-1), peptide tyrosine-tyrosine (PYY) and oxyntomodulin (OXM) following gastric bypass may mediate these favourable effects. GLP-1 promotes insulin secretion in response to an oral glucose load. PYY also increases insulin sensitivity in rodents, and OXM increases energy expenditure (EE). All three hormones increase satiety. Work in this thesis investigates the contributions of these hormones both alone and in combination on the favourable effects on food intake, body weight, and glycaemic control following gastric bypass surgery.

Acute co-infusion of GLP-1 and glucagon (as a surrogate for OXM) as well as glucagon alone increased EE by around 150Kcal per day, an effect that was mediated via the glucagon receptor. GLP-1 did not increase EE but was able to partially counter the hyperglycaemia induced by high doses of glucagon. Acute co-infusion of GLP-1 and glucagon at subanorectic doses reduced food intake by 13% compared to placebo, with no effect seen following administration of either peptide alone. GLP-1 neutralised the hyperglycaemic effect of glucagon at these subanorectic doses.

Acute infusion of GLP-1 and PYY(3-36) alone and in combination demonstrated that GLP-1 augments the acute insulin response to glucose (AIRg) as assessed by an intravenous glucose tolerance test. PYY did not modify the AIRg, and neither hormone alone or in combination had any effect on insulin sensitivity or glucose disposal.

Chronic administration of long acting analogues of GLP-1, PYY and OXM in combination to diet induced obese (DIO) mice resulted in a marked and sustained reduction in body weight of approximately 16% compared to placebo. This was independent of a reduction in food intake suggesting an increase in EE, possibly mediated via fibroblast growth factor-21. In addition, the combination of analogues improved glucose tolerance compared to placebo.

These results suggest that a combination of the gut hormones GLP-1, PYY and OXM, or analogues thereof, offers potential as a novel obesity therapy and may be able to mimic the beneficial metabolic effects seen after gastric bypass.

DECLARATION OF CONTRIBUTORS

Work in this thesis was carried out in the main part by the author with the assistance and collaboration of the colleagues detailed below:

Chapter 2: Chronic DIO mice feeding study with injection of peptide analogues, intraperitoneal glucose tolerance test and tissue harvesting were carried out in collaboration with Dr Kath McCullough, Dr James Minnion, Dr Joy Shillito and Dr Vicky Salem, with assistance from members of the G series team. Extraction of mRNA was carried out in collaboration with Dr Kath McCullough, Dr Joy Shillito and Dr Vicky Salem. rtPCR was carried out in collaboration with Dr Kath McCullough. Plasma hormone assays were carried out in collaboration with Dr Kath McCullough and Dr Joy Shillito.

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In house radio-immunoassays were established and maintained by Professor Mohammad Ghatei. The long-acting analogues of GLP-1, Oxyntomodulin and PYY used in Chapter 2 were designed by Professor S R Bloom, and remain the intellectual property of Imperial College London.

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TABLE OF ABBREVIATONS

5-HT	Serotonin
AC	Adenylate cyclase
ACC1	Acetyl CoA carboxylase1
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AgRP	Agouti-related peptide
AIRg	Acute insulin response to glucose
ANOVA	Analysis of variance
AP	Area Postrema
ARC	Arcuate nucleus of the hypothalamus
ATP	Adenosine triphosphate
AU	Arbitrary units
AUC	Area under the curve
B3	Beta-3
BAT	Brown adipose tissue
BBB	Blood brain barrier
BMI	Body Mass Index
BP	Blood pressure
BSA	Bovine serum albumin
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine- regulated transcript
CATS	Catecholamines
CB-1	Cannabinoid-1
cDNA	Complimentary deoxyribonucleic acid
CHO	Carbohydrate
CHOv	Chinese hamster ovary
CLAMS	Comprehensive laboratory animal monitoring system
CNS	Central Nervous System
CO_2	Carbon dioxide
Cq	Quantification cycle
CT	Computed tomography
CV	Coefficient of Variation
CVO	Circumventricular organs
DEBQ	Dutch eating behaviour questionnaire
DI	Disposition index
DIO	Diet-induced obese
DIT	Diet-induced thermogenesis
DLW	Doubly labelled water
DMN	Dorsomedial nucleus of the hypothalamus
DMX	Dorsal motor nucleus of the vagus nerve
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase-4
EE	Energy expenditure
ELISA	Enzyme-linked Immunosorbent assay
EMA	European medicines agency
FDA	Food and drug administration
FDG	^{18}F-fluorodeoxyglucose

FFA	Free fatty acids
FGF-21	Fibroblast growth factor-21
FI	Food intake
FSIVGTT	Frequently sampled intravenous glucose tolerance test
G-6-Pase	Glucose-6-phosphatase
GCG	Glucagon
GCGR	Glucagon receptor
GH	Growth hormone
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLP-2	Glucagon-like peptide-2
GOAT	Ghrelin O-acyltransferase
GP	G-protein coupled receptor
GRPP	Glicentin-related polypeptide
GSH-R	Growth hormone secretogogue receptor
HbA1c	Haemoglobin A1c
HCl	Hydrochloric acid
HEK	Human embryonic kidney
HFD	High fat diet
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
ICV	Intracerebroventricular
IDF	International diabetes federation
IgG	Immunoglobulin G
IM	Intramuscular
IP	Intervening peptide
IPe	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IV	Intravenous
Kcal	Kilocalorie
KG	Kilogram
LAL	Limulus amoebocyte lysate
LDL	Low density lipoprotein
LPBN	Lateral parabrachial nucleus of the pons
MC4R	Melanocortin 4 receptor
ME	Median eminence
MEMRI	Manganese-enhanced magnetic resonance imaging
MI	Myocardial infarction
MM	Milimetres
MPGF	Major proglucagon fragment
MRC	Medical Research Council
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
NA	Noradrenaline
NAFLD	Non-alcoholic fatty liver disease
NEFA	Nonesterified fatty acids
NHANES	National health and nutrition examination survey
NHS	National Health Service
NM	Nanometers

NPY	Neuropeptide Y
NSRI	Norepinephrine and serotonin reuptake inhibitor
NTS	Nucleus tractus solitaries
OGTT	Oral glucose tolerance test
OVLT	Organum vasculosum of the lamina terminalis
OXM	Oxyntomodulin
PEPCK1	Phosphoenolpyruvate carboxykinase1
PET	Positron emission tomography
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PKA	Protein Kinase A
POMC	Pro-opiomelanocortin
PP	Pancreatic Polypeptide
PPARγ	Peroxisome proliferative activated receptor gamma
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide Tyrosine Tyrosine
QC	Quality control
rDNA	recombinant deoxyribonucleic acid
REE	Resting energy expenditure
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPM	Revolutions per minute
RQ	Respiratory quotient
rtPCR	Real time polymerase chain reaction
RYGB	Roux-en-y gastric bypass
SEM	Standard error of the mean
SFO	Subfornical organ
S_i	Insulin sensitivity index
SM	Skeletal muscle
SNS	Sympathetic nervous system
SOS	Swedish Obese Subjects
$T_{1/2}$	Half Life
T3	Tri-iodothyronine
T4	Thyroxine
TC	Total Cholesterol
TEE	Total energy expenditure
TG	Triglycerides
TR	Thyroid hormone receptor
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
UCP-1	Uncoupling protein-1
UCP-3	Uncoupling protein-3
UK	United Kingdom
US	United States
USA	United Sates of America
VAS	Visual analogue scale
VMN	Ventromedial nucleus of the hypothalamus
WAT	White adipose tissue
WHO	World Health Organisation
αMSH	Alpha-melanocyte stimulating hormone
β-hCG	Beta human chorionic gonadotrophin

TABLE OF CONTENTS

ABSTRACT 2
DECLARATION OF CONTRIBUTORS 3
ACKNOWLEDGEMENTS 5
COPYWRIGHT DECLARATION 6
TABLE OF ABBREVIATONS 7
TABLE OF CONTENTS 10
INDEX OF FIGURES 15
INDEX OF TABLES 22
CHAPTER 1: GENERAL INTRODUCTION 23
1.1 Obesity 24
1.1.1 The Obesity Crisis 24
1.1.2 Central Control of Appetite 26
1.1.2.1 Anorexigenic control circuits 26
1.1.2.2 Orexigenic control circuits 28
1.1.2.3 Vagal signals 28
1.1.2.4 Gut hormone interactions with central control circuits 30
1.1.3 Current Obesity Therapies 31
1.1.3.1 Lifestyle 31
1.1.3.2 Medical Therapies 31
1.1.3.3 Gastric Bypass Surgery 33
1.2 Gut Hormones 37
1.2.1 PYY 37
1.2.2 GLP-1 39
1.2.3 OXM 43
1.2.4 Ghrelin 45
1.3 Other Relevant Hormones 47
1.3.1 Glucagon 47
1.3.2 Leptin 49
1.4 Energy Homeostasis 51
1.4.1 The role of selected components in regulation of energy homeostasis 51
1.4.1.1 Adaptive thermogenesis 51

1.4.1.2 Brown Adipose Tissue 52
1.4.1.3 The Hypothalamo-Pituitary-Thyroid Axis 53
1.4.1.3 Skeletal muscle 54
1.4.2 Measuring Energy Expenditure 56
1.4.2.1 Direct Calorimetry 56
1.4.2.2 Indirect Calorimetry 56
1.4.2.3 Non-Calorimetric Methods 57
1.5 Hypotheses 60
1.5 Aims 61
CHAPTER 2: THE CHRONIC EFFECTS OF NOVEL LONG-ACTING ANALOGUES OF PYY, GLP-1 AND GLUCAGON ON ENERGY HOMEOSTASIS IN DIO MICE 62
2.1 Introduction 63
2.2 Hypothesis and Aims 67
2.2.1 Hypothesis 67
2.2.2 Aims 67
2.3 Materials and Methods 68
2.3.1 Animals 68
2.3.2 Peptides 68
2.3.3 Protocol 70
2.3.3.1 Acute Food Intake following administration of long acting analogues of PYY, GLP-1 and OXM 70
2.3.3.2 Food Intake and Body Weight following chronic administration of long acting analogues of PYY, GLP-1 and OXM 71
2.3.3.3 Glucose Homeostasis measurement following chronic administration of long acting analogues of PYY, GLP-1 and OXM 72
2.3.3.4 Analysis of Markers of Energy Homeostasis in Tissue Samples 73
2.3.3.5 Analysis of Markers of Energy Homeostasis in Plasma Samples 80
2.3.4 Statistics 83
2.3.5 Results 84
2.3.5.1 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on food intake in DIO mice 84
2.3.5.2 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on body weight of DIO mice 86
2.3.5.3 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on glucose homeostasis as measured by an intraperitoneal glucose tolerance test in DIO mice 88

2.3.5.4 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on markers of energy homeostasis . 91
Markers of Energy Homeostasis 93
2.3.5 Discussion 99
CHAPTER 3: THE ACUTE EFFECTS OF CO-INFUSION OF GLUCAGON AND GLP-1 ON ENERGY EXPENDITURE AND GLUCOSE HOMEOSTASIS IN HUMANS 104
3.1 Introduction 105
3.2 Hypothesis and Aims 107
3.2.1 Hypothesis 107
3.2.2 Aims 107
3.3 Materials and Methods 108
3.3.1 Subjects 108
3.3.2 Peptides 110
3.3.3 Calorimetry 110
3.3.4 Protocol 112
3.3.5 Assays 117
3.3.6 Statistics 121
3.4 Results 122
3.4.1 The acute effect of co-infusion of glucagon and GLP-1 on energy expenditure 122
3.4.2 The acute effect of co-infusion of glucagon and GLP-1 on substrate oxidation 124
3.4.3 The acute effect of co-infusion of glucagon and GLP-1 on plasma glucagon and GLP-1 concentrations 126
3.4.4 The acute effect of co-infusion of glucagon and GLP-1 on plasma glucose and insulin concentrations 128
3.4.5 The acute effect of co-infusion of glucagon and GLP-1 on plasma levels of total and acyl ghrelin, thyroid hormones, non-esterified fatty acids, and cortisol 132
3.4.6 The acute effect of co-infusion of GLP-1 and glucagon on cardiovascular parameters and core body temperature 138
3.5 Discussion 140
CHAPTER 4: THE ACUTE EFFECT OF CO-INFUSION OF GLUCAGON AND GLP-1 ON FOOD INTAKE 144
4.1 Introduction 145
4.2 Hypothesis and Aims 146
4.2.1 Hypothesis 146
4.2.2 Aims 146
4.3 Materials and Methods 147

4.3.1 Subjects 147
4.3.2 Peptides 150
4.3.3 Calorimetry 150
4.3.4 Protocol 150
4.3.5 Assays 154
4.3.6 Statistics 156
4.4 Results 157
4.4.1 The acute effect of low-dose co-infusion of glucagon and GLP-1 on food intake 157
4.4.2 The acute effect of low dose co-infusion of glucagon and GLP-1 on hunger, satiety, nausea, and palatability 159
4.4.3 The acute effect of low dose co-infusion of glucagon and GLP-1 on plasma glucagon and active GLP-1 concentrations 161
4.4.4 The acute effect of low dose co-infusion of glucagon and GLP-1 on energy expenditure 164
4.4.5 The acute effect of low-dose co-infusion of glucagon and GLP-1 on substrate oxidation 166
4.4.5 The acute effect of low dose co-infusion of glucagon and GLP-1 on plasma glucose and insulin concentrations 168
4.4.6 The acute effect of low dose co-infusion of glucagon and GLP-1 on plasma levels of total and acyl ghrelin 172
4.4.7 The acute effect of low dose co-infusion of glucagon and GLP-1 on cardiovascular parameters 174
4.5 Discussion 176
5.1 Introduction 182
5.2 Hypothesis and Aims 184
5.2.1 Hypothesis 184
5.2.2 Aims 184
5.3 Materials and Methods 185
5.3.1 Subjects 185
5.3.2 Peptides 187
5.3.3 Protocol 188
5.3.4 Assays 191
5.3.5 Statistics 192
5.4 Results 193
5.4.1 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma glucose and insulin concentrations 193
5.4.2 AUC for insulin and glucose for the first 10 minutes following the FSIVGTT glucose bolus during co-infusion of PYY(3-36) and GLP-1 195

5.4.3 AIRg, S_i and DI response to IV glucose during co-infusion of PYY(3-36) and GLP-1 197
5.4.4 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma PYY and GLP-1 concentrations 199
5.4.5 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma cortisol concentration 201
5.4.6 The acute effect of co-infusion of PYY(3-36) and GLP-1 on hunger, satiety and nausea 203
5.4.7 The acute effect of co-infusion of PYY(3-36) and GLP-1 on cardiovascular parameters 205
5.5 Discussion 207
GENERAL DISCUSSION 211
Future Work 222
APPENDICES 224
APPENDIX A Visual Analogue Scale 225
APPENDIX B Food Preference score 226
APPENDIX C Note on imputed values 227
APPENDIX D1 IC_{50} and EC_{50} of long-acting gut hormone analogues 228
APPENDIX D2 Pharmacokinetic profiles of PYY-LA, GLP-1-LA AND OXM-LA 229
REFERENCES 230

INDEX OF FIGURES

FIGURE 1.1. WORLD HEALTH ORGANISATION MAP DENOTING PREVALENCE OF OBESITY IN BOTH MEN AND WOMEN AGED 20 YEARS OR MORE IN 2008(WHO, 2011) 24
FIGURE 1.2. THE GUT-BRAIN AXIS INTERACTION OF PERIPHERAL SIGNALS WITH THE CENTRAL NERVOUS SYSTEM IN THE REGULATION OF FOOD INTAKE. ENTERO-ENDOCRINE L & K-CELLS SENSE NUTRIENTS AND AS A CONSEQUENCE RELEASE GUT HORMONES PEPTIDE YY, GLUCAGON-LIKE PEPTIDE-1 AND OXYNTOMODULIN. THE OREXIGENIC HORMONE GHRELIN IS RELEASED FROM X/A-LIKE CELLS IN THE FUNDUS OF THE STOMACH IN THE FASTED STATE. ALL OF THESE HORMONES ARE SENSED BY AREAS WITHIN THE BRAINSTEM & HYPOTHALAMUS. THE HYPOTHALAMUS CONTAINS BOTH OREXIGENIC NPY/AGRP NEURONS AND ANOREXIGENIC POMC NEURONS WHICH ACT TO CONTROL FOOD INTAKE. LEPTIN, A HORMONE FROM ADIPOSE TISSUE, INTERACTS WITH THE HYPOTHALAMUS AS AN INDICATOR OF LONGER-TERM NUTRITIONAL STATUS. 27
FIGURE 1.3 ROUX-EN-Y GASTRIC BYPASS. A SCHEMATIC OF THE ANATOMY OF THE UPPER GASTROINTESTINAL TRACT BOTH (A) BEFORE SURGERY, AND (B) FOLLOWING THE ROUX-EN-Y GASTRIC BYPASS OPERATION 36
FIGURE 1.4 THE CLEAVAGE PRODUCTS OF PROGLUCAGON. PROGLUCAGON EXPRESSION IS CONCENTRATED IN THE PANCREAS, L CELLS OF THE GI TRACT AND BRAIN. GLUCAGON IS THE PRIMARY PRODUCT IN THE PANCREAS, WHEREAS GLP-1 AND OXM SECRETION TAKE PLACE PREDOMINANTLY IN THE L CELLS OF THE GI TRACT AND IN THE BRAIN. LOCATION IN THE PROGLUCAGON PEPTIDE SEQUENCE IS INDICATED BY NUMBERS. ADAPTED FROM FIELD ET AL 2010(FIELD ET AL., 2010A) 42
FIGURE 1.5 AMINO ACID SEQUENCES OF THE PEPTIDE HORMONES GLUCAGON, OXM, GLP-1, PYY AND GHRELIN. GLUCAGON, GLP-1 AND OXM ALL BELONG TO THE SECRETIN FAMILY OF PEPTIDE HORMONES AND EXHIBIT SIGNIFICANT SEQUENCE HOMOLOGY (HIGHLIGHTED IN ORANGE)..... 50
FIGURE 1.6 THE ACTIVATION AND ROLE OF BROWN ADIPOSE TISSUE (BAT) IN HEAT PRODUCTION. TEMPERATURE IS SENSED PERIPHERALLY, AND INFORMATION RELAYED TO THE HYPOTHALAMUS VIA THE LATERAL PARABRACHIAL NUCLEUS (LPBN) OF THE PONS. BAT IS SUBSEQUENTLY ACTIVATED VIA THE SYMPATHETIC NERVOUS SYSTEM (SNS) CARRYING AFFERENTS FROM THE HYPOTHALAMUS AND BRAINSTEM. THE HYPOTHALAMUS ALSO STIMULATES RELEASE OF THYROID HORMONES VIA THE PITUITARY GLAND. NORADRENALINE (NA) BINDS TO G-PROTEIN COUPLED (GP) BETA-3 (B3) RECEPTORS ON BAT TO ACTIVATE THE TISSUE VIA THE PATHWAY SHOWN. HEAT ENERGY IS PRODUCED BY THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA IN THE BAT CELL. THYROID HORMONES BIND TO THE THYROID HORMONE RECEPTOR (TR) ON BAT CELLS, AND CAN ALSO STIMULATE HEAT PRODUCTION VIA THIS UNCOUPLING. THESE REACTIONS ARE CARRIED OUT IN THE MITOCHONDRION VIA UNCOUPLING PROTEIN-1 (UCP-1) FOUND ON THE INNER MITOCHONDRIAL MEMBRANE. ABBREVIATIONS: AC ADENYLATE CYCLASE; ATP ADENOSINE TRIPHOSPHATE; CAMP CYCLIC ADENOSINE MONOPHOSPHATE; PKA PROTEIN KINASE A; ADP ADENOSINE DIPHOSPHATE; HSL HORMONE SENSITIVE LIPASE; TG TRIGLYCERIDE; FFA FREE FATTY ACIDS. 59
FIGURE 2.1 CUMULATIVE FOOD INTAKE IN (G) AFTER 70 DAYS OF THE CHRONIC FEEDING STUDY. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. FOOD INTAKE WAS MEASURED EVERY 1-2 DAYS THROUGHOUT THE STUDY, JUST PRIOR TO INJECTION OF THE MICE AT 1500H. THERE WAS NO STATISTICALLY SIGNIFICANT DIFFERENCE IN FOOD INTAKE BETWEEN THE GROUPS. DATA WAS ANALYSED WITH A ONE WAY ANOVA AND TUKEY'S POST-HOC TEST. N=10-12 PER GROUP. 85
FIGURE 2.2 BODY WEIGHT AFTER 70 DAYS OF THE CHRONIC FEEDING STUDY EXPRESSED AS A: ABSOLUTE BODY WEIGHT CHANGE, AND B: PERCENTAGE BODY WEIGHT CHANGE, BASELINE CORRECTED. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. BODY WEIGHT WAS MEASURED EVERY 1-2 DAYS THROUGHOUT THE STUDY, JUST PRIOR TO INJECTION OF THE MICE AT 1500H. THERE WAS A SIGNIFICANT REDUCTION IN BODY WEIGHT IN THE GLP-1-LA GROUP VS PLACEBO

(*P<0.05, **P<0.01); IN THE PYY-LA GROUP VS PLACEBO (*P<0.05, **P<0.01); IN THE GLP-1-LA GROUP VS TRIPLE COMBINATION (***P<0.001); IN THE OXM-LA GROUP VS THE TRIPLE COMBINATION (*P<0.05,**P<0.01, ***P<0.001); IN THE PYY-LA GROUP VS THE TRIPLE COMBINATION (##P<0.01, ###P<0.001) AND IN THE TRIPLE COMBINATION GROUP VS PLACEBO (*P<0.05, **P<0.01, ***P<0.001). ALL DATA WAS ANALYSED BY A TWO-WAY ANOVA WITH BONFERRONI POST-HOC TEST. N=10-12 PER GROUP. 87
FIGURE 2.3 A: INSULIN AND B: GLUCOSE CONCENTRATIONS FOLLOWING IPGTT IN DIO MICE RECEIVING ONE OF PLACEBO, GLP-1-LA, OXM-LA OR PYY-LA. THE IPGTT WAS PERFORMED IN ANIMALS THAT HAD BEEN INJECTED WITH ANALOGUE AND FASTED FROM 6 HOURS PRIOR TO THE IPGTT. BLOOD SAMPLES WERE OBTAINED VIA A TAIL TIP CUT FOR GLUCOSE AT T=-30, 0, 30, 60, 90 AND 120 MINUTES AND FOR INSULIN AT 0, 30, 60 AND 120 MINUTES. INSULIN LEVELS AT T=0 WERE SIGNIFICANTLY HIGHER IN THE PLACEBO GROUP VS OXM-LA (#P<0.05) AND IN THE PLACEBO, GLP-1-LA AND PYY-LA GROUPS COMPARED TO THE TRIPLE COMBINATION GROUP ($$$ P<0.001, && P<0.01, AND *P<0.05 RESPECTIVELY). GLUCOSE LEVELS WERE SIGNIFICANTLY LOWER IN THE COMBINATION GROUP COMPARED TO PLACEBO (&&& P<0.001); GLP-1-LA (% P<0.05, %%P<0.01); OXM-LA (^P<0.05) AND PYY-LA ($$$P<0.001). IN ADDITION, COMPARED TO BOTH THE PLACEBO GROUP AND THE PYY-LA GROUP, GLUCOSE WAS SIGNIFICANTLY LOWER IN THE GLP-1-LA GROUP (## P<0.01, ## P<0.001), AND (+P<0.05, +++P<0.001) RESPECTIVELY AND IN THE OXM-LA GROUP (£££P<0.001), AND (**P<0.01***P<0.001) RESPECTIVELY. ANALYSIS WAS BY TWO-WAY ANOVA WITH BONFERRONI POST-HOC TEST. N=10-12 PER GROUP ……. ….88
FIGURE 2.4 AREA UNDER THE CURVE (AUC) FOR A: INSULIN AND B: GLUCOSE FOLLOWING AN IPGTT IN DIO MICE RECEIVING ONE OF PLACEBO, GLP-1-LA, OXM-LA OR PYY-LA. THE IPGTT WAS PERFORMED IN ANIMALS THAT HAD BEEN INJECTED WITH ANALOGUE AND FASTED FROM 6 HOURS PRIOR TO THE IPGTT. BLOOD SAMPLES WERE OBTAINED VIA A TAIL TIP CUT FOR GLUCOSE AT T=-30, 0, 30, 60, 90 AND 120 MINUTES AND FOR INSULIN AT 0, 30, 60 AND 120 MINUTES. GLUCOSE LEVELS WERE SIGNIFICANTLY HIGHER IN THE PLACEBO AND PYY-LA GROUPS COMPARED TO THE GLP-1-LA, OXM-LA AND TRIPLE COMBINATION GROUPS (*P<0.05, **P<0.01, ***P<0.001). THERE WAS NO STATISTICAL SIGNIFICANCE BETWEEN THE INSULIN LEVELS IN ANY OF THE GROUPS. ANALYSIS WAS BY A ONE-WAY ANOVA WITH TUKEY'S POST HOC TEST. N=10-12 90
FIGURE 2.5 HARVESTED TISSUE WEIGHTS FROM A: LIVER B: INTERSCAPULAR BAT AND C: EPIDYDIMAL WAT EXPRESSED AS A PERCENTAGE OF TOTAL BODY WEIGHT. LIVER WEIGHT AS A PERCENTAGE OF TOTAL BODY WEIGHT WAS SIGNIFICANTLY REDUCED IN THE GROUP RECEIVING GLP-1-LA COMPARED TO THE GROUPS RECEIVING PLACEBO AND OXM-LA **P<0.01. BAT WEIGHT AS A PERCENTAGE OF TOTAL BODY WEIGHT WAS REDUCED IN THE GLP-1-LA (*P<0.05) AND TRIPLE COMBINATION (***P<0.001) GROUPS COMPARED TO PLACEBO. EPIDIDYMAL WAT WEIGHT AS A PERCENTAGE OF TOTAL BODY WEIGHT WAS REDUCED IN THE PLACEBO GROUP COMPARED TO THE GLP-1-LA (*P<0.05). ALL DATA WERE ANALYSED BY ONE-WAY ANOVA WITH TUKEY'S POST-HOC TEST. N=10-12. 92
FIGURE 2.6 MRNA EXPRESSION IN LIVER TISSUE IN DIO MICE FOLLOWING THE CHRONIC FEEDING STUDY. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. FGF-21 EXPRESSION IN LIVER WAS SIGNIFICANTLY INCREASED IN THE GROUPS RECEIVING OXM-LA AND THE TRIPLE COMBINATION COMPARED TO PLACEBO (*P<0.05). A: PHOSPHOENOLPYRUVATE CARBOXYKINASE 1 (PEPCK) B: FIBROBLAST GROWTH FACTOR-21 (FGF-21), C: GLUCOSE-6-PHOSPHATASE (G-6-PASE) AND D: ACETYL-COENZYME A CARBOXYLASE ALPHA (ACC1). NONE OF THE OTHER MEASURED MRNA EXPRESSION PROFILES OF INTEREST WERE SIGNIFICANTLY ALTERED BY THE INTERVENTIONS ABOVE. DATA WERE ANALYSED BY ONE WAY ANOVA WITH DUNNETT'S POST-HOC TEST. N=7-10 94
FIGURE 2.7 MRNA EXPRESSION IN BAT IN DIO MICE FOLLOWING A CHRONIC FEEDING STUDY. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. A: UNCOUPLING PROTEIN-1 (UCP-1), B: PEROXISOME PROLIFERATIVE ACTIVATED RECEPTOR GAMMA COACTIVATOR 1 ALPHA (PGC1A) C: PEROXISOME PROLIFERATIVE ACTIVATED RECEPTOR GAMMA (PPARΓ). NONE OF THE MEASURED MRNA EXPRESSION PROFILES OF INTEREST WERE SIGNIFICANTLY ALTERED BY THE

INTERVENTIONS ABOVE. DATA WERE ANALYSED BY ONE WAY ANOVA WITH DUNNETT'S POST-HOC TEST. N=7-10 ....95MRNA EXPRESSION IN WAT IN DIO MICE FOLLOWING A CHRONIC FEEDING STUDY. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. A: UNCOUPLING PROTEIN-1 (UCP-1), B: HORMONE SENSITIVE LIPASE (HSL), C: PEROXISOME PROLIFERATIVE ACTIVATED RECEPTOR GAMMA (PPARΓ). NONE OF THE MEASURED MRNA EXPRESSION PROFILES OF INTEREST WERE SIGNIFICANTLY ALTERED BY THE INTERVENTIONS ABOVE. DATA WERE ANALYSED BY ONE WAY ANOVA WITH DUNNETT'S POST-HOC TEST. N=2-6 964
FIGURE 2.8 MRNA EXPRESSION IN WAT IN DIO MICE FOLLOWING A CHRONIC FEEDING STUDY. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. A: UNCOUPLING PROTEIN-1 (UCP-1), B: HORMONE SENSITIVE LIPASE (HSL), C: PEROXISOME PROLIFERATIVE ACTIVATED RECEPTOR GAMMA (PPARΓ). NONE OF THE MEASURED MRNA EXPRESSION PROFILES OF INTEREST WERE SIGNIFICANTLY ALTERED BY THE INTERVENTIONS ABOVE. DATA WERE ANALYSED BY ONE WAY ANOVA WITH DUNNETT'S POST-HOC TEST. N=2-6 96
FIGURE 2.9 HARVESTED PLASMA FROM DIO MICE FOLLOWING A CHRONIC FEEDING STUDY WAS ANALYSED FOR MARKERS OF ENERGY HOMEOSTASIS. DIO MICE RECEIVED EITHER PLACEBO, GLP-1-LA, OXM-LA, OR PYY-LA ALONE OR IN COMBINATION. A: LEPTIN, B FREE TRI-IODOTHYRONINE (FT3), C: TRIGLYCERIDES (TG), D: ADIPONECTIN AND E: BETA-HYDROXYBUTYRATE (B-HB) WERE MEASURED IN THE PLASMA SAMPLES. THE TRIPLE COMBINATION OF ANALOGUES REDUCED LEPTIN COMPARED TO PLACEBO AND EACH ANALOGUE ALONE (*P<0.05, ***P<0.001). TRIGLYCERIDE LEVELS WERE SIGNIFICANTLY ELEVATED IN THE GROUP RECEIVING PYY-LA VS GLP-1-LA (*P<0.05). ALL DATA WERE ANALYSED WITH A ONE-WAY ANOVA AND TUKEY'S POST-HOC TEST. N=10-12 98
FIGURE 3.1 THE INDIRECT CALORIMETER. VOLUNTEERS WERE ASKED TO LIE ON A BED IN A SEMI-RECUMBENT POSITION AND THE CALORIMETER HOOD WAS PLACED OVER THEIR HEAD. EXPIRED AIR WAS COLLECTED VIA THE TUBE EXITING THE HOOD AND ITS COMPOSITION ANALYSED BY THE CALORIMETER. WITH KIND PERMISSION FROM DR E.S. CHAMBERS 111
FIGURE 3.2 THE ACUTE EFFECT OF CO-INFUSION OF GLUCAGON AND GLP-1 ON EE AND BLOOD GLUCOSE: STUDY PROTOCOL. PARTICIPANTS ARRIVED AT T=0, WHEN THEY WERE CANNULATED AND CALORIMETRY MEASUREMENTS COMMENCED. THE PEPTIDE HORMONE INFUSION WAS COMMENCED AT T=45 AND CONTINUED UNTIL T=90. BLOOD SAMPLES WERE DRAWN AS INDICATED BY THE BLUE (GUT HORMONES) AND GREEN (GLUCOSE AND INSULIN) MARKERS 116
FIGURE 3.3 ENERGY EXPENDITURE CHANGE FROM BASELINE KCAL/DAY. PARTICIPANTS WERE ADMINISTERED INFUSION OF PLACEBO (GELOFUSINE), GLP-1 AT 0.8PMOL/KG/MIN, GLUCAGON AT 50NG/KG/MIN AND GLP-1 PLUS GLUCAGON IN COMBINATION AT THE ABOVE DOSES. MEASUREMENT OF REE WAS PERFORMED PRIOR TO THE START OF THE INFUSION, AND 30 MINUTES INTO THE INFUSION ONCE PLASMA PEPTIDE LEVELS HAD ATTAINED STEADY STATE. STATISTICAL ANALYSIS WAS PERFORMED USING GRAPHPAD PRISM, USING A ONE-WAY ANOVA WITH TUKEY'S POST HOC TEST. N=10. ***P<0.001 VS PLACEBO ### P<0.001 VS GLP-1 123
FIGURE 3.4 THE ACUTE EFFECT OF CO-INFUSION OF GLUCAGON AND GLP-1 ON SUBSTRATE OXIDATION RATES. PARTICIPANTS WERE ADMINISTERED INFUSION OF PLACEBO (GELOFUSINE), GLP-1 AT 0.8PMOL/KG/MIN, GLUCAGON AT 50NG/KG/MIN AND GLP-1 PLUS GLUCAGON IN COMBINATION AT THE ABOVE DOSES. FAT AND CARBOHYDRATE OXIDATION RATES WERE CALCULATED FROM VO_2 AND VCO_2 MEASUREMENTS ACHIEVED DURING INDIRECT CALORIMETRY, AND PROTEIN OXIDATION WAS CALCULATED USING URINE UREA AND URINE VOLUME MEASUREMENTS. DATA WAS ANALYSED USING GRAPHPAD PRISM, BY A ONE-WAY ANOVA WITH TUKEY'S POST HOC TEST. N=10. ***P<0.001 PLACEBO VS GLUCAGON + GLP-1. ## P<0.01 GLP-1 VS GLUCAGON +GLP-1, *P<0.05 PLACEBO VS GLUCAGON + GLP-1, #P<0.05 GLUCAGON VS GLUCAGON + GLP-1. 125
FIGURE 3.5 GLP-1 (A) AND GLUCAGON (B) PLASMA HORMONE CONCENTRATIONS FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE

DOSES. THE INFUSION COMMENCED AT T=45 MINUTE, AND CONTINUED FOR 45 MINUTES (GREY BAR). PLASMA SAMPLES WERE DRAWN AT T=30,45,60,75, 90 AND 105 MINUTES FOR ANALYSIS OF HORMONE PROFILE. 127
FIGURE 3.6 A & B THE ACUTE EFFECT OF CO-INFUSION OF GLUCAGON AND GLP-1 ON PLASMA INSULIN (A) AND GLUCOSE (B) CONCENTRATIONS FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. THE INFUSION COMMENCED AT T=45 MINUTES, AND CONTINUED FOR 45 MINUTES (GREY BAR). PLASMA SAMPLES WERE DRAWN AT T=30,45,60,75,90 AND 105 MINUTES FOR ANALYSIS. COMPARED TO PLACEBO, THE COMBINATION OF GLUCAGON + GLP-1 CAUSED A SIGNIFICANT INCREASE IN SERUM INSULIN (***P<0.001). COMPARED TO GLP-1, BOTH GLUCAGON ALONE (*P<0.05), AND THE COMBINATION OF GLUCAGON +GLP-1(### P<0.001) CAUSED A SIGNIFICANT RISE IN SERUM INSULIN. THE COMBINATION INFUSION ALSO CAUSED A SIGNIFICANT RISE IN INSULIN VS GLUCAGON ALONE ($ P<0.05, $$ P<0.01, $$$ P<0.001). GLP-1, GLUCAGON AND THE COMBINATION OF GLP-1/GLUCAGON ALL CAUSED A SIGNIFICANT RISE IN PLASMA GLUCOSE CONCENTRATION COMPARED TO PLACEBO (*P<0.05, **P<0.01, ***P<0.001, # P<0.05). GLUCAGON AND THE COMBINATION OF GLP-1/GLUCAGON CAUSED A RISE IN GLUCOSE COMPARED TO GLP-1 (### P<0.001, $$$ P<0.001), AND GLUCAGON ALONE CAUSED A RISE IN GLUCOSE COMPARED TO THE COMBINATION INFUSION (£££ P<0.01, £££ P<0.001). N=10. DATA ANALYSIS WAS BY A TWO-WAY REPEATED MEASURES ANOVA WITH BONFERRONI POST-HOC TEST. 130
FIGURE 3.7 A & B AREA UNDER THE CURVE FOR INSULIN (A) AND GLUCOSE (B) FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. THE COMBINATION INFUSION RESULTED IN A SIGNIFICANT RISE IN SERUM INSULIN COMPARED TO BOTH PLACEBO AND GLP-1 (P<0.001). GLUCAGON ALONE INCREASED PLASMA GLUCOSE COMPARED TO BOTH PLACEBO AND GLP-1 (P<0.001), AND THE COMBINATION INFUSION ALSO INCREASED PLASMA GLUCOSE COMPARED TO GLP-1 ALONE (P<0.01). N=10. ANALYSIS BY ONE WAY ANOVA WITH TUKEY'S POST-HOC TEST. 131
FIGURE 3.8 A & B TOTAL AND ACYL GHRELIN LEVELS PRIOR TO AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. THE INFUSION OF A COMBINATION OF GLP-1 AND GLUCAGON SIGNIFICANTLY REDUCES BOTH TOTAL AND ACYL GHRELIN LEVELS (*P<0.05, **P<0.01 RESPECTIVELY). N=10. ANALYSIS WAS BY TWO-WAY REPEATED MEASURES ANOVA WITH BONFERRONI POST-HOC TEST. 134
FIGURE 3.9 CORTISOL LEVEL PRIOR TO AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. DATA WAS ANALYSED BY A TWO-WAY REPEATED MEASURES ANOVA WITH BONFERRONI POST-HOC TEST. N=10. THERE WAS NO SIGNIFICANT DIFFERENCE IN PLASMA CORTISOL IN ANY OF THE GROUPS WHEN POST INFUSION LEVELS WERE COMPARED TO BASELINE. 135
FIGURE 3.10 NEFA CONCENTRATIONS PRIOR TO AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. DATA WAS ANALYSED BY A TWO-WAY REPEATED MEASURES ANOVA WITH BONFERRONI POST-HOC TEST. N=10. INFUSION OF GLUCAGON ALONE (***P<0.001) AND THE COMBINATION OF GLP-1 + GLUCAGON (***P<0.001) BOTH CAUSED A SIGNIFICANT REDUCTION IN CIRCULATING LEVELS OF PLASMA NEFAS. . 136
FIGURE 3.11 TSH, FREE T4 AND FREE T3 PLASMA CONCENTRATIONS PRIOR TO AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. DATA WAS ANALYSED BY A TWO-WAY REPEATED MEASURES ANOVA WITH BONFERRONI POST-

HOC TEST. N=10. INFUSION OF GLUCAGON ALONE CAUSED A REDUCTION IN TSH (**P<0.01) AND FT3 (*P<0.05) BUT NO OTHER INFUSION CAUSED ANY SIGNIFICANT CHANGES IN THYROID HORMONE LEVELS. 137
FIGURE 3.12 CHANGE FROM BASELINE FOR PULSE AND BLOOD PRESSURE MEASUREMENT FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE (B.BRAUN, CRAWLEY, UK)), GLP-1 ALONE AT 0.8PMOL/KG/MIN, GLUCAGON ALONE AT 50NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. DATA ANALYSIS WAS BY A ONE-WAY ANOVA WITH TUKEY'S POST HOC TEST. N=10. THERE WAS NO SIGNIFICANT CHANGE FROM BASELINE IN CARDIOVASCULAR PARAMETERS FOLLOWING ANY OF THE INFUSIONS. 139
FIGURE 4.1 'THE ACUTE EFFECT OF CO-INFUSION OF GLUCAGON AND GLP-1 ON FOOD INTAKE': STUDY PROTOCOL. PARTICIPANTS ARRIVED AT T=-60, WHEN THEY WERE CANNULATED. CALORIMETRY MEASUREMENTS WERE COMMENCED AT T=-30 UNTIL T=0. THE PEPTIDE HORMONE INFUSION WAS COMMENCED AT T=0 AND CONTINUED UNTIL T=120. FURTHER CALORIMETRY TOOK PLACE AT T=40 TO T=70. AN AD-LIBITUM MEAL WAS SERVED AT T=90. THE INFUSION WAS STOPPED AT T=120 AND PARTICIPANTS DISCHARGED 1 HOUR LATER. BLOOD SAMPLES WERE DRAWN AS INDICATED BY THE BLUE (GUT HORMONES), GREEN (GLUCOSE AND INSULIN) AND PURPLE (GHRELIN) MARKERS 153
FIGURE 4.2 A: THE ACUTE EFFECT OF LOW-DOSE CO-INFUSION OF GLUCAGON AND GLP-1 ON FOOD INTAKE FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. ANALYSIS WAS BY A REPEATED MEASURES ANOVA WITH TUKEY'S POST HOC TEST. THE COMBINATION OF GLP-1 AND GLUCAGON SIGNIFICANTLY REDUCED FI VS PLACEBO (*P<0.05), AND GLUCAGON ALONE ($ P<0.05) BUT NOT GLP-1. N=13 B: ENERGY INTAKE AS A PERCENTAGE CHANGE OF THE PLACEBO VISIT N=13. 158
FIGURE 4.3 THE ACUTE EFFECT OF LOW DOSE CO-INFUSION OF GLUCAGON AND GLP-1 ON A: HUNGER, B: SATIETY, C: NAUSEA AND D: PALATABILITY OF AN AD-LIBITUM STUDY MEAL. VAS SCORES WERE RECORDED DURING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. NAUSEA WAS INCREASED AT T=120 MINUTES (POSTPRANDIALLY) WITH THE COMBINATION INFUSION, BUT THIS WAS NOT SIGNIFICANT. A, B & C ANALYSED WITH A REPEATED MEASURES NON-PARAMETRIC FRIEDMAN'S TEST WITH DUNN'S MULTIPLE COMPARISON POST-HOC TEST, AND D ANALYSED WITH A REPEATED MEASURES ONE-WAY ANOVA WITH TUKEY'S POST-HOC TEST. N=13 160
FIGURE 4.4 A & B THE ACUTE EFFECT OF LOW-DOSE CO-INFUSION OF GLUCAGON AND GLP-1 ON A: PLASMA ACTIVE GLP-1 AND B: GLUCAGON LEVELS FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. THE INFUSION COMMENCED AT T=0 AND LASTED FOR 120 MINUTES. BLOOD SAMPLES WERE DRAWN AT T=-60, -30, 0, 40, 70, 90, 120, 150 AND 180 FOR ANALYSIS OF PLASMA HORMONE PROFILES. 163
FIGURE 4.5 THE ACUTE EFFECT OF LOW-DOSE CO-INFUSION OF GLUCAGON AND GLP-1 ON RESTING ENERGY EXPENDITURE BOTH PRIOR TO, AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. THERE WAS NO STATISTICALLY SIGNIFICANT CHANGE IN REE DETECTED BETWEEN THE GROUPS ON ANALYSIS WITH REPEATED MEASURES ONE WAY ANOVA AND TUKEY'S POST-HOC TEST. N=12. 165
FIGURE 4.6 THE ACUTE EFFECT OF LOW DOSE CO-INFUSION OF GLUCAGON AND GLP-1 ON A:CARBOHYDRATE B: FAT AND C:PROTEIN OXIDATION. OXIDATION RATES WERE CALCULATED BOTH PRIOR TO, AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. FAT AND CARBOHYDRATE OXIDATION RATES WERE CALCULATED

FROM VO_2 AND VCO_2 MEASUREMENTS ACHIEVED DURING INDIRECT CALORIMETRY, AND PROTEIN OXIDATION WAS CALCULATED USING URINE UREA AND URINE VOLUME MEASUREMENTS. DATA WAS ANALYSED BY A ONE-WAY REPEATED MEASURES ANOVA WITH TUKEY'S POST HOC TEST. N=12 (ONE DATA SET MISSING). A: **P<0.05 PLACEBO VS GLUCAGON, ££ P<0.05 GLUCAGON VS GLUCAGON + GLP-1, ### P<0.001 GLP-1 VS GLUCAGON, ***P<0.001 GLP-1 VS GLUCAGON + GLP-1, $$$P<0.001 PLACEBO VS GLUCAGON + GLP-1. B:*P<0.05 GLUCAGON VS GLUCAGON + GLP-1, **P<0.01 PLACEBO VS GLUCAGON, ###P<0.001 PLACEBO VS GLUCAGON + GLP-1, $$$ P<0.001 GLP-1 VS GLUCAGON ALONE, ***P<0.001 GLP-1 ALONE VS GLUCAGON + GLP-1. C: P<0.05 GLP-1 VS GLUCAGON. 167

FIGURE 4.7 A & B. THE ACUTE EFFECT OF CO-INFUSION OF LOW DOSE GLUCAGON AND GLP-1 ON A:PLASMA GLUCOSE AND B:INSULIN CONCENTRATIONS. SAMPLES WERE TAKEN DURING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. GLUCAGON CAUSED A SIGNIFICANT RISE IN GLUCOSE LEVELS COMPARED TO PLACEBO (***P<0.001, *P<0.05), GLP-1 (###P<0.001), AND THE COMBINATION INFUSION (£P<0.05). IN ADDITION, THE COMBINATION CAUSED A SIGNIFICANT RISE IN GLUCOSE COMPARED TO GLP-1 ALONE (&& P<0.01). THE COMBINATION INFUSION OF GLUCAGON AND GLP_1 CAUSED A SIGNIFICANT RISE OF INSULIN COMPARED TO PLACEBO (**P<0.01), AND GLP-1 ALONE (*P<0.05). ALL DATA WERE ANALYSED WITH REPEATED MEASURES TWO-WAY ANOVA WITH BONFERRONI POST-HOC TEST. N=13 170

FIGURE 4.8 A & B AREA UNDER THE CURVE FOR INSULIN (A) AND GLUCOSE (B) FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. A: THE COMBINATION INFUSION RESULTED IN A SIGNIFICANT RISE IN SERUM INSULIN COMPARED TO ALL OTHER GROUPS (PLACEBO $$$ P<0.001; GLP-1 ###P<0.001; GLUCAGON *P<0.05). GLUCAGON ALONE ALSO SIGNIFICANTLY INCREASED INSULIN SECRETION COMPARED TO PLACEBO AND GLP-1 ALONE (***P<0.001, £££P<0.001 RESPECTIVELY). B: GLUCAGON ALONE INCREASED PLASMA GLUCOSE COMPARED TO BOTH PLACEBO AND GLP-1 (***P<0.001 AND $$$P<0.001 RESPECTIVELY), AND THE COMBINATION INFUSION ALSO INCREASED PLASMA GLUCOSE COMPARED TO GLP-1 ALONE (##P<0.01) AND GLUCAGON ALONE (*P<0.05) N=10. ANALYSIS BY REPEATED MEASURES ONE WAY ANOVA WITH TUKEY'S POST-HOC TEST. 171

FIGURE 4.9 THE ACUTE EFFECT OF LOW DOSE CO-INFUSION OF GLUCAGON AND GLP-1 ON A: ACYLATED AND B: TOTAL GHRELIN LEVELS. SAMPLES WERE TAKEN BOTH BEFORE, AND FOLLOWING INFUSION OF EACH OF PLACEBO (GELOFUSINE, B.BRAUN, CRAWLEY, UK), GLP-1 ALONE AT 0.4 PMOL/KG/MIN, GLUCAGON ALONE AT 10 NG/KG/MIN, AND A COMBINATION OF GLP-1 AND GLUCAGON AT THE ABOVE DOSES. INFUSION OF THE HORMONES EITHER ALONE OR IN COMBINATION HAD NO SIGNIFICANT EFFECT ON GHRELIN CONCENTRATIONS. DATA WERE ANALYSED USING A REPEATED MEASURES TWO-WAY ANOVA WITH BONFERRONI POST-HOC TEST. N=13 173

FIGURE 4.10 THE ACUTE EFFECT OF CO-INFUSION OF LOW DOSE GLUCAGON + GLP-1 ON CARDIOVASCULAR PARAMETERS (A: PULSE, B: SYSTOLIC BLOOD PRESSURE AND C: DIASTOLIC BLOOD PRESSURE). THERE WAS NO STATISTICAL SIGNIFICANCE BETWEEN THE GROUPS FOR ANY OF THESE PARAMETERS. ANALYSIS WITH TWO-WAY REPEATED MEASURES ANOVA WITH TUKEY'S POST HOC TEST N=13. 175

FIGURE 5.1 'THE ACUTE EFFECT OF CO-INFUSION OF PYY(3-36) AND GLP-1 ON GLUCOSE HOMEOSTASIS': STUDY PROTOCOL. PARTICIPANTS ARRIVED AT T=-30, WHEN THEY WERE CANNULATED. THE PEPTIDE HORMONE INFUSION WAS COMMENCED AT T=0 AND CONTINUED UNTIL T=240. AT T=60 A 0.3G/KG BOLUS OF IV GLUCOSE WAS ADMINISTERED AND A FSIVGTT WAS PERFORMED. BLOOD SAMPLES WERE DRAWN AS INDICATED BY THE BLUE (GUT HORMONES), AND GREEN (GLUCOSE AND INSULIN) MARKERS 190

FIGURE 5.2A: THE ACUTE EFFECT OF CO-INFUSION OF PYY(3-36) AND GLP-1 ON SERUM INSULIN CONCENTRATIONS FOLLOWING INFUSION OF GELOFUSINE ALONE, GLP-1(7-36 AMIDE) AT 0.2PMOL/KG/MIN, PYY(3-36) AT 0.15PMOL/KG/MIN AND PYY(3-36) AND GLP-1(7-36 AMIDE) COMBINED

AT THE ABOVE DOSES. DATA WERE ANALYSED WITH A TWO-WAY REPEATED MEASURES ANOVA AND BONFERRONI POST-HOC TEST. THERE WAS NO STATISTICAL SIGNIFICANCE BETWEEN THE GROUPS. N=14. B: THE ACUTE EFFECT OF CO-INFUSION OF PYY(3-36) AND GLP-1 ON PLASMA GLUCOSE CONCENTRATIONS FOLLOWING INFUSION OF GELOFUSINE ALONE, GLP-1(7-36 AMIDE) AT 0.2PMOL/KG/MIN, PYY(3-36) AT 0.15PMOL/KG/MIN AND PYY(3-36) AND GLP-1(7-36 AMIDE) COMBINED AT THE ABOVE DOSES. DATA WERE ANALYSED WITH A TWO-WAY REPEATED MEASURES ANOVA AND BONFERRONI POST-HOC TEST. THERE WAS NO STATISTICAL SIGNIFICANCE BETWEEN THE GROUPS. N=14 194

FIGURE 5.3 AUC FOR A: INSULIN AND B: GLUCOSE IN THE FIRST 10 MINUTES FOLLOWING THE INTRAVENOUS GLUCOSE BOLUS IN THE FSIVGTT. THERE WAS NO STATISTICALLY SIGNIFICANT CHANGE IN PLASMA GLUCOSE LEVELS DURING ANY OF THE INFUSIONS. THERE WAS A SIGNIFICANT INCREASE IN INSULIN WHEN PARTICIPANTS RECEIVED AN INFUSION OF GLP-1 ALONE (*P<0.05), BUT NOT THE OTHER INFUSIONS. DATA WERE ANALYSED USING A ONE WAY REPEATED MEASURES ANOVA WITH TUKEY'S POST-HOC TEST. N=14 196

FIGURE 5.4 AIRG, S_I AND DI FOLLOWING INFUSION OF GELOFUSINE ALONE, GLP-1(7-36 AMIDE) AT 0.2PMOL/KG/MIN PYY(3-36) AT 0.15PMOL/KG/MIN AND PYY(3-36) PLUS GLP-1(7-36 AMIDE) COMBINED AT THE ABOVE DOSES. GLP-1 CAUSES A SIGNIFICANT INCREASE IN A: AIRG VS PLACEBO (*P<0.05), AND FOR C: DI VS PLACEBO (*P<0.05) BUT THERE WAS NO STATISTICAL SIGNIFICANCE BETWEEN ANY OF THE GROUPS FOR S_I. DATA WERE ANALYSED BY ONE-WAY ANOVA WITH TUKEY'S POST HOC TEST N=14 198

FIGURE 5.5 THE ACUTE EFFECT OF CO-INFUSION OF PYY(3-36) AND GLP-1 ON A: PLASMA GLP-1 CONCENTRATIONS AND B: PLASMA PYY CONCENTRATIONS FOLLOWING INFUSION OF GELOFUSINE ALONE, GLP-1(7-36 AMIDE) AT 0.2PMOL/KG/MIN, PYY(3-36) AT 0.15PMOL/KG/MIN AND PYY(3-36) AND GLP-1(7-36 AMIDE) COMBINED AT THE ABOVE DOSES. THE INFUSION COMMENCED AT T=0 AND LASTED FOR 240 MINUTES. BLOOD SAMPLES WERE DRAWN AT T=0, 20, 40, 60, 80, 100, 160 AND 240 MINUTES FOR ANALYSIS OF PLASMA HORMONE PROFILES. N=13 200

FIGURE 5.6 THE ACUTE EFFECT OF CO-INFUSION OF PYY(3-36) AND GLP-1 ON PLASMA CORTISOL LEVELS FOLLOWING INFUSION OF GELOFUSINE ALONE, GLP-1(7-36 AMIDE) AT 0.2PMOL/KG/MIN, PYY(3-36) AT 0.15PMOL/KG/MIN AND PYY(3-36) AND GLP-1(7-36 AMIDE) COMBINED AT THE ABOVE DOSES. GLP-1 ALONE (*P<0.05, **P<0.01) AND THE COMBINATION OF PYY(3-36) + GLP-1 (#P<0.05, ##P<0.01) CAUSED AN ELEVATION OF PLASMA CORTISOL COMPARED TO PLACEBO. DATA ANALYSED WITH A TWO WAY REPEATED MEASURES ANOVA AND BONFERRONI POST-HOC TEST. N=14 202

FIGURE 5.7 THE ACUTE EFFECT OF CO-INFUSION OF PYY(3-36) AND GLP-1 ON A: HUNGER, B:NAUSEA, C:SATIETY. DATA ARE PRESENTED AS CHANGE FROM BASELINE IN MM. THERE WAS A SMALL BUT SIGNIFICANT DIFFERENCE IN NAUSEA SCORE BETWEEN THE PLACEBO AND PYY(3-36) GROUPS (P<0.05), BUT NO SIGNIFICANT DIFFERENCE BETWEEN THE OTHER GROUPS AT ANY TIME POINT, DATA WERE ANALYSED USING A REPEATED MEASURES NON-PARAMETRIC FRIEDMAN'S TEST WITH DUNN'S MULTIPLE COMPARISON POST-HOC TEST. N=12..... 203

FIGURE 5.8 THE ACUTE EFFECT ON CARDIOVASCULAR PARAMETERS A: PULSE, B: SYSTOLIC BLOOD PRESSURE (SBP), C: DIASTOLIC BLOOD PRESSURE (DBP) FOLLOWING FSIVGTT AND INFUSION OF GELOFUSINE ALONE, GLP-1(7-36 AMIDE) AT 0.2PMOL/KG/MIN, PYY(3-36) AT 0.15PMOL/KG/MIN AND PYY(3-36) AND GLP-1(7-36 AMIDE) COMBINED AT THE ABOVE DOSES. THERE WAS NO STATISTICAL SIGNIFICANCE BETWEEN CARDIOVASCULAR PARAMETERS IN ANY OF THE INFUSION GROUPS. DATA WERE ANALYSED USING A REPEATED MEASURES TWO-WAY ANOVA WITH BONFERRONI POST-HOC TEST. N=12 206

INDEX OF TABLES

TABLE 2.1 TISSUES HARVESTED AND RESPECTIVE BIOCHEMICAL MARKERS OF ENERGY HOMEOSTASIS ANALYSED 73
TABLE 2.2 PROBES EMPLOYED IN THE QUANTIFICATION OF DNA BY RTPCR. ALL PROBES WERE OBTAINED FROM APPLIED BIOSYSTEMS, UK. FOR ABBREVIATIONS SEE TABLE 2.1 79
TABLE 3.1 DEMOGRAPHIC DETAILS AND MEANS OF ALL VOLUNTEERS INCLUDED IN THE STUDY 'THE ACUTE EFFECTS OF CO-INFUSION OF GLUCAGON AND GLP-1 ON EE AND GLUCOSE HOMEOSTASIS' 109
TABLE 3.2 COLLECTION DETAILS OF SAMPLES OBTAINED DURING STUDY VISIT 115
TABLE 4.1 DEMOGRAPHIC DETAILS AND MEANS OF ALL VOLUNTEERS INCLUDED IN THE STUDY 'THE ACUTE EFFECTS OF CO-INFUSION OF GLUCAGON AND GLP-1 ON FOOD INTAKE' 149
TABLE 5.2 DEMOGRAPHIC DETAILS AND MEANS OF ALL VOLUNTEERS INCLUDED IN THE STUDY 'THE ACUTE EFFECTS OF CO-INFUSION OF PYY AND GLP-1 ON GLUCOSE HOMEOSTASIS' (DM = DATA MISSING) 186

CHAPTER 1: GENERAL INTRODUCTION

1.1 Obesity

1.1.1 The Obesity Crisis

Obesity has reached epidemic proportions worldwide, with World Health Organisation (WHO) figures from 2008 indicating a prevalence of overweight in greater than 1.4 billion adults, 500 million of whom were categorised as obese(WHO, 2014). Overweight is classed as a Body Mass Index (BMI) of 25-30kg/m^2, and obesity as a BMI ≥30kg/m^2. The problem is exaggerated in the western world, due to increasingly sedentary lifestyles and the ready availability of high calorie, high fat foods but continues to grow globally.

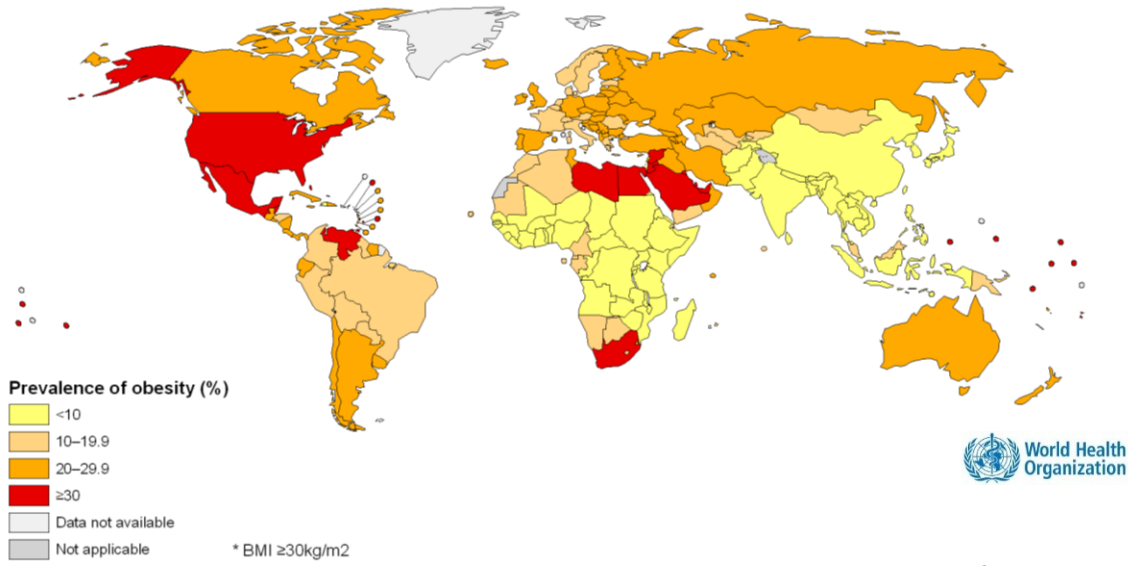


Figure 1.1. World Health Organisation map denoting prevalence of obesity in both men and women aged 20 years or more in 2008(WHO, 2011)

Figures from England alone suggest that the population prevalence of obesity has almost doubled over the last twenty years from 13.2% to 24.4% in men, and from 16.4% to 25.1% in women. Similarly, the proportion of people who are overweight has increased over the same period from 57.6% to 66.6% in men, and from 48.6% to 57.2% in women (Lifestyles statistics team, 2014). Obesity places a huge

burden on healthcare providers both financially, with projected costs to the National Health Service (NHS) predicted to rise to £8.3 billion by 2025 (Foresight, 2007), and clinically due to the number of medical conditions for which obesity is a risk factor. It is implicated in the development of type 2 diabetes, ischaemic heart disease, stroke and cancer, and increases the risk of both cause-specific and all-cause mortality (Ringback Weitoft et al., 2008, Whitlock et al., 2009, Zheng W et al., 2011). Interestingly, although obesity per se clearly carries health risks, recent data from Denmark suggests a shift in the goalposts in terms of the optimal BMI range. Whilst a BMI between 20-25 kg/m^2 has traditionally been accepted as carrying the fewest health risks, the Danish study suggests that a BMI of 27 kg/m^2 is now associated with the lowest rate of all cause mortality. (Afzal et al., 2016)

1.1.2 Central Control of Appetite

There are complex interactions between the gut and peripheral tissues and the central nervous system (CNS) involved in the control of appetite (Figure 1.2).

Within the CNS, the hypothalamus and brainstem are integral to the control of appetite. They detect peripheral signals in the circulation such as nutrients and gut hormones as well as hormones from adipose tissue such as leptin. The brain and peripheral circulation are separated by the blood brain barrier (BBB), but there are areas where the BBB is incomplete and leaky and at these points, peripheral signals have access to the central nervous system. These areas are called circumventricular organs, or CVOs. There are three such areas in proximity to the hypothalamus: the median eminence (ME), the subfornical organ (SFO), and the organum vasculosum of the lamina terminalis (OVLT). The area postrema (AP), another CVO, is adjacent to the brainstem.

Within the hypothalamus, the arcuate nucleus of the hypothalamus (ARC) is a key area involved in appetite control. It is closely related to the ME and therefore has access to, and is able to process peripheral satiety signals directly. It contains two populations of neurons which are particularly important in relation to appetite (Fig 1.2).

1.1.2.1 Anorexigenic control circuits

The first population is anorexigenic, i.e. its role is to suppress food intake. This neuronal population expresses cocaine and amphetamine regulated transcript protein (CART) and pro-opiomelanocortin (POMC)(Lambert et al., 1998). POMC is cleaved to produce alpha-melanocyte stimulating hormone (αMSH), the ligand for the melanocortin-4 receptor (MC4R). The melanocortin-4 receptor is associated with appetite and body habitus, and humans with loss of function mutations in the MC4R are obese (Farooqi et al., 2000) as are MC4R knockout mice(Huszar et al., 1997). Furthermore, administration of MC4R agonists has been shown to reduce food intake(Fan et al., 1997).

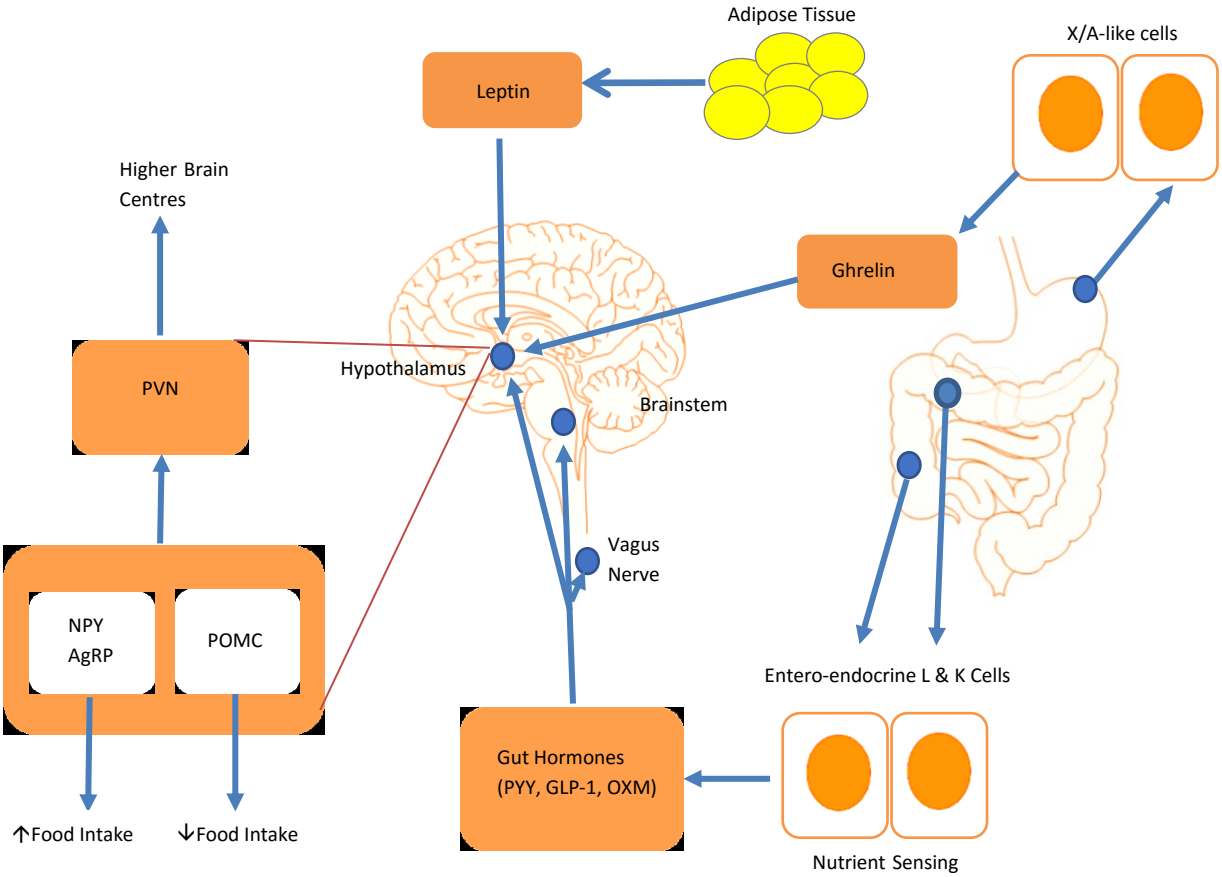


Figure 1.2. The gut-brain axis Interaction of peripheral signals with the central nervous system in the regulation of food intake. Entero-endocrine L & K-cells sense nutrients and as a consequence release gut hormones Peptide YY, Glucagon-like Peptide-1 and Oxyntomodulin. The orexigenic hormone Ghrelin is released from X/A-like cells in the fundus of the stomach in the fasted state. All of these hormones are sensed by areas within the brainstem & hypothalamus. The hypothalamus contains both orexigenic NPY/AgRP neurons and anorexigenic POMC neurons which act to control food intake. Leptin, a hormone from adipose tissue, interacts with the hypothalamus as an indicator of longer-term nutritional status.

1.1.2.2 Orexigenic control circuits

The second neuronal population of interest in the ARC is orexigenic, or appetite stimulating. These neurons express agouti-related peptide (AgRP) and neuropeptide Y (NPY). AgRP is an antagonist of αMSH at the MC4R (Ollmann et al., 1997) and causes hyperphagia when injected directly into the paraventricular nucleus of the hypothalamus (PVN)(Hagan et al., 2000). The PVN is an area which receives projections from the ARC and is important in energy regulation (Légrádi and Lechan, 1999, Martin et al., 2006).

NPY is a member of the 'Pancreatic Polypeptide fold' (PP fold) family of proteins, which also includes Peptide Tyrosine-Tyrosine (Peptide YY, or PYY) and Pancreatic Polypeptide (PP). This group of proteins exert their effect through binding to the seven-transmembrane-domain G protein coupled 'Y' receptors of the NPY receptor family. In humans, there are functionally four Y receptors, Y1, Y2, Y4 and Y5. The Y3 receptor initially was postulated as a subtype with a higher affinity for NPY compared to PYY, but was never subsequently cloned, with no specific agonists/ antagonists for this receptor subtype identified. The Y6 receptor has been identified in mice and rabbits(Larhammar D, 2001) but in humans a frame shift mutation has inactivated the Y6 gene on chromosome 5, seemingly rendering it a pseudogene. (Ballantyne, 2006).

NPY acts mainly on Y1 and Y5 receptors in the hypothalamus to exert its orexigenic effects (Duhault et al., 2000). When injected directly into the central nervous system NPY causes hyperphagia, and following chronic administration this results in obesity (Zarjevski et al., 1993). Ablation of NPY/AgRP neurons by transgenic techniques results in a lean, hypophagic phenotype(Bewick et al., 2005), supporting the role of these peptides as orexigenic agents.

1.1.2.3 Vagal signals

In addition to their direct central effects, gut hormones can activate receptors on vagal afferent neurons which transmit satiety signals to appetite-related areas in the brainstem such as the nucleus

tractus solitarius (NTS). The NTS is known to contain neurons expressing POMC in the rat (Kawai et al., 1984), and moreover, the dorsal motor nucleus of the vagus nerve (DMX), adjacent to the NTS, exhibits an extremely high density of MC4R (Mountjoy et al., 1994). This implies that the DMX may mediate anorexia via activation of NTS POMC neurons (Millington, 2007). In addition, the NTS also contains NPY neurons which project onto the PVN (Sawchenko et al., 1985). The NTS can therefore mediate anorexic or orexigenic effects following stimulus from the vagus.

Such vagal afferent signals are triggered on eating by gastric distension (Wang et al., 2008) and by gut hormone action on receptors on the vagus nerve itself (Dockray, 2009)(Fig 1.2). Both GLP-1 and PYY(3-36) (Abbott et al., 2005, Burdyga et al., 2008, Bucinskaite et al., 2009) as well as ghrelin (Date et al., 2002) have been shown to act in this manner as well as potentially having direct mechanisms of action on central areas of appetite control such as the ARC and the brainstem via circumventricular organs (see section 1.1.2). Receptor expression on the vagus nerve itself is dynamic, displaying a certain degree of plasticity. In the fasted state, or if food intake is reduced by as little as 10% per day, the Y2 receptors for PYY on the vagus appear to be down-regulated, with a five-fold reduction in Y2R transcripts (Burdyga et al., 2008), thus reducing satiety signals and increasing the stimulus to eat. In models of obesity, this ability of the vagal neurons to adjust their phenotype is lost. Obese rats develop an inability to up-regulate vagal Y2 receptors and as such exhibit a blunted sensitivity to satiety signals (de Lartigue et al., 2012) . Conversely, the effect of the orexigenic hormone ghrelin is enhanced in the fasted state(Kentish et al., 2012), with a demonstrable up-regulation in vagal afferents of the nodose ganglia of the expression of ghrelin receptor mRNA compared to the fed state(Kentish et al., 2012).

1.1.2.4 Gut hormone interactions with central control circuits

Evidence that the gut hormones PYY, GLP-1 and OXM exert their effects on satiety via nuclei in the brainstem and hypothalamus is widespread. PYY(3-36), the active form of the hormone, has been shown to increase *c-Fos* expression (a marker of neuronal activation) in the hypothalamic and brainstem regions of the ARC, NTS and AP (Blevins et al., 2008, Bonaz et al., 1993, Halatchev et al., 2004), and the Y2 receptor is widely expressed within hypothalamic nuclei (Fetissov et al., 2004, Jacques et al., 1997). In addition, some change in hypothalamic signal intensity on manganese-enhanced magnetic resonance imaging (MEMRI) was observed after peripheral administration of PYY(3-36), implying some degree of hormone action at this site, although whether this is direct or indirectly mediated via the vagus is not apparent (Hankir et al., 2011). PYY expression in the brainstem can be regulated in rodents at least in part by nutritional status, starvation and weight loss surgery, with a correlation also demonstrated between PYY expression, weight loss and leptin levels (Gelegen et al., 2012). Similarly, peripheral administration of oxyntomodulin (OXM) has also been shown to increase *c-Fos* expression in the ARC (Dakin et al., 2004), as well as causing an alteration of signal intensity within the AP on MEMRI (Parkinson et al., 2009). To support this evidence of OXM action in the hypothalamus and brainstem, Vrang et al demonstrated that preproglucagon (the precursor to both OXM and GLP-1) expressing neurons in the NTS project onto specific hypothalamic targets (Vrang et al., 2007). GLP-1 itself has a high density of receptor binding sites within the hypothalamus (Kanse et al., 1988). Tauchi et al demonstrated GLP-1 immunoreactivity in the PVN (Tauchi et al., 2008), and Katsurada et al showed that GLP-1 neurons from the NTS project directly onto the PVN, indicating that endogenous GLP-1 does indeed target the PVN in the control of feeding (Katsurada et al., 2014). In addition to the effects of PYY(3-36) and OXM on *c-Fos* in areas key to the control of appetite, both GLP-1 and its analogue, Exendin-4, have been demonstrated, albeit at supraphysiological levels, to induce *c-Fos* in the brainstem, the ARC and the PVN (Baraboi et al., 2011, Parker et al., 2013), reinforcing the role of these areas in GLP-1 mediated control of feeding and appetite.

1.1.3 Current Obesity Therapies

1.1.3.1 Lifestyle

In the face of this growing problem of obesity, there is a huge need for effective treatment strategies. In some cases, intensive lifestyle and dietary modification can achieve weight loss which is sustained at least in the short term. Indeed, Carney et al showed an initial reduction in body weight of 14.4% using a combination approach of meal replacements plus weekly classes. Participants in the study achieved a sustained weight loss of 12.9% of initial weight (compared to around 5% seen with most medical therapies – see section 1.1.3.2) which persisted 2 years after the interventions ceased, with little evidence of patients regaining their lost weight (Carney et al., 2015). However, in a further large review conducted in the USA, looking at data from the 1999-2002 National Health and Nutrition Examination Survey (NHANES), at least a third of overweight/obese adults aged 20-84 years who had achieved substantial weight loss (classed as 10% less than their maximum body weight 1 year prior to survey) had regained more than 5% of their body weight over time (Weiss et al., 2007).

1.1.3.2 Medical Therapies

Lifestyle and dietary modification alone are inadequate for the majority of obese individuals, but despite an increasingly high demand for intervention, the field of obesity therapeutics traditionally has limited options to offer the obese patient. Medical therapies are few and far between. Previously available therapies include Sibutramine, a norepinephrine and serotonin reuptake inhibitor (NSRI), and Rimonabant, which acts as an antagonist at the cannabinoid-1 (CB-1) receptor. Sibutramine was withdrawn as it increased blood pressure and the risk of cardiovascular events (James et al., 2010), whilst Rimonabant was withdrawn amidst concerns regarding adverse psychiatric events (Christensen et al., 2007).

Until recently, the only licensed product in the UK was Orlistat, a pancreatic lipase inhibitor which prevents fat absorption and confers a modest weight loss of 2.9 kg more than placebo (Rucker et al.,

2007). In addition to weight loss, it reduces the incidence of diabetes as well as improving glycaemic control in patients with diabetes. It also has a beneficial effect on concentrations of total cholesterol (TC) and low density lipoprotein cholesterol (LDL), as well as blood pressure. In more recent years, a few selected novel therapies have been brought to the market, which have been cautiously approved for use in weight management. The first was Lorcaserin (Belviq®, Eisai), which stimulates 5HT-2C (serotonin) receptors on the anorectic hypothalamic POMC neurons to produce an average 3-3.6% weight loss compared to placebo (Fidler et al., 2011, Smith et al., 2010). Lorcaserin has been approved by the Food and Drug Administration (FDA) in the USA, but not its European counterpart, the European Medicines Agency (EMA), due to concerns over the potential for adverse effects including cardiac valvulopathies, seen with earlier drugs acting on the 5-HT system. The next drug to be approved by the FDA was a fixed dose combination of Phentermine and Topiramate (Qsymia®, Vivus). Phentermine was in use as a short term weight management adjunct, with actions similar to that of amphetamines, but was not licensed for longer term use due to the perceived risk of addiction (Colman, 2005). Topiramate is a drug used therapeutically for seizures and migraine, which has also incidentally been shown to confer an anorectic effect (Ben-Menachem et al., 1996). The combination of these two drugs at a low dose achieved an average 6.6% weight loss above and beyond that seen with placebo. At a higher dose, weight loss was greater but use was limited by side effects, in particular psychiatric and cognitive effects such as depression, anxiety or attention disturbance. (Gadde et al., 2011). Administering the combination of two drugs allowed smaller doses to be given, and hence reduced disadvantageous side effects. Once again, despite approval from the FDA, the EMA has not granted a license for the use of Qsymia® (Vivus), with concerns centering on teratogenicity, cardiovascular effects, and potential neuropsychiatric consequences.

The third novel therapy to be recently approved, this time both in the USA (Contrave®, Takeda) and Europe (Mysimba®, Orexigen) is another drug combination, this time of bupropion and naltrexone. Individually, they are respectively a mixed dopamine/norepinephrine reuptake inhibitor used as an antidepressant and for smoking cessation (Stahl SM et al., 2004) and an opioid receptor antagonist,

used in the treatment of alcohol dependence, acting via dopaminergic reward pathways(Johnson, 2008). This combination therapy achieved a weight loss at one year of 3.2 - 5.2% compared to placebo in three separate trials (Wadden et al., 2011, Apovian et al., 2013, Greenway et al.). Use continues with careful monitoring of cardiovascular and psychiatric safety profiles.

The scarcity of treatment options makes the prospect of employing gut hormones to aid weight loss an exciting one. Whether it is to activate the anorexigenic pathways via the use of PYY, GLP-1 and OXM, or to block the orexigenic stimulus of Ghrelin, much research is being carried out in this field. Indeed, in the USA the FDA recently approved the use of a GLP-1 mimetic, Saxenda® (NovoNordisk, generic name Liraglutide), for long term weight management after treatment with a dose of 3.0 mg a day was able to induce an average 5.4% weight loss compared to placebo (FDA, 2015, Pi-Sunyer et al., 2015). The potential of individual gut hormones as weight loss therapies is explored in greater detail below.

1.1.3.3 Gastric Bypass Surgery

The only obesity treatment that has been shown to confer long term, sustained weight loss is bariatric surgery. The most common procedure has been the Roux-en-Y gastric bypass (RYGB) (Fig 1.3), accounting for up to 75% of all bariatric surgeries (Smith et al., 2008). RYGB not only reduces stomach size, but also bypasses part of the small intestine, resulting in some reduced nutrient absorption but seemingly not enough to be clinically significant (MacLean et al., 2001). There is little consensus as to the optimal length of the Roux (alimentary) limb, with recent reviews of a long (150cm) vs short (100cm) Roux limb revealing similar weight loss, metabolic benefits and incidence of complications between the two groups(Dogan et al., 2017, Ramos et al., 2016). A significant proportion of gastric bypass procedures are now performed laparoscopically, which is associated with a reduced incidence of complications (Morgan et al., 2015). Overall, we have seen a rise in the numbers of people undergoing bariatric surgery from about 470 in 2003/4 to over 6500 in 2009/10 (NICE, 2014). The eligibility criteria for bariatric surgery were altered by NICE in 2014. In addition to offering surgery to

all patients with a BMI greater than 40kg/m^2 or more, or between 35 kg/m^2 and 40 kg/m^2 with a comorbidity such as diabetes or hypertension, specific new recommendations were put in place regarding patients with recent onset type 2 diabetes. It is now advised that patients with recent onset type 2 diabetes with a BMI greater than 30kg/m^2 should be considered for surgery, and possibly at even lower BMI measurements for those of Asian origin (NICE, 2014).

The Swedish Obese Subjects (SOS) trial is a long-running prospective study of the long-term outcomes following weight reduction surgery (Sjöström, 2013). In excess of 2000 participants underwent one of three procedures: gastric bypass (13%), gastric banding (19%) and vertical banded gastroplasty (68%). This invaluable resource has demonstrated that at 20 years post-surgery (for all 3 procedures) a mean weight loss of 18% was maintained, compared to a 1% sustained weight loss in the control group. The patients who underwent RYGB achieved a greater initial weight loss than the other two groups, although by 20 years this difference was not significant. The combined surgical groups showed a long-term reduction in all-cause mortality, incidence of diabetes, cancer, stroke and myocardial infarction (MI) compared to controls. The SOS study also supported the findings of other groups, who showed that diabetes remission may be observed following gastric bypass surgery. In the SOS study, this remission was seen in 72% of patients at 2 years (Sjöström, 2013), and Mingrone et al demonstrated a similar diabetes remission rate (defined as a fasting glucose <5.6mmol/l and a HBA1c of < 6.5% off medication) of 75% two years after gastric bypass (Mingrone et al., 2012). Brethauer et al used a more stringent cut off of HBA1c <6% and fasting glucose <5.6mmol/l off medication to define remission of diabetes, but still reported initial remission rates of 52% 2 years post-operatively for RYGB. Interestingly, in the study by Mingrone et al, neither the pre-operative BMI nor the degree of post-operative weight loss were predictors of diabetes remission, a finding that has also been reported in other studies (Rubino et al., 2006, Pories et al., 1987). Indeed, the remission of diabetes occurs very soon after surgery before any significant weight loss occurs and so is likely at least initially to be independent of weight loss. Pournaras et al demonstrated both an increase in insulin secretion and a reduction in insulin resistance within the first week after surgery. The latter is unexplained, but the

former is associated with enhanced GLP-1 prandial responses, which maybe contributory. (Pournaras et al., 2010b). Longer term maintenance of diabetes remission is more likely to be linked to weight loss and the improved insulin sensitivity that this confers. Unfortunately, the remission of diabetes is not always sustained, and by 10 years post-operatively up to 50% of the SOS study patients who had achieved diabetes remission had relapsed (Sjöström, 2013). Brethauer et al found that type 2 diabetes recurred in 17% of their patients after an initial remission rate of 52% at 2 years following gastric bypass surgery. They showed that relapse was associated with a longer duration of pre-operative diabetes, smaller excess weight loss after surgery, and greater weight regain (Brethauer et al., 2013).

Studies have demonstrated an increase in the post-prandial secretion of satiety gut hormones after RYGB (Beckman et al., 2011, Le Roux et al., 2007, Pournaras et al., 2010a, Le Roux et al., 2006), nominally PYY, GLP-1 and OXM. These changes are not seen after gastric banding – a restrictive procedure producing early satiety but not reduced absorption (Korner et al., 2006) – and the change in secretion of gut hormones is thought to be one of the mechanisms which may explain the superior and sustained weight loss as well as the improvement in blood glucose observed after RYGB. The post-operative changes in gut hormones suggest that these hormones could be targets for novel obesity therapies, and as such they have generated a large amount of research interest.

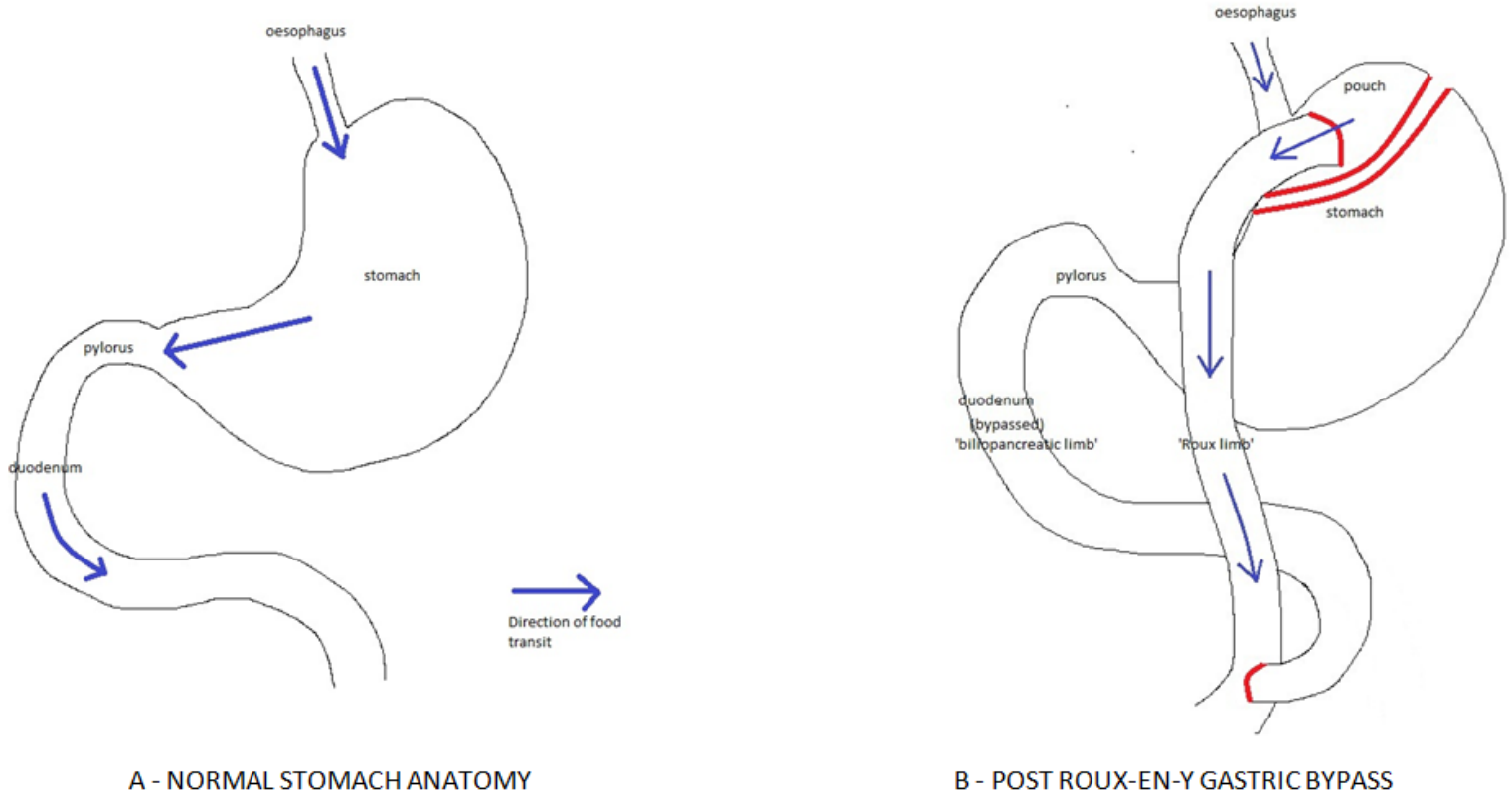


Figure 1.3 Roux-en-Y gastric bypass. A schematic of the anatomy of the upper gastrointestinal tract both (A) before surgery, and (B) following the Roux-en-Y gastric bypass operation

1.2 Gut Hormones

1.2.1 PYY

PYY, a 36 amino acid peptide, is secreted from entero-endocrine L cells of the distal gut in response to sensing oral intake of nutrients. PYY is part of the 'PP fold' family of peptides which includes PP, and NPY. These peptides are named after their tertiary structure, which forms a hairpin-like U shaped fold, and PYY is specifically named for the tyrosine residues at each terminal (Fig 1.5).

PYY secretion is promoted by ingestion of all different classes of nutrients; however, opinions are divided as to which macronutrient causes the greatest induction of PYY release. In rodents, protein appears to be the most potent stimulus for PYY secretion (Batterham et al., 2006), whereas a high carbohydrate, low fat diet was demonstrated to give the greatest rise in PYY levels in obese humans (Essah et al., 2007). Interestingly, PYY levels start to rise rapidly (within about 15 minutes) following any nutrient ingestion (Gibbons et al., 2013), and certainly long before the nutrients themselves might be expected to reach the distal gut, implying other neural or hormonal mechanisms involved in its release. The native hormone is secreted as PYY (1-36), and is then processed by the enzyme dipeptidyl-peptidase-4 (DPP-4) to give the active form, PYY(3-36). PYY, along with other gut hormones such as OXM and GLP-1, exhibit enhanced postprandial levels in subjects who have undergone gastric bypass surgery (Borg et al., 2006, Laferrère et al., 2010, Le Roux et al., 2006). This post-surgical rise in PYY is thought, at least in part, to be responsible for the significant and maintained weight loss seen in this patient group.

PYY exerts its effects via the Y2 receptor, one of the members of the NPY receptor family, of which there are 4 in humans– Y1, Y2, Y4 and Y5 (Troke et al., 2013). Y2 receptors are found throughout both the peripheral and central nervous systems, with a high concentration in the ARC and are therefore very likely to be significant in the control of appetite. In addition to its central effects on appetite, the actions of PYY include a reduction in gastric emptying as well as a delay in intestinal transit and as a consequence PYY has a potent inhibitory effect on food intake in both rodents and humans (Batterham

et al., 2002). Infusion of PYY has also been shown to reduce levels of the gastric hormone ghrelin, which is well characterised as an orexigenic agent (Batterham et al., 2003). This adds a further potential mechanism to explain the anorexic potential of PYY. Baseline PYY levels are lower in obese compared to lean subjects and, interestingly, in obese people there is a blunting of the postprandial PYY rise (Batterham et al., 2003), suggesting that a lack, or at least reduction of endogenous PYY secretion could be associated with the development of obesity - either as cause or effect. However, despite the lower PYY levels found in the obese, infusion of PYY resulted in a reduction in hunger and caloric intake in both obese and lean individuals (Batterham et al., 2003), implying that exogenous PYY could be considered as a therapeutic intervention for obesity. However, when considering its therapeutic potential, it must be noted that the half-life of PYY is very short at just 7-10 minutes (Lluis et al., 1989), and its functional half- life (defined by the authors as 'the time of administration prior to re-feeding that gives half-maximal biological response') is measured at just 3 hours (Shechter et al., 2005), although Batterham et al did show a sustained reduction in food intake over the subsequent 24 hours following a short, 90 minute infusion of PYY (Batterham et al., 2002). This short duration of action presents a significant obstacle to the development of PYY as a treatment. In addition, the supraphysiological peak PYY level achieved when PYY is administered experimentally has resulted in nausea – a further limitation to its therapeutic use (Gantz et al., 2007).

1.2.2 GLP-1

GLP-1 is a peptide hormone which acts as an incretin, stimulating release of insulin from the pancreatic β cells in response to an oral nutrient load. GLP-1 is released mainly from the entero-endocrine L cells in the small intestine (Herrmann et al., 1995), and is a product of post translational processing of the glucagon precursor, proglucagon (Orskov et al., 1989) (fig.1.3)

GLP-1 has two major biologically active forms, GLP-1(7-36) amide and GLP-1(7-37), the predominant one being GLP-1(7-36) amide. GLP-1 is rapidly broken down in vivo by the enzyme dipeptidyl peptidase-4 (DPP-4) to inactive metabolites, and therefore has a short circulating half-life, rendering native GLP-1 inappropriate for therapeutic use. GLP-1 acts via its own distinct receptor, a G protein coupled receptor, which has a varied tissue distribution in humans. Amongst others, GLP-1 receptors are found in the pancreas, gut and brain, in particular the hypothalamus, NTS and AP, all areas closely related to appetite regulation (Merchenthaler et al., 1999). In addition to its incretin effect, actions of GLP-1 include suppression of glucagon secretion from α cells of the pancreas (Creutzfeldt et al., 1996), delaying gastric emptying (Schirra et al., 2006), and suppression of appetite (Punjabi et al., 2011).

GLP-1 is currently one of the most successful gut hormones to be exploited for therapeutic purposes in humans. It is used for treatment of type 2 diabetes due to its incretin effect, and to date several preparations are available. The first molecule to be developed as a drug was Exenatide (Byetta®, AstraZeneca). It was first approved by the FDA in 2005, and was derived from the saliva of the Gila monster lizard, *Heloderma suspectum.* It shares only 53% homology with human GLP-1. Whilst it binds to the GLP-1 receptor, it is resistant to degradation by DPP-4, lengthening the half-life and allowing twice-daily injection (Robles and Singh-Franco, 2009). Following this, other manipulations of GLP-1 were made to extend the half-life, and provide a more acceptable injection schedule. Liraglutide (Victoza®, NovoNordisk) was developed as a once-a-day injection alternative to Exenatide. Liraglutide has a much closer homology (97%) to human GLP-1, but has been modified by the addition of a 16-

carbon fatty acid chain which allows it to bind to albumin (Sisson, 2011). This slows breakdown and clearance and allows a more desirable once daily administration. In a head to head study, Liraglutide performed better than Exenatide in terms of glycaemic improvement, but the degree of body weight loss was similar in both groups, and modest at ~3kg in 26 weeks (Buse et al., 2009). Nausea is the main side effect of these GLP-1 receptor agonists, and can limit the administration of higher doses which might have greater effects on weight loss. However, the recent SCALE trial successfully gave 3mg of Liraglutide per day which resulted in 8.4 ± 7.3 kg weight loss at 56 weeks compared to 2.8 ± 6.5 kg in the placebo group. This dose did cause mild to moderate nausea, but it was mostly limited to the first 4-8 weeks after which the nausea improved(Pi-Sunyer et al., 2015).

Further progress has been made with the advent of once weekly Exenatide LAR or Bydureon®(AstraZeneca). Bydureon is essentially Exenatide incorporated in microspheres which consist of poly(D,L lactic-co-glycolic acid), which allows for a gradual delivery of the drug through slow release technology (Drucker et al., 2008). In addition to good effects on glycaemic control, the overall amount of weight loss over a 30 week study was similar to that achieved with Exenatide itself (Drucker et al., 2008). Since the success of these GLP-1 receptor agonists in the treatment of diabetes, many others are in the pipeline, with prolonged half-lives due to variations in molecular structure. Phase III trials are currently underway for an osmotic minipump, which provides a constant subcutaneous delivery of exenatide (Tibble et al., 2013, Intarcia Therapeutics), hopefully avoiding the peak in peptide levels that appears to cause the commonest side effect, nausea. Phase II studies of this technology reported encouraging weight loss, and so results of the phase III study will be revealing. Despite encouraging results for diabetes control, the utility of these GLP-1 analogues as weight loss therapies initially seemed limited, with little hope that GLP-1 monotherapy would be licenced as a treatment for obesity. However, in 2015 the FDA approved Saxenda®(NovoNordisk, Liraglutide [rDNA origin]) as a potential treatment for chronic weight management alongside diet and exercise (FDA, 2015), after it demonstrated an average weight loss of 5.4% at one year compared to placebo (Pi-Sunyer et al., 2015).

Previous work has suggested that GLP-1 causes stimulation of islet cell neogenesis and reduced beta cell apoptosis (Perfetti and Hui, 2004, Brubaker and Drucker, 2004), hence its insulinotropic effect. Furthermore, several studies have shown that liraglutide increases insulin secretion and pancreatic beta cell sensitivity to glucose (Kim et al., 2014, Takabe et al., 2012, Schwasinger-Schmidt et al., 2013). In addition, the recent LIBRA trial confirmed that Liraglutide enhanced beta cell function over a 48 week study period but that this effect was lost on stopping the medication(Retnakaran et al., 2014). However, as with all emerging therapies, the longer term effects must be monitored. Recent work by Abdulreda et al in humanised mice transplanted with human pancreatic beta islet cells and receiving long term Liraglutide treatment showed an initial improvement followed by a progressive deterioration of pancreatic beta cell function over time, associated with escape of glycaemic control (Abdulreda et al., 2016). The mechanisms remain to be elucidated.

In addition to Abdulreda's findings, other concerns have been raised over the safety of GLP-1 receptor agonists. It is suggested that both DPP-4 inhibitors (which prevent breakdown of GLP-1), and GLP-1 receptor agonists increase the potential risk of pancreatitis, pancreatic cancer, hyperplasia of the exocrine pancreas, and thyroid C-cell hyperplasia, potentially a precursor of medullary thyroid cancer (Butler et al., 2013). However, these findings remain under investigation, and no conclusion with regards to causality has yet been drawn. The risk-benefit ratio of the use of these agents must also be considered (Nauck, 2013).

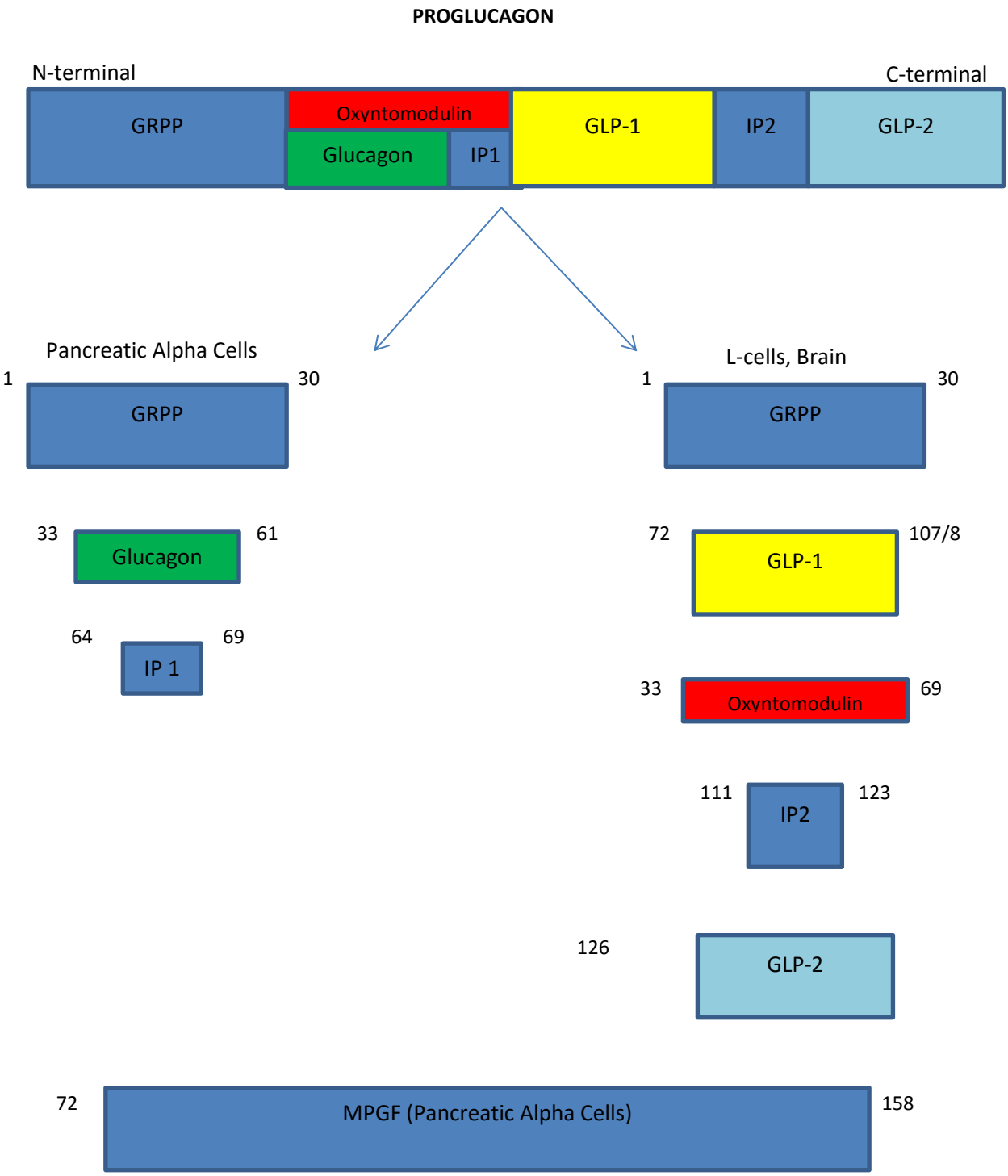


Figure 1.4 The Cleavage products of Proglucagon. Proglucagon expression is concentrated in the pancreas, L cells of the GI tract and brain. Glucagon is the primary product in the pancreas, whereas GLP-1 and OXM secretion take place predominantly in the L cells of the GI tract and in the brain. Location in the proglucagon peptide sequence is indicated by numbers. Adapted from Field et al 2010(Field et al., 2010a)

1.2.3 OXM

OXM is a 37 amino acid peptide hormone. It is processed similarly to GLP-1 via the post-translational modification of proglucagon, and also like GLP-1 it is secreted from the entero-endocrine L cells of the distal gut in response to a meal. OXM incorporates the whole 29-amino acid sequence of glucagon, but in addition has a further 8-amino acids at the carboxy-terminal due to differential processing of the proglucagon precursor (Fig 1.3) A specific receptor for OXM has not been identified, but the peptide has been shown to bind both the GLP-1 (GLP-1R) and glucagon (GCGR) receptors (Baldissera et al., 1988, Baggio et al., 2004, Pocai et al., 2009, Kosinski JR et al., 2012). It does, however, have a low affinity for these receptors compared to the cognate ligands (Holst, 1997, Baldissera et al., 1988, Gros et al., 1993). It seems likely that OXM exerts the majority of its effect on appetite via the GLP-1 receptor, as administration of a GLP-1 receptor antagonist alongside OXM blocks its anorectic actions (Dakin et al., 2001).

OXM has clear effects on reducing food intake, and increasing energy expenditure. Central injection of OXM into the ARC causes a sustained reduction in food intake (Dakin et al., 2004). Healthy human volunteers receiving intravenous infusion of OXM have also been reported to decrease their food intake (Cohen et al., 2003) both during, and in the 12 hours subsequent to the end of the infusion. Furthermore, self-administration of OXM as a subcutaneous injection given three times a day over a 4 week period resulted in a 2.4% reduction in body weight, and reduced food intake in overweight and obese volunteers (Wynne et al., 2005).

When given peripherally in rats, OXM significantly decreases food intake and reduces body weight gain compared to pair fed animals (Dakin et al., 2004). As the pair fed and treatment groups in this study by Dakin et al received identical amounts of food, the OXM treated group must therefore have an additional stimulus for weight loss, as it cannot be explained by decreased food intake alone. On post mortem examination, the epididymal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) fat pads of both groups of animals were weighed. The fat pad weight was lower

in the OXM treated animals, suggesting an increase in energy expenditure to be the added stimulus to weight loss with the OXM group utilising fat stores as an energy substrate. OXM has been demonstrated to increase energy expenditure (Wynne et al., 2006) (Dakin et al., 2002) which appears, at least in human volunteers, to be mediated via an increase in activity related energy expenditure (EE). As discussed in this thesis, it has now been shown that exogenously administered glucagon reliably increases resting EE in healthy human volunteers (Tan et al., 2013), and as OXM has dual actions at both the glucagon and GLP-1 receptors, it is likely that it exerts its effects on energy expenditure via the glucagon receptor.

OXM presents an interesting prospect for the treatment of obesity due to its dual effects on reducing food intake, and increasing energy expenditure – a two-pronged attack. It appears to act via the GCGR to increase energy expenditure with none of the negative effects on glycaemic control that might be expected as a consequence of glucagon receptor agonism. A combination approach, using peptides like OXM which exhibit action at both the glucagon and GLP-1 receptors has also been successful in rats. These studies used peptide analogues which were dual agonists of these two systems, and reported weight loss with no deterioration of glycaemic control and an improved lipid profile in diet-induced obese animals (Day et al., 2009, Pocai et al., 2009). This is encouraging when contemplating the development of novel obesity therapies.

1.2.4 Ghrelin

Ghrelin is the only hormone to have been identified as orexigenic. It is a 28 amino acid peptide hormone (Fig 1.5) secreted from X/A-like cells in the fundus of the stomach (Fig 1.2), and acts through a G-protein coupled receptor, the 'growth hormone secretogogue' receptor (GSH-R)(Kojima M et al., 1999). Initially, ghrelin was found to stimulate the release of growth hormone (GH) from the pituitary gland (Peino et al., 2000), and its name derives from this action as a Growth Hormone Releasing Peptide. Only later were its potent effects on appetite elucidated. Ghrelin exists in two main forms, the inactive, non-acylated from, and the active, acylated form, converted from non-acylated ghrelin by the enzyme Ghrelin O-acyltransferase (GOAT).

Administration of ghrelin, both peripheral and central, has been shown to increase food intake and cause weight gain in rodents (Wren et al., 2000, Tschop et al., 2000, Lawrence et al., 2002). Peripheral intravenous administration is also associated with an increase in both gastric motility and secretion (Masuda et al., 2000). In normal healthy subjects, ghrelin levels are highest pre-prandially, in the fasted state (Toshinai et al., 2001) supporting its role as a stimulus to eat. Levels are seen to be higher in people with either planned weight loss due to dietary restriction or very low body weight due to the eating disorder anorexia nervosa (Ariyasu et al., 2001, Shiiya et al., 2002, Cummings et al., 2002), again reinforcing the role of the hormone as an orexigen. Conversely, as one would expect, ghrelin levels are low in hyperglycaemia and after meals (Ariyasu et al., 2001, Tschöp et al., 2001), as the drive to eat diminishes. They are also lower in obese subjects (Shiiya et al., 2002). Interestingly, plasma concentration of ghrelin is inconsistent after gastric bypass surgery, and therefore its role in weight loss in such patients is unclear. Initial studies suggested it was reduced (Cummings et al., 2002, Geloneze et al., 2003), but subsequent studies have either shown no effect, or an increase in ghrelin post-surgery(Terra et al., 2013, Perez-Romero et al., 2010, Mancini et al., 2006). Ghrelin is secreted from cells in the fundus of the stomach which is directly involved in the surgical procedure, but this does not appear to have any specific effect on post-operative plasma levels of the hormone.

Ghrelin, like other gut hormones, exerts actions in the central appetite control areas of the brainstem and hypothalamus. Ghrelin receptors are found in the ARC, and *c-fos* expression is increased in the ARC after peripheral administration of ghrelin (Hewson and Dickson, 2000). In rodents who had undergone ablation of the ARC, administration of ghrelin no longer stimulated food intake (Tamura et al., 2002), supporting the ARC as a key component in ghrelin's mechanism of action. When given centrally, ghrelin also stimulates *c-fos* expression in other nuclei known to be involved in appetite control including the PVN, dorsomedial nucleus (DMN), and lateral hypothalamus as well as in the AP and NTS in the brainstem(Lawrence et al., 2002). Hypothalamic levels of NPY and agouti-related protein mRNA were also increased after central administration of Ghrelin (Kamegai et al., 2001), both of which are known to be orexigenic.

In addition to the central effects, ghrelin also acts via the vagus to exert its effects. Both the hormone and its receptor are expressed in vagal afferents in mice(Page et al., 2007), and vagotomy in rats reduces feeding and *c-fos* expression in the ARC after ghrelin administration (Date et al., 2002). Central administration of ghrelin has been shown to increase pancreatic exocrine secretion via vagal signals in anaesthetised rats (Sato et al., 2003).

1.3 Other Relevant Hormones

1.3.1 Glucagon

Glucagon is a 29 amino acid peptide, derived from the cleavage of proglucagon and secreted from the α-cells of the pancreatic islets (Fig 1.4). It is released into the hepatic portal vein in hypoglycaemic/fasted states and also in response to exercise(Unger et al., 1978), and acts on the liver to promote hepatic glycogenolysis and gluconeogenesis to maintain glucose homeostasis (Striffler et al., 1981, Studer et al., 1984, Stevenson et al., 1987, Wasserman et al., 1989). Secretion is inhibited by insulin, somatostatin and hyperglycaemia(Johnson et al., 1975, Unger et al., 1978). Glucagon mediates its effects via the glucagon receptor (GCGR), a 7-transmembrane G-protein coupled receptor that has a wide tissue distribution. The GCGR is expressed in the gut, adrenal glands, brain, heart, pancreas, spleen and in adipocytes, but is predominantly found in the liver and kidney (Svoboda et al., 1994). This broad tissue distribution supports the probability that glucagon plays a significant role in general energy homeostasis and not simply glucose regulation. Physiologically, circulating levels are highest before meals as would be expected, as glucagon promotes an increase in blood glucose in this fasted state. Conversely, secretion of glucagon is suppressed in hyperglycaemia (Unger et al., 1978), and glucagon has been demonstrated to directly increase insulin secretion (Huypens et al., 2000, Kawai et al., 1995, Svoboda et al., 1994). As a prospective treatment for obesity, glucagon has been shown to both increase energy expenditure (Davidson et al., 1960, Nair, 1987), and to significantly reduce food intake, with a subjective reduction of appetite in man (Schulman et al., 1957), as well as an objective reduction in meal size in rats following infusion of glucagon into the portal vein (Geary et al., 1993, Le Sauter et al., 1991). These satiety effects of glucagon are thought to be mediated via vagal afferents (Geary and Smith, 1983). The mechanism of action of the observed rise in EE is unknown, and is likely to be multifactorial, but potential candidates include an increase in oxygen consumption and heat production in brown adipose tissue (Billington et al., 1987), or alternatively the positive chronotropic and inotropic effects on the heart seen on administration of glucagon (Glick et al., 1968). However,

whilst glucagon has the demonstrable beneficial effects of increasing EE and reducing food intake, there are the potential negative effects to consider – the predominant concern being that of inducing hyperglycaemia. This concern is addressed in the following thesis (Tan et al., 2013).

1.3.2 Leptin

Like glucagon, leptin is not a gut hormone. It is an adipokine, a 167 amino-acid adipocyte-derived hormone. Nonetheless, leptin plays an important role in appetite and regulation of food intake. It exerts its effects via the ARC, acting on the two neuronal populations described above. It causes activation of anorectic POMC neurons, and inhibition of the orexigenic NPY/AgRP neurons. The overall result of this is to decrease food intake and mediate appetite (Cowley et al., 2001, Elias et al., 1999). Whilst the gut hormones described above act in the short term to mediate appetite and food intake, leptin can be described as a more 'long-term' indicator of nutritional status. Circulating leptin levels are proportional to body fat mass, and act as an indicator of peripheral energy status.

Leptin is encoded by the obese (*ob*) gene(Zhang et al., 1994), and rodents with no leptin (*ob/ob*) are obese and hyperphagic(Coleman, 1978). Injection of leptin into these *ob/ob* mice results in a 30% reduction in body weight, mediated by a reduction of food intake and concurrent increase in energy expenditure (Halaas et al., 1995). Like rodents, humans who have leptin deficiency are similarly obese and hyperphagic (Montague et al., 1997).

A decrease in body fat results in a reduction in plasma leptin concentration. This drop in leptin levels acts as a starvation signal, prompting an increase in food intake and a reduction in energy expenditure(Ahima et al., 1996). In view of this, one would expect a raised circulating leptin concentration in obese individuals - which is indeed the case. However, unlike mice injected with leptin, obese individuals with high leptin levels do not exhibit an increase in energy expenditure and a decrease in food intake, suggesting an insensitivity, or resistance to endogenous leptin in the obese state (Maffei et al., 1995). Unfortunately, this leptin 'resistance' means that leptin is currently of limited use as a target for obesity therapy.

AA SEQUENCE POSITION	1				5					10					15					20					25					30					35		
HORMONE																																				
GLUCAGON	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr								
OXM	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asp	Thr	Lys	Arg	Asp	Arg	Asp	Asp	Ile	Arg
GLP-1 (7-36)	His	Ala	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly	Gln	Ala	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg							
PYY	Tyr	Pro	Ala	Lys	Pro	Glu	Ala	Pro	Gly	Glu	Asp	Ala	Ser	Pro	Glu	Glu	Leu	Ser	Arg	Tyr	Tyr	Ala	Ser	Leu	Arg	His	Tyr	leu	Asn	Leu	Val	Thr	Arg	Gln	Arg	Tyr	
GHRELIN	Gly	Ser	Ser	Phe	Leu	Ser	Pro	Glu	His	Gln	Arg	Val	Gln	Gln	Arg	Lys	Glu	Ser	Lys	Lys	Pro	Pro	Ala	Lys	Leu	Gln	Pro	Arg								

Figure 1.5 Amino Acid sequences of the peptide hormones Glucagon, OXM, GLP-1, PYY and Ghrelin. Glucagon, GLP-1 and OXM all belong to the secretin family of peptide hormones and exhibit significant sequence homology (highlighted in orange).

1.4 Energy Homeostasis

Energy expenditure can broadly be divided into three key components. The first is resting energy expenditure (REE), which is roughly equivalent to the Basal Metabolic Rate (BMR). This is the energy used by the body to perform all 'normal' cellular functions. It is measured at rest, at a neutral temperature and in the fasted state. The second component is diet-induced thermogenesis (DIT), the heat energy produced by metabolising ingested nutrients, and the third is activity-related EE which is particularly difficult to measure. These account for roughly 65- 70%, 10% and 20% of total EE (TEE) respectively.

1.4.1 The role of selected components in regulation of energy homeostasis

1.4.1.1 Adaptive thermogenesis

On exposure to cold, initial mechanisms such as shivering are activated to generate heat. Alongside this shivering is also seen an increase in oxygen consumption, indicating an increase in EE. However, following prolonged cold exposure shivering gradually decreases. Despite this reduction in shivering, there is no reduction in oxygen consumption, suggesting that there is adaptation to develop methods of non-shivering thermogenesis (Davis, 1961). It has subsequently been shown that cutaneous thermoreceptors located in nerve endings in the skin transmit afferent signals to central temperature regulatory nuclei in the hypothalamus (the exact hypothalamic nuclei involved remain unclear) via the lateral parabrachial nucleus of the pons (Kobayashi and Osaka, 2003) (Morrison et al., 2012). The hypothalamus in turn activates efferent pathways of the sympathetic nervous system (SNS) (Minokoshi et al., 1986, Perkins et al., 1981). The SNS then innervates thermogenic target tissues such as skeletal muscle and brown adipose tissue (BAT) to promote thermogenesis. (Chernogubova et al., 2004). This role of the SNS in the innervation of BAT is supported by several studies showing that blockade of the SNS for example with beta-antagonist drugs such as propranolol (beta-blockers) reduces BAT activity (Chernogubova et al., 2004, Soderlund et al., 2007, Tatsumi et al., 2004, Parysow

et al., 2007). Conversely, stimulation of the SNS by administration of noradrenaline (NA) causes an increase in BAT activity (Chernogubova et al., 2004), and in patients with phaeochromocytoma who have an excess of catecholamines, increased ^{18}F-fluorodeoxyglucose (FDG - a labelled form of glucose), uptake in BAT can be observed on positron emission tomography (PET) imaging(Yamaga et al., 2008).

1.4.1.2 Brown Adipose Tissue

Brown adipose tissue (BAT) and white adipose tissue (WAT) are the two types of fat tissue found in humans. WAT is predominantly concerned with storage of energy and makes up the adipose deposits associated with obesity. Conversely, BAT is involved in energy production, and is found in greatest volume in neonates as well as hibernating mammals, both of whom need to generate heat via non-shivering thermogenesis (as described in section 1.4.1.1). BAT cells contain a large number of mitochondria, which express the BAT-specific uncoupling protein-1 (UCP-1). Conventionally, it was thought that adult humans had very little BAT, but recent studies demonstrating significant active BAT deposits in adults have renewed research interest in this tissue (Virtanen et al., 2009, Cypess et al., 2009, Au-Yong et al., 2009). BAT deposits in adult humans are found in the supraclavicular and paracervical regions, and the amount of BAT appears to be inversely correlated with body weight (Cypess et al., 2009) suggesting a significant role in energy homeostasis. Crucially, it appears that BAT in adult humans can be both recruited and activated to increase EE (Blondin et al., 2014, Chen et al., 2013, Izzi-Engbeaya et al., 2015), raising the potential for the manipulation of BAT in developing new therapies for obesity.

The SNS plays a pivotal role in the activation of BAT. Noradrenaline, a catecholamine and neurotransmitter in SNS efferents binds to the beta-3 receptor on the BAT cell (Figure 1.6). The beta-3 receptor is a G protein coupled stimulatory receptor, which activates downstream signalling via cAMP and Protein Kinase A (PKA). This pathway directly activates UCP-1, but also stimulates lipolysis via hormone sensitive lipase (HSL) resulting in an increase in free fatty acids (FFAs) which act as a substrate for heat production and also themselves regulate UCP-1 activity (Cannon and Nedergaard,

2004). UCP-1 is a BAT-specific protein, which dissociates or 'uncouples' oxidative phosphorylation from ATP synthesis, and dissipates the resulting energy as heat. It appears to act as an anion transporter across the mitochondrial membrane, possibly using FFAs as cofactors, facilitating proton (H+) transport.

In addition to established BAT deposits, recent studies have demonstrated the 'recruitment' of UCP-1 expressing cells from white adipocyte depots (Petrovic et al., 2010, Wu et al., 2012). Whilst UCP-1 is specific to BAT cells, these 'Brite' (Brown-white) or 'beige' adipocytes not only express UCP-1 but also exhibit significant heat production on stimulation with NA. They are not classic BAT cells, but the existence of these inducible cells presents an interesting avenue of research in the field of obesity therapeutics.

1.4.1.3 The Hypothalamo-Pituitary-Thyroid Axis

Like gut hormone and temperature regulation, the hypothalamus is also central to the control of thyroid hormones. The PVN secretes thyrotropin-releasing hormone (TRH) which acts on the pituitary gland to stimulate the release of thyroid-stimulating hormone (TSH). This in turn acts on the thyroid gland itself to secrete the thyroid hormone thyroxine (T4). Thyroxine is the major circulating form of thyroid hormone, which is converted by de-iodinase enzymes to tri-iodothyronine (T3) in tissues. T3 is the predominant active form of the hormone. Thyroid hormones are intrinsic in the control of metabolism and energy homeostasis, with a significant reduction in EE seen in the hypothyroid state (Oppenheimer et al., 1987). Furthermore, the reduction in EE seen as expected in fasting/caloric restriction is associated with a reduction in thyroid hormones (Vella et al., 2011).

Thyroid hormones are also critical in BAT activation (Broeders et al., 2016). This can occur both centrally, with stereotaxic administration of T3 directly into the ventromedial nucleus of the hypothalamus (VMN) increasing BAT sympathetic activity (Lopez et al., 2010), or peripherally via the thyroid hormone receptor (TR) on the BAT cell (Figure 1.6). BAT cells contain cytoplasmic de-iodinase

type D2, which converts T4 into T3, and is stimulated by the SNS to increase T3 production. In this way, the SNS can enhance the thermogenic response(Silva and Larsen, 1983). In addition to the presence of the D2 de-iodinase, BAT also contains nuclear T3 receptors in a quantity similar to that seen in the thyroid hormone-sensitive pituitary and hepatic tissues (Bianco and Silva, 1987). Indeed, UCP-1 requires the presence of T3 to respond fully to SNS stimulation (Bianco and Silva, 1987).

1.4.1.3 Skeletal muscle

Skeletal muscle (SM) accounts for around 40% of total body weight in non-obese subjects, and thus can be responsible for between 20 to 30% of resting oxygen uptake (Zurlo et al., 1990). It is well known for generation of heat from shivering thermogenesis, with heat produced from contraction-relaxation of muscle fibres on acute cold exposure. Skeletal muscle also exhibits significant non-shivering thermogenesis on stimulation by catecholamines, increasing total body oxygen consumption by up to 40% (Lundholm and Svedmyr, 1965, Astrup et al., 1985), but the mechanism for this remains unclear.

Skeletal muscle is known to express uncoupling protein-3 (UCP-3), a member of the same family of proteins as UCP-1 which facilitates adaptive thermogenesis in BAT. Interestingly, despite clear evidence of mitochondrial uncoupling in skeletal muscle as a consequence of cold-exposure, there was no concurrent up-regulation of UCP-3 (Wijers et al., 2008), throwing doubt on its role in adaptive thermogenesis in skeletal muscle. Obesity, insulin resistance and aging have all been associated with a lower level of skeletal muscle mitochondrial uncoupling (Mogensen et al., 2007, Ritov et al., 2010, Iossa et al., 2004, Van den Berg et al., 2010), supporting the important role of this tissue in regulating EE. The prospect of being able to stimulate adaptive thermogenesis in SM is interesting. The adult human has much more SM than BAT, and so control of adaptive thermogenesis in SM could be a prospective target for obesity therapy. Skeletal muscle does show evidence of mitochondrial uncoupling albeit not due to the action of UCP-3. One candidate pathway for this uncoupling is via

sarcolipin, a regulator of the calcium Ca^{2+}-ATPase (Serca) pump on the sarcoplasmic reticulum. It is involved in SM based thermogenesis; mice with sarcolipin knockout were unable to maintain their core temperature, and were obese (Bal et al., 2012). It is suggested that sarcolipin can uncouple calcium transport from the SERCA pump and cause futile cycling, resulting in a net consumption of ATP but no product utilised (see section 3.5).

1.4.2 Measuring Energy Expenditure

1.4.2.1 Direct Calorimetry

Direct calorimetry is a measurement of the heat losses from an organism. The technique was first invented in the 1700s, by Laplace and Lavoisier (Lavoisier and Laplace, 1783). Direct heat losses have been measured in man in the past, through use of suits (Webb, 1980, Hambraeus et al., 1994) or chambers (Seale et al., 1991) in which a subject would be placed and thermal losses directly measured. Direct calorimeters are extremely expensive to build and run (Levine, 2005), and their use has largely diminished since the advent of indirect calorimetry, a cheaper and simpler way to measure EE based on measurement of inspired and expired gases, which gives equivalent results to direct calorimetry (Seale et al., 1990).

1.4.2.2 Indirect Calorimetry

The basis of indirect calorimetry is to measure the amount of oxygen consumed (VO_2) and the amount of carbon dioxide (VCO_2) produced by a subject. The amount of energy required to convert one unit of oxygen to one unit of carbon dioxide is known, and energy expenditure can therefore be calculated using a modified Weir equation (Weir, 1949) (see also section 3.3.3). Thus:

EE (Kcal/min) = (3.94xVO_2 (l/min)) + (1.11xVCO_2 (l/min)) – (2.17 x nitrogen excretion)

Nitrogen excretion can be estimated from a measurement of urinary urea excretion, as roughly 80% of urine nitrogen is excreted as urine urea. Carbohydrate and fat oxidation can be calculated from the VO_2 and VCO_2, but protein metabolism is not reflected in the gas analysis thus the equation requires a measure of nitrogen excretion to calculate EE.

Indirect calorimetry can be carried out via a face mask, a ventilation hood (Figure 3.1), or a respiratory chamber. Face masks can be restrictive and uncomfortable, causing anxiety and hence influencing EE. Similarly, ventilation hoods can be quite claustrophobic which could also cause anxiety, activate the

sympathetic nervous system and bias EE measurement. The gold standard is to use a respiratory chamber or room. The subject is mobile and not restrained by gas measuring equipment, and the environment can be kept constant. A respiratory chamber is also really the only practical way to obtain long term (i.e. 24 hour) calorimetry measurement incorporating all three components of energy expenditure, i.e. resting EE, diet induced thermogenesis and activity related energy expenditure. However, it is expensive, time consuming, and fails to measure EE under truly free living conditions.

1.4.2.3 Non-Calorimetric Methods

Doubly labelled water (DLW) is considered a gold standard when measuring EE. Like indirect calorimetry the technique is based on measuring the amount of oxygen consumed and the amount of carbon dioxide produced, and then calculating EE. However unlike indirect calorimetry it can be considered a 'free living' method of EE measurement. In short, labelled water is administered to subjects. This water has been labelled with stable, non-radioactive isotopes at both the hydrogen (^{2}H - deuterium) and the oxygen (^{18}O) atoms, thus when these labelled atoms are excreted they can be measured. The labelled water mixes with total body water. The ^{2}H isotopes are simply excreted in body water, but the ^{18}O will mix and be excreted both in body water and as part of carbon dioxide (CO_2). Urine/saliva samples are taken at baseline then at subsequent time points up to 21 days for isotope measurement. The difference between the rate of loss of ^{2}H and ^{18}O can be used to assess the production of CO_2 (Park et al., 2014), and from this EE can be determined (Brage et al., 2015).

Other ways to measure 'free living' energy expenditure are in development, in order to achieve a more representative measurement of a subject's true total energy homeostasis. Calorimetry has limited utility in measuring 'free living' activity related EE which accounts for a reasonable proportion of TEE (~20%). Measurement of heart rate shows a correlation to EE and can be of some use, but is more valuable when used in conjunction with other techniques. Activity logs are often used, and documented activity correlated to a known energy expenditure for said activity (Levine, 2005). This

relies heavily on the individual being assessed and hence is subject to reporting and recall bias. 3-D accelerometers, or activity monitors, can also be used as a surrogate measure of free living EE. These measure movement and physical activity, and increasingly can also simultaneously record heart rate and respiratory rate. When validated against DLW these devices generally perform well (Bouten et al., 1996), but may slightly over-or underestimate TEE (Brazeau et al., 2016) depending on the specific device.

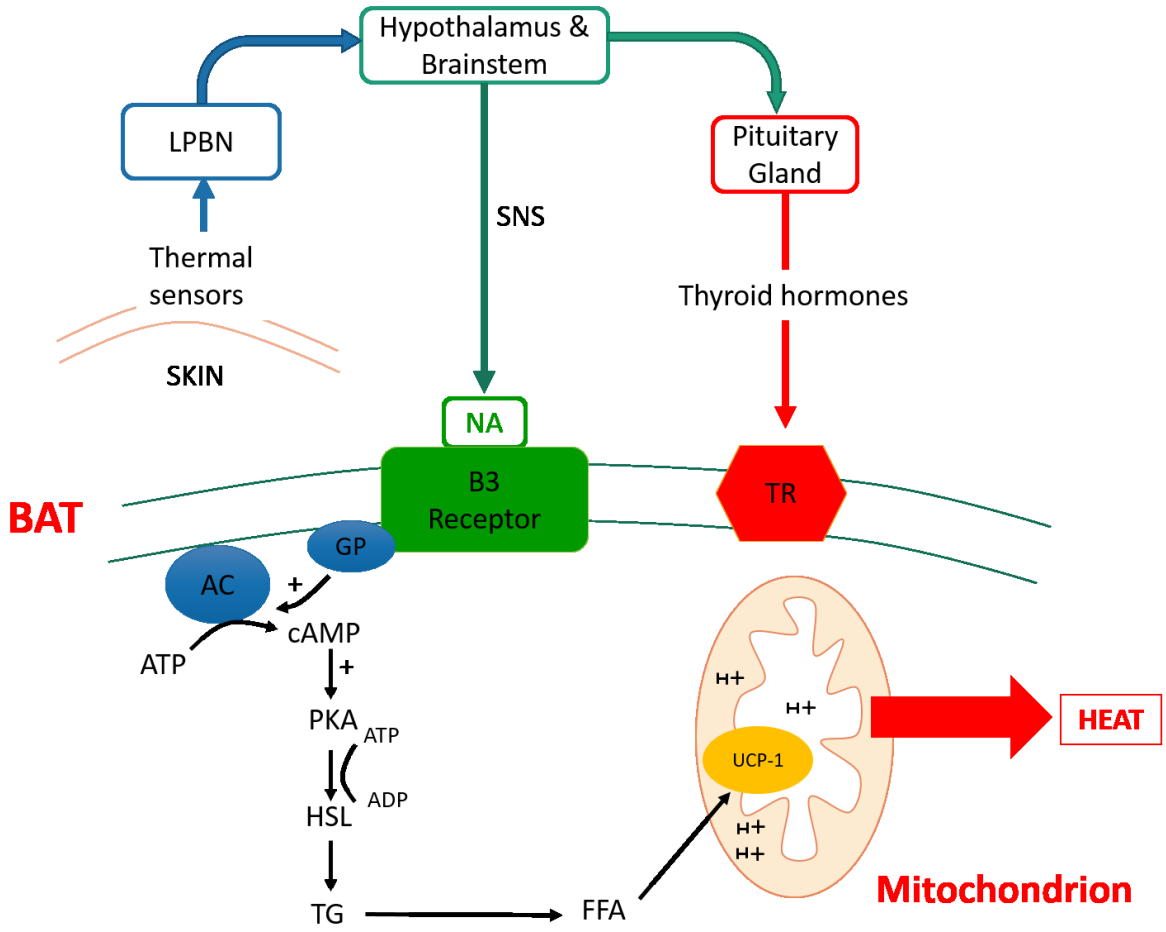


Figure 1.6 The activation and role of Brown Adipose Tissue (BAT) in heat production. Temperature is sensed peripherally, and information relayed to the hypothalamus via the lateral parabrachial nucleus (LPBN) of the Pons. BAT is subsequently activated via the sympathetic nervous system (SNS) carrying afferents from the hypothalamus and brainstem. The hypothalamus also stimulates release of thyroid hormones via the pituitary gland. Noradrenaline (NA) binds to G-protein coupled (GP) Beta-3 (B3) receptors on BAT to activate the tissue via the pathway shown. Heat energy is produced by the uncoupling of oxidative phosphorylation in mitochondria in the BAT cell. Thyroid hormones bind to the thyroid hormone receptor (TR) on BAT cells, and can also stimulate heat production via this uncoupling. These reactions are carried out in the mitochondrion via uncoupling protein-1 (UCP-1) found on the inner mitochondrial membrane. Abbreviations: AC Adenylate Cyclase; ATP Adenosine Triphosphate; cAMP cyclic Adenosine monophosphate; PKA Protein Kinase A; ADP Adenosine diphosphate; HSL hormone sensitive lipase; TG triglyceride; FFA Free fatty acids.

1.5 Hypotheses

The elevated levels of the hormones PYY, GLP-1 and OXM which are well documented to occur following gastric bypass surgery may underlie many of the favourable and sustained body weight and glycaemic changes observed post-operatively. This thesis will examine the physiological effects of these hormones and their analogues on energy expenditure, energy intake, and glucose homeostasis.

I hypothesise that:

- In Diet-induced Obese (DIO) mice, chronic administration of a combination of novel analogues of PYY, GLP-1 and OXM will result in weight loss, a decrease in food intake and an increase in energy expenditure that is greater than the effect of placebo or any of these analogues alone
- In healthy overweight human volunteers an infusion of glucagon will increase energy expenditure and reduce food intake, and any accompanying rise in glucose will be ameliorated by co-infusion of GLP-1
- In healthy overweight human volunteers an infusion of GLP-1 will enhance the acute insulin response to glucose and co-administration with PYY will not attenuate this response

1.5 Aims

- To investigate the effect of novel analogues of PYY, GLP-1 and OXM on body weight, food intake, glucose homeostasis, and energy expenditure when administered chronically to DIO mice
- To investigate the effect of co-infusion of GLP-1 and glucagon on energy intake, energy expenditure and glucose regulation in healthy overweight human volunteers
- To investigate the effect of co-infusion of PYY and GLP-1 on the acute insulin response to glucose in healthy overweight human volunteers

CHAPTER 2: THE CHRONIC EFFECTS OF NOVEL LONG-ACTING ANALOGUES OF PYY, GLP-1 AND GLUCAGON ON ENERGY HOMEOSTASIS IN DIO MICE

2.1 Introduction

The approach of using more than one agent in combination has been successful in the development of new therapies for obesity, and of those that have recently been licenced for human use, two are combination therapies – Phentermine/topiramate and bupropion/naltrexone (see section 1.1.3). Whilst these are not peptide hormones, their development lends weight to the potential of using combined peptide hormones therapeutically for obesity, to replicate at least in part the hormonal milieu seen after gastric bypass surgery. Peptides, or their analogues, offer the advantage over small molecules of being generally more specific for purpose. The close structural similarity of peptide therapies to their bioactive native parent molecule minimises the potential for adverse drug reactions or toxicity. Specific hormones of interest that are raised following bariatric surgery are PYY, GLP-1 and oxyntomodulin. These peptide hormones have been administered acutely in combination by our department and others. Neary et al demonstrated an additive food reduction in both rodents and man following administration of GLP-1(7-36) and PYY(3-36)(Neary et al., 2005), and Field et al showed a similar additive effect on food intake with co-infusion of PYY(3-36) and OXM, achieving a 42% reduction compared to placebo (Field et al., 2010b). Administration of GLP-1 with glucagon (as a surrogate for OXM, which is a dual agonist at both receptors) is able to reduce food intake and increase energy expenditure in healthy volunteers (Tan et al., 2013, Cegla et al., 2014). As discussed in chapter 1, the short half-lives of the peptide hormones render them of limited therapeutic use - PYY has a $t_{1/2}$ of just 7-10 minutes(Lluis et al., 1989) (although the functional half-life may be longer(Shechter et al., 2005)), the half-life of GLP-1 is measured at 1.5-5 minutes (Hui et al., 2002), and that of OXM is 12 minutes (Schjoldager et al., 1988).

In studies by Pocai et al and Day et al, long acting analogues were developed which were combination dual agonists at the glucagon and GLP-1 receptors. These were administered chronically to DIO mice, and significantly improved body weight, lipids and plasma glucose (Day et al., 2009, Pocai et al., 2009).

This study uses long acting analogues of the hormones PYY (PYY-LA), GLP-1 (GLP-1-LA) and oxyntomodulin, (OXM-LA) which were developed by Professor S R Bloom in the Department of Investigative Medicine, Imperial College London. These analogues had 82, 57 and 84% homology with their native peptides respectively, and they were modified to negate breakdown by the ubiquitous enzyme DPP-4. The aim was to mimic, at least in part, the hormonal changes seen after gastric bypass surgery in order to investigate the individual and combined effects of these gut hormones on food intake, body weight and glycaemic control as well as energy expenditure as OXM (and analogues thereof) is known to increase EE (Wynne et al., 2006, Liu et al., 2010). The peptides were administered chronically to DIO mice. Following the initial part of the study, post-mortem studies were carried out on the mice to examine various markers of energy homeostasis, investigating potential mechanistic contributions to the study outcomes.

These markers of energy homeostasis include leptin, a 'long term' indicator of nutritional status, which exhibits blood levels proportional to fat mass (see section 1.3.2), and free tri-iodothyronine (FT3), which is integral to BAT-mediated thermogenesis and essential for the normal response of UCP-1 to SNS stimulation (see section 1.4.1). Both leptin and FT3 concentrations were measured in plasma. UCP-1 mRNA expression (quantified by rtPCR) was measured in brown and white adipose tissue samples (see section 1.4.1.2).

I also measured Fibroblast-growth-factor-21 (FGF-21) mRNA expression in harvested liver tissue. FGF-21 is a hormone predominantly produced in the liver with intrinsic actions on glucose and lipid homeostasis. It is known to have a role in fatty acid oxidation under fasted conditions, and exogenous administration reduces body weight and adiposity as well as increasing EE (Xu et al., 2009). Circulating levels of FGF-21 are increased following exogenous administration of glucagon (Habegger et al., 2013), an observation supported by Pocai et al who demonstrated a rise in FGF-21 expression following use of a dual agonist peptide at the GLP-1 and glucagon receptors (Pocai et al., 2009). FGF-21 is a downstream target of, and major regulator of the physiological effects of peroxisome proliferative

activated receptor gamma (PPARγ), a nuclear receptor that is a transcriptional regulator of adipogenesis (Dutchak et al., 2012). PPARγ agonists improve insulin sensitivity and are in clinical use as therapy for type 2 diabetes. PPARγ mRNA expression was therefore measured in BAT and WAT. Adiponectin, an insulin sensitising adipokine with levels inversely correlated to body fat has been shown to be a downstream effector for PPARγ, and more recently Lin et al have shown that FGF-21 induces adiponectin synthesis – making adiponectin a downstream effector of FGF-21 as well (Lin et al., 2013). In this study, Adiponectin concentration was measured by ELISA in plasma samples.

Glucose-6-Phosphatase (G-6-P-ase), phosphoenolpyruvate carboxykinase (PEPCK1), and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) are all enzymes with a role in glycolysis and gluconeogenesis downstream of the glucagon receptor in hepatocytes. G-6-Pase converts G-6-P into glucose during glycogen breakdown, PEPCK1 catalyses an important rate limiting step in gluconeogenesis, and glucagon is thought to increase PEPCK and G-6-Pase transcription through a mechanism involving PCG1α (Jiang and Zhang, 2003). Therefore, any effect of the long acting analogues on expression of these enzymes could regulate hepatic glucose homeostasis. G-6-Pase and PEPCK1 mRNA expression were measured in liver tissue. PGC1α mRNA expression was measured in BAT.

Glucagon activates hormone sensitive lipase (HSL), which converts triglycerides into free fatty acids (FFAs) and glycerol. This is supported by data from Day et al, who showed that chronic administration of a glucagon-GLP-1 dual agonist peptide increased phosphorylation of HSL in WAT (Day et al., 2009). Further downstream, FFAs undergo β-oxidation to provide the substrate acetyl-Coenzyme A (Acetyl-CoA) for the Krebs cycle and energy production. Acetyl-CoA generated in this way by β-oxidation may overwhelm the capacity of the Krebs Cycle and be alternatively processed in the biosynthesis of ketone bodies (Pocai et al., 2009). In order to assess this, β hydroxybutyrate was measured in plasma. A key enzyme involved in fatty acid oxidation is ACC1, or acetyl coA carboxylase 1 (Fillmore et al., 2012). Any up-or down regulation of these enzymes by the long acting analogues could affect energy production

and hence EE, and subsequently body weight. In this study, HSL mRNA expression was measured in WAT, triglyceride concentration in plasma and ACC1 mRNA expression in liver samples.

2.2 Hypothesis and Aims

2.2.1 Hypothesis

- Once daily administration of long acting analogues of PYY and GLP-1/OXM in combination will cause weight loss and increased energy expenditure in diet-induced obese mice
- The combination of peptide analogues will cause more weight loss and reduction in food intake than any analogue alone
- Any hyperglycaemia resulting from agonism at the glucagon receptor by the OXM analogue will be countered by the insulinotropic effects of the GLP-1 analogue without any deterioration in glucose homeostasis

2.2.2 Aims

To investigate the effect of analogues of the gut hormones PYY, GLP-1 and OXM on:

- Food intake and body weight
- Glycaemic control
- Energy expenditure
- Markers of energy homeostasis

2.3 Materials and Methods

2.3.1 Animals

All animal procedures were carried out under the project licence (70/6402) and were performed in accordance with the British Home Office Animal (Scientific Procedures) Act 1986.

Studies were performed using adult male (8 weeks old) C57BL/6 mice (Charles River, Margate, UK) with a mean baseline body weight of 25g. They were group housed for 8 weeks and were rendered obese by administration of an ad-libitum high fat diet (HFD – 60% kcal from fat, Research Diets, New Jersey, USA). After 8 weeks, they were singly housed for a further two weeks before the start of the study, with continued ad-libitum access to HFD under a 12/12h light/dark cycle, lights on at 0700, with a controlled environmental temperature of 21-23^{o}C. All animals underwent regular handling and were acclimatised to any interventions to avoid stress during the course of the study. At the start of the study, animals had a mean baseline weight of 38g (36-45g).

2.3.2 Peptides

Long acting analogues of the hormones PYY (PYY-LA), GLP-1 (GLP-1-LA) and oxyntomodulin, (OXM-LA) were developed by Professor S R Bloom in the Department of Investigative Medicine, Imperial College London. Peptides were custom synthesised using solid-phase synthesis by Insight Biotechnology Ltd, UK and subsequently purified by reverse-phase high-performance liquid chromatography. The analogues had 82, 57 and 84% homology with their native peptides respectively, and they were modified to negate breakdown by the ubiquitous enzyme DPP-4. All three had an amidated C-terminal which was designed for better peptide solubility. Analogue amino acid sequences are currently intellectual property and not further discussed here.

Receptor binding assays were performed to assess the affinity of the analogues for the receptor of their parent compound (IC_{50}). Abililty of peptides to activate the receptor (EC_{50}) was assessed by cAMP enzyme-linked immunosorbent assay (ELISA). Peptide pharmacokinetic profiles were also examined to ensure a prolonged half-life in plasma. This was performed in Wistar rats, by administration of

1mg/20µl/rat. This work was carried out by other members of the research team, and results can be viewed in appendix D.

PYY-LA bound to its receptor with a comparable affinity to its parent peptide. GLP-1-LA did not exhibit as good affinity for its receptor as the parent peptide, with a receptor binding affinity roughly 7-fold lower than GLP-1 itself. OXM-LA bound with similar affinity to the GLP-1 receptor as OXM itself, but had approximately 4-fold greater affinity for the glucagon receptor than OXM. Thus the ratio of binding affinity to the two receptors for OXM was (GLP: Glucagon) 18:1, and for OXM-LA this was 99:1.

2.3.3 Protocol

2.3.3.1 Acute Food Intake following administration of long acting analogues of PYY, GLP-1 and OXM

An initial acute food intake study was undertaken over 24 hours in order to determine the doses of the long acting analogues to be used in the main part of this study. The aim was to determine a dose of analogue which conferred anorexia lasting >6 hours with no signs of malaise in the rodents.

Adult male C57B1/6 mice with a mean body weight of 38g, mean age 18 weeks, and with n=9-10 per treatment group were used in this study. They were fasted from 1600h the night before the acute study, and then were injected subcutaneously at the start of the light phase with placebo (0.9% saline, (Bayer, Haywards Heath, UK)), PYY-LA, GLP-1-LA or OXM-LA at ascending doses. Following this, animals were returned to their cages and food intake was measured at regular intervals over the next 24 hours. Data is not shown, but subsequent to this acute food intake study, the following doses were calculated to move forward to the chronic experiment: PYY analogue to be given at a dose of 30 nmol/kg, the GLP-1 analogue at a dose of 5 nmol/kg, and the OXM analogue at a dose of 20 nmol/kg.

2.3.3.2 Food Intake and Body Weight following chronic administration of long acting analogues of PYY, GLP-1 and OXM

Following the dose finding step of the acute feeding study, DIO mice with a mean body weight of 38g, mean age of 18 weeks, and with n=10-12 per treatment group were used in this study. They were injected subcutaneously once a day at 1500h for 70 days. Dose interval was determined by pharmacokinetic studies (Appendix D2). Animals were singly housed.

Animals were randomised to receive one of five treatment interventions: placebo (0.9% saline, Bayer, Haywards Heath, UK); PYY-LA alone at a dose of 30 nmol/kg, GLP-1-LA alone at a dose of 5 nmol/kg, OXM-LA alone at a dose of 20 nmol/kg, or a combination of all three analogues at the above doses. The interventions were well tolerated by the rodents, and on day 6 doses of the analogues were titrated to double the starting dose. Subsequently on day 16, the dose of OXM-LA was doubled again. The final doses of PYY-LA 60 nmol/kg, GLP-1-LA 10 nmol/kg and OXM-LA 80 nmol/kg remained well tolerated and were continued to the termination of the study.

Mice were weighed and food intake calculated every 1-2 days immediately prior to peptide administration throughout the study. Mice were given ad-libitum access to high fat diet throughout.

2.3.3.3 Glucose Homeostasis measurement following chronic administration of long acting analogues of PYY, GLP-1 and OXM

On day 70, at the termination of the study and prior to the animals being culled, an intraperitoneal glucose tolerance test (IPGTT) was performed to assess clearance of a glucose load from the plasma of these DIO mice. The investigation was performed over 3 consecutive days due to the number of mice to be tested. This allowed maintenance of a consistent interval from analogue injection/commencement of fasting to performance of the IPGTT in all animals.

Animals were injected with peptide or placebo at 0700h on the study day. The previously ad-libitum fed animals were then fasted. The IPGTT commenced at 1300h after a 6 hour fast. 2g/kg of intraperitoneal glucose were injected at T=0. Whole blood samples were obtained via a tail tip cut at t=-30, 0, 30, 60, 90 and 120 minutes. Additional samples for measurement of insulin were taken at t=-30, 30, 60 and 120 minutes. Samples for glucose measurement were analysed immediately with point-of-care testing using a handheld glucometer (Contour, Bayer, UK). Samples for insulin were collected into 500µl Eppendorf tubes, and immediately spun and separated. Following separation, the samples were stored at -20 ^{o}C until they were analysed using an insulin ELISA (Milipore, UK)

2.3.3.4 Analysis of Markers of Energy Homeostasis in Tissue Samples

At 70 days, following performance of the IPGTT, animals were culled. Mice were injected as normal with placebo or peptide at 1500h, and were then culled by CO_2 asphyxiation the following day at the onset of the light phase.

The liver, interscapular brown adipose tissue (BAT) and bilateral epididymal white adipose tissue (WAT), were removed, weighed and snap frozen in liquid nitrogen. They were subsequently stored at -80^{o}C until analysis.

Samples were analysed for the following biochemical markers of energy homeostasis, which were specifically markers of energy expenditure, lipid oxidation and gluconeogenesis:

Tissue	Biochemical markers
Plasma	Leptin Free T3 Triglycerides Adiponectin B-hydroxybutyrate.
Liver	Glucose-6-phosphatase (G-6-Pase) Phosphoenolpyruvate carboxykinase 1 (PEPCK1) Acetyl-coenzyme A carboxylase alpha (ACC1) Fibroblast growth factor-21 (FGF-21)
BAT	Uncoupling protein -1 (UCP-1) Peroxisome proliferative activated receptor gamma (PPARγ) Peroxisome proliferative activated receptor gamma co-activator 1-alpha (PGC1α)
WAT	Uncoupling protein -1 (UCP-1) Peroxisome proliferative activated receptor gamma (PPARγ) Hormone sensitive lipase (HSL)

Table 2.1 Tissues harvested and respective biochemical markers of energy homeostasis analysed

RNA extraction from tissues

In order to quantify the gene expression for the above markers of energy expenditure, tissue mRNA expression was quantified by real-time polymerase chain reaction (rt-PCR). Initially, total RNA was extracted from tissues by the Trireagent (guanidinium thiocyanate-phenol-chloroform method) method as follows:

- Tissue samples of approximately 50 mg were ground to a powder in a pestle and mortar in liquid nitrogen
- Ground sample was added to 1ml Tri-reagent and homogenised, with the subsequent homogenate transferred to Eppendorf tubes, and incubated for 10 minutes at room temperature
- 100 µl of bromo-chloropropane was added and the sample shaken vigorously for 15 seconds. Samples then underwent a further 10 minute room temperature incubation
- Tubes were then centrifuged at 14000g at 4^{o}C for 10 minutes
- The upper phase (aqueous) was separated off into an autoclaved Eppendorf tube and transferred to -20^{o}C storage. RNA remains in the aqueous phase, whereas DNA and proteins are separated into the interphase and the organic phase.

RNA purification

Purelink RNA Mini Kit and Purelink DNase set were obtained from Invitrogen, (Paisley, UK) and procedures carried out according to the manufacturer's instruction:

400 µl of 70% ethanol was added to the thawed upper aqueous phase as described above. This was then mixed thoroughly by hand and 700 µl of the sample was transferred to the spin cartridge. The cartridge was centrifuged at 12000g for 15 seconds at room temperature. The flow through from the spin cartridge was discarded and the RNA remained bound to the cartridge membrane. If there was more than 700 µl of sample, the above steps were repeated until the entire sample was processed.

The Purelink DNase protocol was then adopted to purify DNA free total RNA. 350 µL of Wash Buffer I was added to the spin cartridge with the bound RNA. The cartridge was then centrifuged at 12000g for 15 seconds at room temperature. The flow-through and collection tube were discarded. The spin cartridge was inserted into a fresh collection tube. 80 µl of PureLink® DNase mixture (prepared as per manufacturer's instruction) was pipetted directly onto the surface of the spin cartridge membrane containing the bound RNA and incubated at room temperature for 15 minutes. A further 350 µl of Wash Buffer I was then added to the spin cartridge which was centrifuged at 12000g for 15 seconds at room temperature. The flow through and collection tube were again discarded and the spin cartridge inserted into a new collection tube. 500 µl of Wash Buffer II with ethanol was then added to the spin cartridge and a further centrifugation step of 12000g for 15 seconds was undertaken at room temperature. The flow-through was discarded and the spin cartridge reinserted into the same collection tube. This last centrifugation and discard step was repeated once. The spin cartridge was then centrifuged at 12000g for 1 minute to dry the bound RNA onto the membrane. The collection tube was then discarded and the spin cartridge inserted into a recovery tube. 30 µl–100 µl of RNase-Free Water was added to the centre of the spin cartridge, and incubated for 1 minute at room temperature. The cartridge and recovery tube was then spun for 1 minute at 12000g at room temperature and the purified RNA collected in the recovery tube. Samples were then stored at -80^{o}C until analysis.

RNA Quantification

RNA concentration and purity was assessed by UV spectroscopy, using an 1101 spectrophotometer (Biochrom, Cambridge, UK). Samples were diluted to 1:50, and placed in a quartz cuvette. Absorption was read at 260 nm and 280 nm. The 280 nm reading gives an assessment of purity of the sample. The ratio for pure RNA $A_{260/280}$ should be approximately 2.

Concentration of the RNA was calculated using the following formula:

Concentration (µg/ml) = (absorbance 260 x dilution factor) x 40

A minimum concentration of 50 µg/ml of total RNA was needed for the subsequent amplification step (rtPCR).

The liver samples contained adequate RNA, but the RNA content of the BAT and WAT samples was inadequate. To adjust the RNA concentration of the BAT and WAT samples, Ethanol precipitation was employed (it was not necessary to use ethanol precipitation for the liver samples):

- 1 µl of glycogen (5mg/ml, Ambion, UK), 0.1x volume of 2M sodium acetate and 2.5x volume 100% ethanol were added to samples. Tubes were vigorously mixed by hand
- Samples were incubated at -20^{o}C for 60 minutes then centrifuged at 14000g for 7 minutes
- Supernatant was discarded and the remaining pellet was air dried for 2-3 hours in the fume hood.
- Pellets were re-suspended with autoclaved distilled water according to their original measured RNA concentration to give a final yield of 50 µg/ml.
- They were then stored at -20^{o}C

<u>Reverse transcription of purified samples</u>

This was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK), according to manufacturer's instructions.

Kit components were thawed, and the Master Mix (containing buffer, nucleotides, primers and reverse transcriptase) prepared and mixed on ice. 10 µl of 2× Master Mix was then pipetted into Eppendorf tubes. To this, 10 µl of RNA sample was added in each well, and mixed. The tubes were sealed and centrifuged briefly to spin contents to the bottom of the wells and remove air bubbles. They were then placed on ice.

Following preparation of the Eppendorf tubes, the thermal cycler (PTC-100 7307 Programmable Thermal Controller, MJ Research Inc., US) under was programmed for a reaction volume of 20 µl (i.e. 500 ng of RNA) under the following conditions:

Step 1 25^{o}C for 10 minutes

↓

Step 2 37^{o}C for 120 minutes

↓

Step 3 85^{o}C for 5 minutes

↓

Step 4 4^{o}C ∞

The reverse transcription run was then commenced. At the end of the run, cDNA samples were stored at -20^{o}C until they were required for the rtPCR reaction.

Real time (rt) PCR

A Taqman gene expression assay was used (Applied Biosystems, UK) for rtPCR reactions. A PCR reaction mix was prepared in a micro centrifuge tube consisting of 1 µl of 20x Taqman gene expression probe, 10 µl of Taqman universal PCR Master Mix, 1 µl of cDNA, and 8 µl of autoclaved distilled water. This was mixed well by inversion, and the tube centrifuged briefly. 20 µl of PCR reaction mix was transferred into each well of a 384 well plate, which was then sealed, centrifuged briefly and loaded into the rtPCR machine (7900HT Fast Real-Time PCR System, Applied Biosystems, Warrington, UK).

PCR was then performed as follows:

Step 1 50^{o}C for 2 minutes

↓

Step 2 95^{o}C for 10 minutes

↓

Step 3 95^{o}C for 10 minutes

↓

Thermal cycling: 95^{o}C for 15 seconds followed by 60^{o}C for 1 minute

This was repeated for 40 cycles

rtPCR quantifies DNA by use of a reporter probe labelled with a fluorophore. As the reaction continues, the reporter probe and the reaction primers anneal to the DNA target. As the polymerisation reaction continues, the polymerase enzyme travels along the DNA strand. When it encounters the probe, the polymerase degrades the probe, releasing the fluorescent reporter from its quencher. This gives a measurable increase in fluorescence which is detected by the PCR machine. The increase in fluorescence can be plotted against the number of cycles on a logarithmic scale. The cycle number at which fluorescence exceeds the threshold for detection is called the quantification cycle, or C_q (Bustin et al., 2009).

DNA was quantified using the $\Delta\Delta C_q$ method (SDS v2.3 software, Applied Biosystems, UK) which is based on normalisation with a single reference gene, in this case 18S. The difference between the target gene and the reference gene was assessed by calculating the difference in C_q values (ΔC_q). The ΔC_qs of the samples may then be directly compared(Bustin et al., 2009), and reported as a fold-change compared to vehicle.

The following fluorophore tagged reporter probes were obtained from Applied Biosystems, UK, and were employed for quantification of DNA in this study:

Gene of interest and tissue source	Probe
G-6-Pase (Liver	Mm00839363_m1
PEPCK1 (Liver)	Mm01247058_m1
PGC1α (BAT)	Mm01208835_m1
UCP-1 (BAT & WAT)	Mm01244861_m1
FGF-21 (Liver)	Mm00840165_g1
ACC1 (Liver)	Mm01304257_m1
PPARγ (BAT & WAT)	Mm01184322_m1
HSL (WAT)	Mm00495359_m1

Table 2.2 Probes employed in the quantification of DNA by rtPCR. All probes were obtained from Applied Biosystems, UK. For abbreviations see Table 2.1

Probes were validated before the experiment, comparing a random selection of samples from each tissue to the 18S control.

2.3.3.5 Analysis of Markers of Energy Homeostasis in Plasma Samples

Blood samples were obtained by cardiocentesis and collected into heparinised Eppendorf tubes. The tubes were immediately spun at 10000rpm for 10 minutes, and the plasma separated and stored at -80^{o}C until analysis.

Leptin

A murine leptin ELISA was obtained from Crystal Chem Inc., USA, and performed according to manufacturer's instructions. The assay is a sandwich ELISA, wherein wells in a 96-well plate were coated with monoclonal rabbit anti-leptin 'anchoring' antibodies. 50 µl of guinea-pig anti-mouse leptin IgG antibody, (the 'capture' antibody) was added to the wells alongside 5µl of plasma sample or standard. The capture antibody forms a complex with leptin in the samples, and forms a secondary complex with the immobilised anchoring antibodies on the plate. The assay was incubated overnight at 4 ^{o}C to allow all reagents to reach equilibrium. Wells were washed five times to remove unbound reagents, and then 100 µl of anti-guinea pig IgG enzyme conjugate was added per well. This is a 'detection antibody' which conjugates with horseradish peroxidase (HRP), an enzyme whose activity can be measured in the presence of the substrate (3, 3', 5, 5'-tetramethylbenzidine). 100 µl of this substrate was added per well following seven wash steps to remove excess HRP, and the plate was incubated in the dark for a further 30 minutes. The reaction was then stopped with stop solution (1M sulphuric acid) and absorbance measured. Absorbance was measured spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) at wavelengths of 450 and 630 nanometers and the latter subtracted from the former. A standard curve was then constructed to enable calculation of leptin concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

FT3

An ELISA for murine free T3 was obtained from Cusabio Biotech, China. This was a competitive inhibition enzyme immunoassay and was carried out in accordance with the manufacturer's

instruction. The wells of a 96 well microtitre plate were coated with an antibody specific to T3. Standards or samples (50 µl) plus biotin-conjugated T3 were added to the appropriate wells of the plate, and competitive inhibition occurred between sample and the biotin-conjugated T3 for the antibody coating the plate. The plate was incubated for one hour to allow equilibrium to develop and then washed to remove excess reagents. Horse radish peroxidase (HRP) was then added to the wells. 3,3', 5, 5'-tetramethylbenzidine, a substrate for HRP was added to the wells, and colour developed dependent on the amount of bound biotin-conjugated T3 in the well. Stop solution (0.3M HCl) was applied to all wells, and the colour intensity assessed spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) by measuring absorbance at the 450nm wavelength. A standard curve was then constructed to enable calculation of free T3 concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

Triglycerides

Triglycerides were measured using a triglyceride quantification kit (Abcam, Cambridge, UK). A standard curve was set up by diluting 40 µl of the 1 mM Triglyceride standard into 160 µl of the provided Assay Buffer. This was then mixed to generate a triglyceride standard at 0.2 nM concentration. Standards were plated in ascending concentrations from 0 to 50 µl.

Following plating of the standards, serum samples were added to the appropriate wells at a volume of 50 µl. 2 µl of Lipase was then added to all wells and incubated for 20 minutes at room temperature in order to break triglycerides down into glycerol and fatty acids. 50 µl of reaction mix (triglyceride assay buffer, triglyceride probe and triglyceride enzyme mix) was then added to each well, and the plate incubated for 60 minutes in the dark. The assay was then measured spectrophotometrically (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) by determining absorption at the 560nm wavelength. A standard curve was then constructed to enable calculation of free T3 concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

Adiponectin

Adiponectin was measured by ELISA (Abcam, Cambridge, UK). A 96 well plate was pre-coated with anti-adiponectin monoclonal 'capture' antibody. 50 µl of standard or sample was added to the appropriate wells, and the plate was incubated for two hours. After 5 wash steps, 50 µl of adiponectin specific biotinylated detection antibody was added to each well and the plate incubated for one hour. A further five wash steps were performed and then 50 µl of streptavidin-HRP conjugate was added to each well. The plate was incubated for 30 minutes and a further five wash steps performed again. Chromagen substrate was added and the plate incubated for 10 minutes. 50 µl of acidic stop solution was then added to each well and the colour of the assay changed from blue to yellow. Absorbance was read immediately at a wavelength of 450nm (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA). A standard curve was then constructed to enable calculation of adiponectin concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

<u>B-hydroxybutyrate</u>

A β-hydroxybutyrate assay kit was used (Abcam, Cambridge, UK) for quantification and the assay was performed according to manufacturer's instructions.

A standard curve was set up in a 96 well plate in volumes of 50 µl. Samples were added to remaining appropriate wells and made up to a reaction volume of 50 µl with assay buffer. 50 µl of reaction mix (β-HB Assay Buffer, β-HB Enzyme Mix, and β-HB Substrate Mix) was then added to each well. The assay plate was then incubated in the dark for 30 minutes, and the assay subsequently read at a wavelength of 450nm on a microplate reader (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA). A standard curve was then constructed to enable calculation of β-hydroxybutyrate concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

2.3.4 Statistics

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). A two-way repeated measures ANOVA with Bonferroni post-hoc test was used to assess differences in body weight, insulin and glucose during the IPGTT.

Food intake, the AUC for insulin and glucose obtained from the IPGTT, dissected tissue weights, plasma assays for markers of energy homeostasis were analysed using a one-way ANOVA with Tukey's post-hoc test. Tissue mRNA expression was analysed by a one-way ANOVA with Dunnett's post-hoc test.

All results are described as mean ± SEM, and statistical significance taken at $p<0.05$ unless otherwise stated.

2.3.5 Results

2.3.5.1 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on food intake in DIO mice

Food intake was measured by food weight every 1-2 days throughout the 70 day chronic feeding study, and food intake for each DIO mouse documented in a cumulative fashion. Food weight was measured immediately prior to subcutaneous injection. Each animal received daily subcutaneous injection of either placebo (0.9% Saline, Bayer, Haywards Heath, UK), GLP-1-LA (5 nmol/kg), OXM-LA (20 nm/kg) or PYY-LA (30 nmol/kg), or a combination of all three at the above doses. These doses were doubled after 6 days, and the dose of OXM-LA was further doubled on day 16, and the final dosing regime was: PYY-LA 60 nmol/kg, GLP-1-LA 10 nmol/kg and OXM-LA 80 nmol/kg. This was continued to the termination of the study.

There was no statistically significant difference in food intake between any of the treatment groups either alone or in combination. Analysis was performed using a one-way ANOVA with Tukey's post-hoc test. N=10-12 per group.

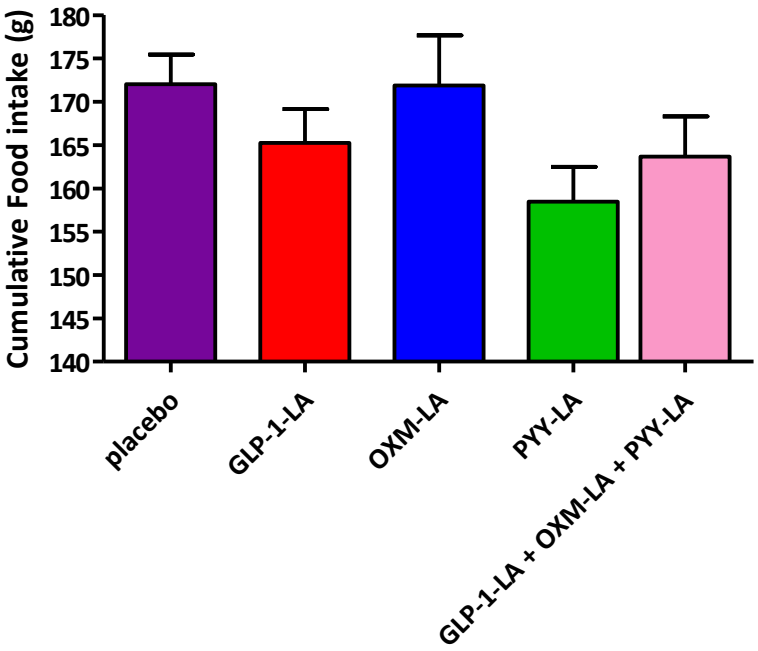


Figure 2.1 Cumulative food intake in (g) after 70 days of the chronic feeding study. DIO mice received either placebo, GLP-1-LA, OXM-LA, or PYY-LA alone or in combination. Food intake was measured every 1-2 days throughout the study, just prior to injection of the mice at 1500h. There was no statistically significant difference in food intake between the groups. Data was analysed with a one way ANOVA and Tukey's post-hoc test. N=10-12 per group.

2.3.5.2 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on body weight of DIO mice

Body weight was measured every 1-2 days throughout the 70 day chronic feeding study, immediately prior to subcutaneous injection of either placebo (0.9% saline, Bayer, Haywards Heath, UK), GLP-1-LA (10 nmol/kg), OXM-LA (80 nm/kg) or PYY-LA (60 nmol/kg), or a combination of all three at the above final doses.

There was a significant reduction in body weight in the GLP-1-LA group vs placebo (*p<0.05, **p<0.01); in the PYY-LA group vs placebo (*p<0.05, **p<0.01); in the GLP-1-LA group vs triple combination (***p<0.001); in the OXM-LA group vs the triple combination (*p<0.05,**p<0.01, ***p<0.001); in the PYY-LA group vs the triple combination (##p<0.01, ###p<0.001) and in the triple combination group vs placebo (*p<0.05, **p<0.01, ***p<0.001). All data was analysed by a two-way ANOVA with Bonferroni post-hoc test. N=10-12 per group.

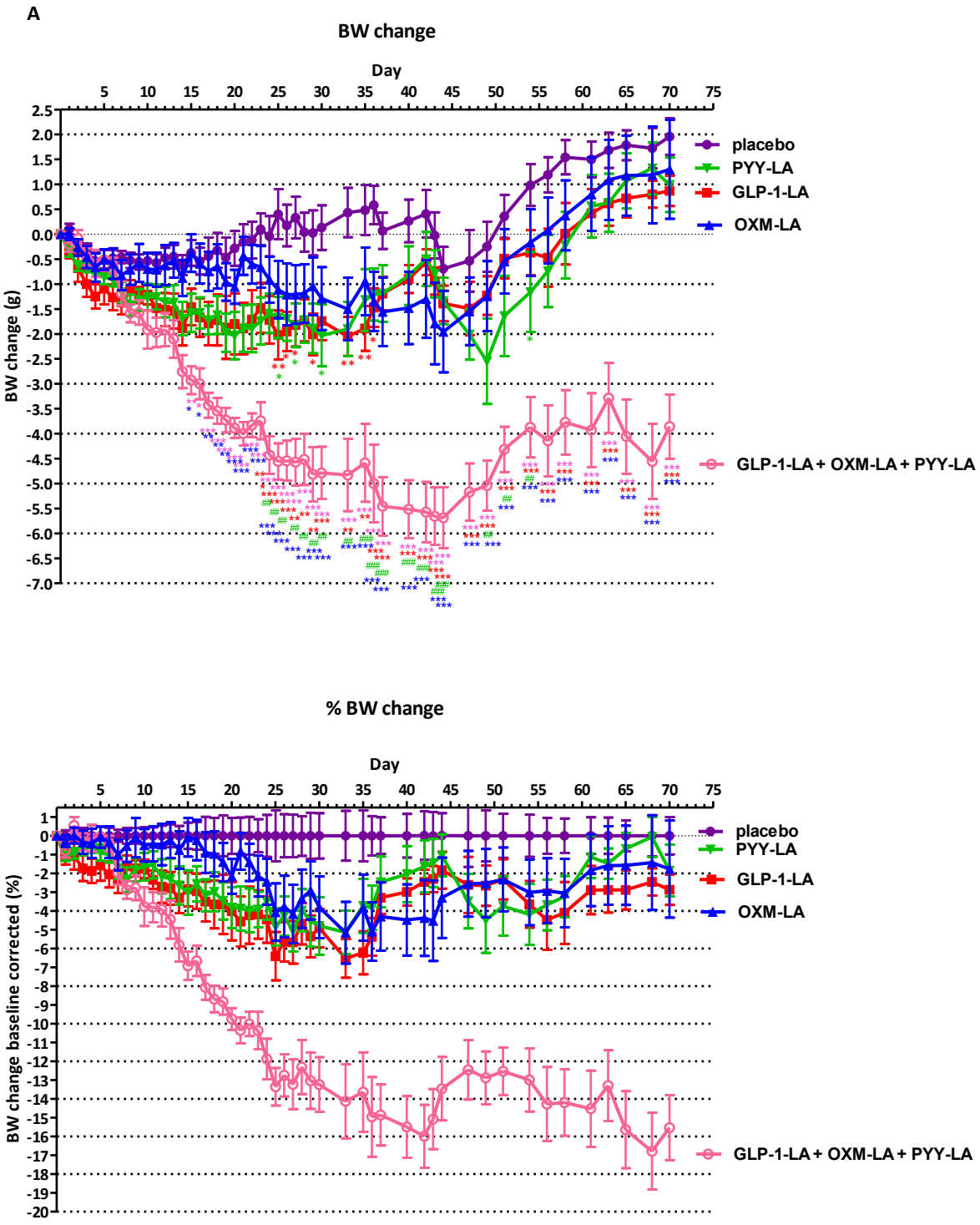


Figure 2.2 Body weight after 70 days of the chronic feeding study expressed as A: absolute body weight change, and B: percentage body weight change, baseline corrected. DIO mice received either placebo, GLP-1-LA, OXM-LA, or PYY-LA alone or in combination. Body weight was measured every 1-2 days throughout the study, just prior to injection of the mice at 1500h. There was a significant reduction in body weight in the GLP-1-LA group vs placebo (*p<0.05, **p<0.01); in the PYY-LA group vs placebo (*p<0.05, **p<0.01); in the GLP-1-LA group vs triple combination (*p<0.001); in the OXM-LA group vs the triple combination (*p<0.05,**p<0.01, ***p<0.001); in the PYY-LA group vs the triple combination (##p<0.01, ###p<0.001) and in the triple combination group vs placebo (*p<0.05, **p<0.01, ***p<0.001). All data was analysed by a two-way ANOVA with Bonferroni post-hoc test. N=10-12 per group.**

2.3.5.3 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on glucose homeostasis as measured by an intraperitoneal glucose tolerance test in DIO mice

Glucose homeostasis was assessed by means of an intraperitoneal glucose tolerance test (IPGTT). The IPGTT was performed in animals that had been injected with analogue and fasted from 6 hours prior to the procedure. Blood samples were obtained via a tail tip cut for glucose at t=-30, 0, 30, 60, 90 and 120 minutes and for insulin at 0, 30, 60 and 120 minutes. Insulin levels at t=0 were significantly higher in the placebo group vs OXM-LA (10.7 ± 1.13 ng/ml vs 5.7 ± 1.1 ng/ml #p<0.05) and in the placebo, GLP-1-LA and PYY-LA groups compared to the triple combination group (10.7 ± 1.13 ng/ml ($$$ p<0.001); 9.18 ±1.3 ng/ml (&& p<0.01) and 8.5 ±1.5 ng/ml (*p<0.05) respectively vs 3.34 ± 1.15 ng/ml. Glucose levels were not significantly different between groups at baseline. They were, however, significantly lower in the combination group compared to placebo at later time points (&&& p<0.001); GLP-1-LA (% p<0.05, %%p<0.01); OXM-LA (^p<0.05) and PYY-LA ($$$p<0.001). In addition, compared to both the placebo group and the PYY-LA group, glucose was significantly lower in the GLP-1-LA group (## p<0.01, ## p<0.001), and (+p<0.05, +++p<0.001) respectively and in the OXM-LA group (£££p<0.001), and (**p<0.01***p<0.001) respectively. Analysis was by two-way ANOVA with Bonferroni post-hoc test. N=10-12 per group

AUC was calculated for insulin and glucose during the IPGTT. Glucose levels were significantly higher in the placebo and PYY-LA groups compared to the GLP-1-LA, OXM-LA and triple combination groups (*p<0.05, **p<0.01, ***p<0.001). There was no statistical significance between the insulin levels in any of the groups. Analysis was by a one-way ANOVA with Tukey's post hoc test. N=10-12

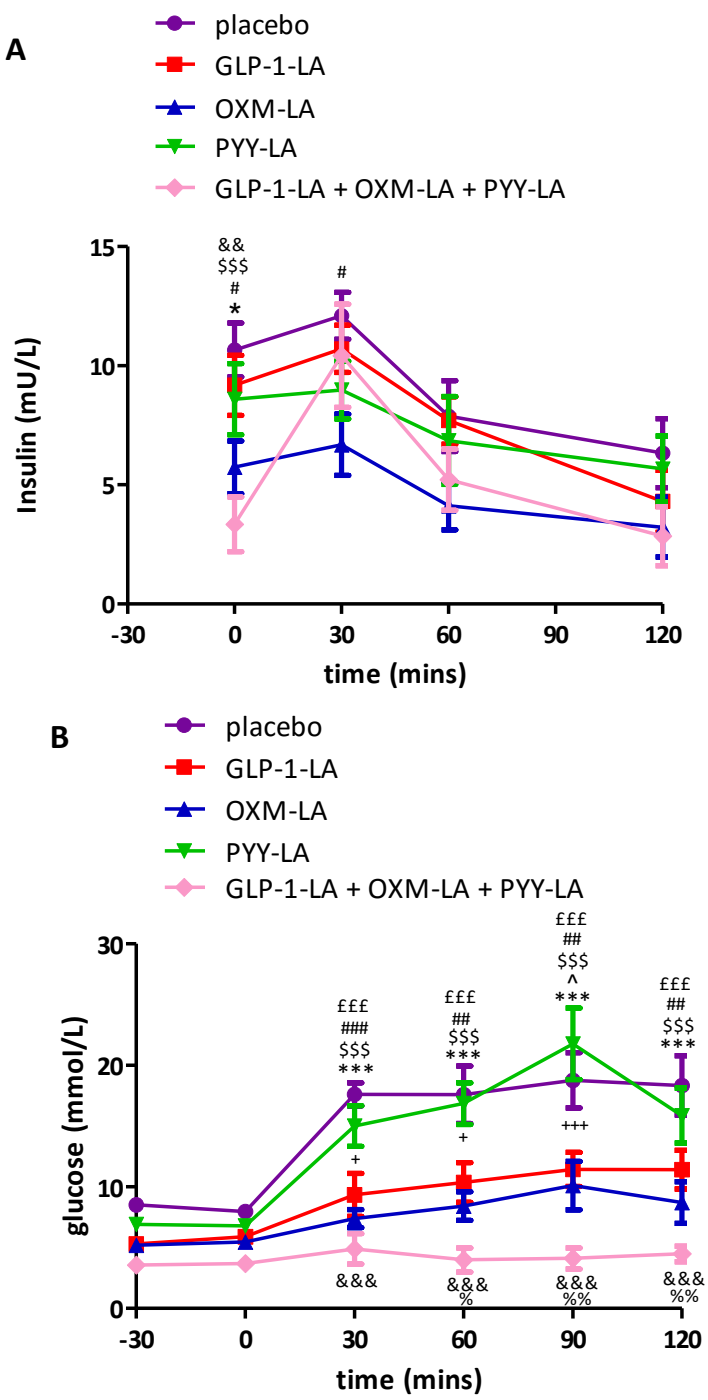


Figure 2.3 A: Insulin and B: glucose concentrations following IPGTT in DIO mice receiving one of placebo, GLP-1-LA, OXM-LA or PYY-LA. The IPGTT was performed in animals that had been injected with analogue and fasted from 6 hours prior to the IPGTT. Blood samples were obtained via a tail tip cut for glucose at t=-30, 0, 30, 60, 90 and 120 minutes and for insulin at 0, 30, 60 and 120 minutes. Insulin levels at t=0 were significantly higher in the placebo group vs OXM-LA (#p<0.05) and in the placebo, GLP-1-LA and PYY-LA groups compared to the triple combination group ($$$ p<0.001, && p<0.01, and *p<0.05 respectively). Glucose levels were significantly lower in the combination group compared to placebo (&&& p<0.001); GLP-1-LA (% p<0.05, %%p<0.01); OXM-LA (^p<0.05) and PYY-LA ($$$p<0.001). In addition, compared to both the placebo group and the PYY-LA group, glucose was significantly lower in the GLP-1-LA group (## p<0.01, ## p<0.001), and (+p<0.05, +++p<0.001) respectively and in the OXM-LA group (£££p<0.001), and (p<0.01***p<0.001) respectively. Analysis was by two-way ANOVA with Bonferroni post-hoc test. N=10-12 per group**

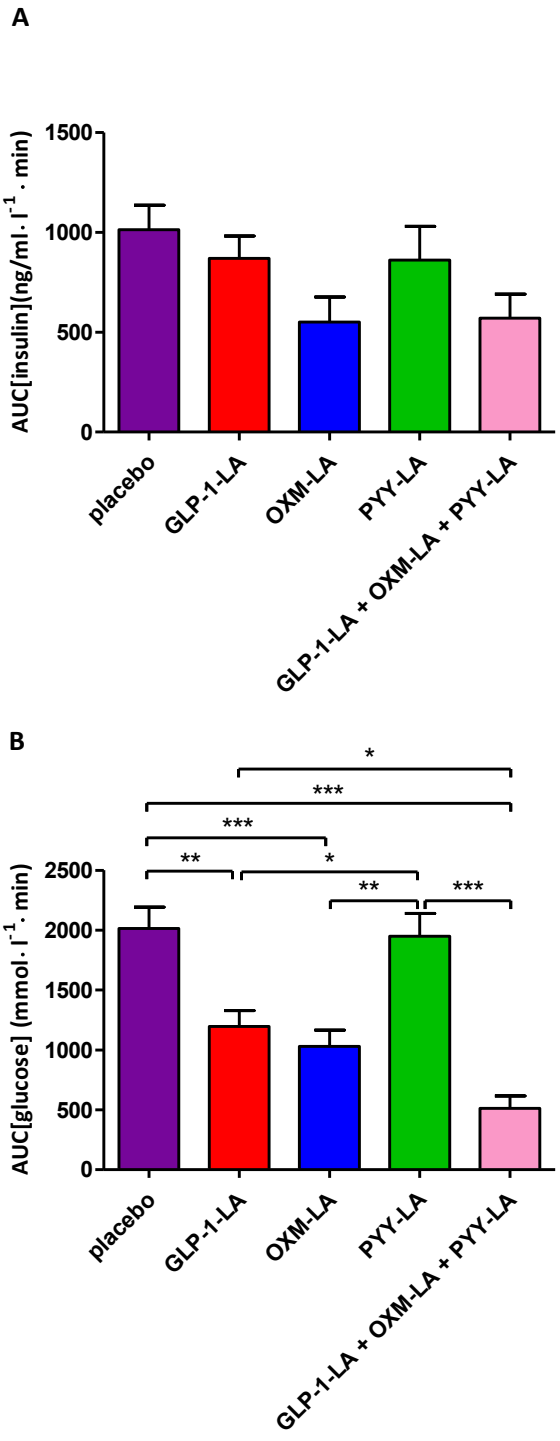


Figure 2.4 Area under the curve (AUC) for A: Insulin and B: Glucose following an IPGTT in DIO mice receiving one of placebo, GLP-1-LA, OXM-LA or PYY-LA. The IPGTT was performed in animals that had been injected with analogue and fasted from 6 hours prior to the IPGTT. Blood samples were obtained via a tail tip cut for glucose at t=-30, 0, 30, 60, 90 and 120 minutes and for insulin at 0, 30, 60 and 120 minutes. Glucose levels were significantly higher in the placebo and PYY-LA groups compared to the GLP-1-LA, OXM-LA and triple combination groups (*p<0.05, **p<0.01, ***p<0.001). There was no statistical significance between the insulin levels in any of the groups. Analysis was by a one-way ANOVA with Tukey's post hoc test. N=10-12 per group

2.3.5.4 The effect of chronic administration of the long acting gut hormone analogues GLP-1-LA, OXM-LA and PYY-LA both alone and in combination on markers of energy homeostasis

Harvested tissue weights from DIO mice following conclusion of the chronic food intake study

At the conclusion of the 70 day food intake study, mice were culled by CO_2 asphyxiation. Liver, interscapular BAT and epididymal WAT deposits were dissected out, weighed and snap frozen in liquid nitrogen prior to analysis for gene markers of energy homeostasis. Tissue weights were expressed as a percentage of whole body weight.

Liver weight as a percentage of total body weight was significantly reduced in the group receiving GLP-1-LA compared to the groups receiving placebo and OXM-LA (4.2 ± 0.21% vs 5.8 ± 0.25% and 5.8 ± 0.32% respectively, **p<0.01). BAT weight as a percentage of total body weight was reduced in the GLP-1-LA (0.72 ±0.05%, *p<0.05) and triple combination (0.56 ± 0.05% ***p<0.001) groups compared to placebo (0.86 ±0.03%). Epididymal WAT weight as a percentage of total body weight was reduced in the placebo group compared to the GLP-1-LA (2.2 ± 0.11% vs 2.9 ± 0.18% *p<0.05).

All data were analysed by one-way ANOVA with Tukey's post-hoc test. N=10-12.

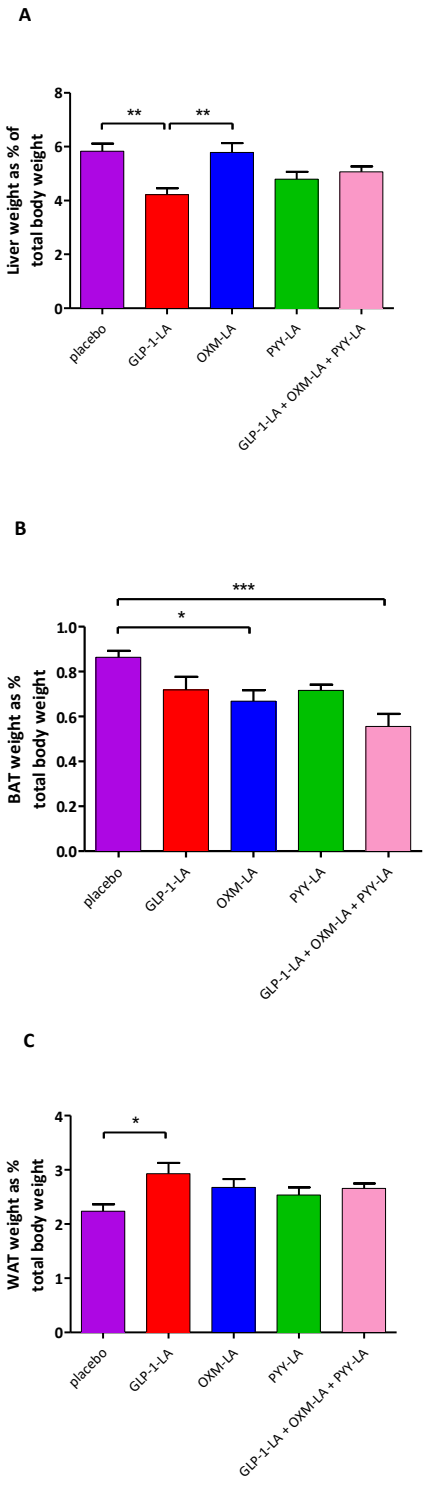


Figure 2.5 Harvested tissue weights from A: Liver B: interscapular BAT and C: epididymal WAT expressed as a percentage of total body weight. Liver weight as a percentage of total body weight was significantly reduced in the group receiving GLP-1-LA compared to the groups receiving placebo and OXM-LA **p<0.01. BAT weight as a percentage of total body weight was reduced in the GLP-1-LA (*p<0.05) and triple combination (*p<0.001) groups compared to placebo. Epididymal WAT weight as a percentage of total body weight was reduced in the placebo group compared to the GLP-1-LA (*p<0.05). All data were analysed by one-way ANOVA with Tukey's post-hoc test. N=10-12 per group.**

Markers of Energy Homeostasis in Tissue Samples

Harvested tissue from liver, BAT and WAT was analysed for expression of mRNA for genes specifically involved in energy homeostasis, thermogenesis, lipolysis and glucose cycling.

In liver tissue, FGF-21 expression was significantly increased in the groups which had received OXM-LA and the triple combination of analogues compared to placebo (2.27 ± 0.67 arbitrary units (AU) and 2.39 ± 0.59 AU vs 0.96 ± 0.12 AU*$p<0.05$).

There was no evidence that any of the interventions altered the expression of any of the other markers of energy homeostasis of interest that were tested here. Of note, the mRNA yield was very low in some of the WAT samples, which posed a challenge in making a meaningful comparison between samples and placebo.

All data were analysed using a one-way ANOVA with Dunnett's post-hoc test. N=7-10 for liver and BAT samples, N=2-6 for WAT samples.

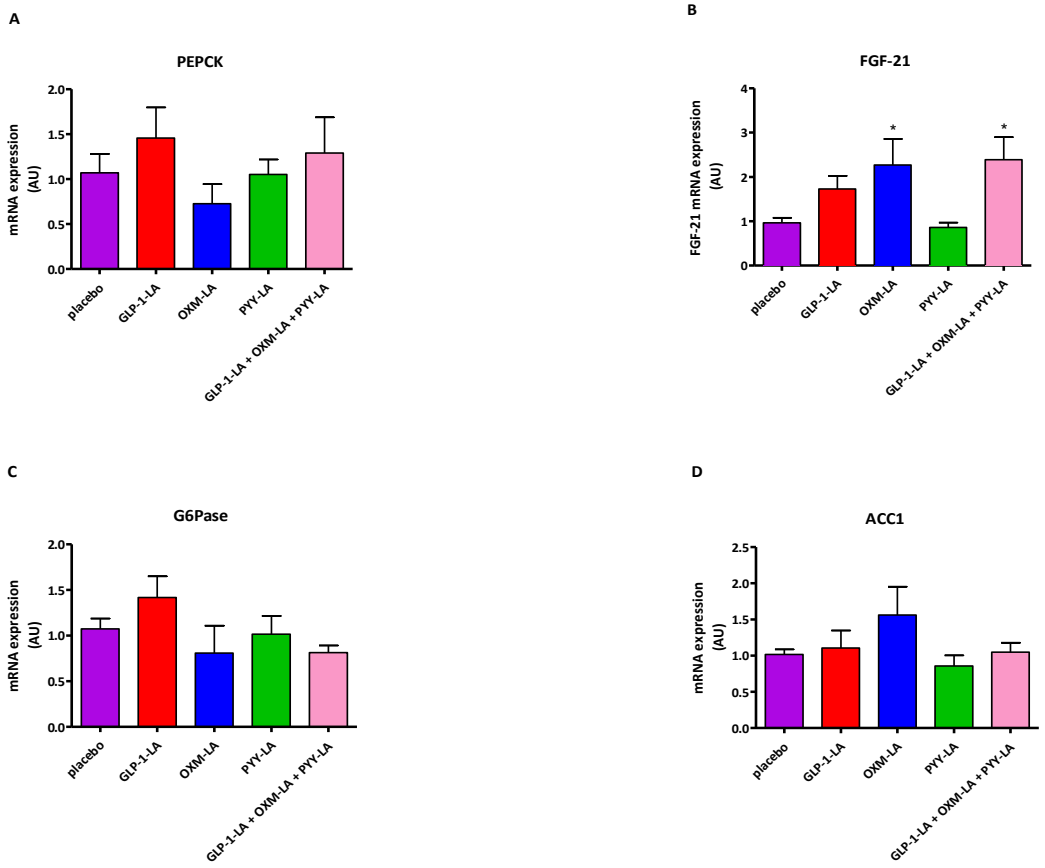


Figure 2.6 mRNA expression in liver tissue in DIO mice following the chronic feeding study. DIO mice received either placebo, GLP-1-LA, OXM-LA, or PYY-LA alone or in combination. FGF-21 expression in liver was significantly increased in the groups receiving OXM-LA and the triple combination compared to placebo (*p<0.05). A: phosphoenolpyruvate carboxykinase 1 (PEPCK) B: Fibroblast growth factor-21 (FGF-21), C: glucose-6-phosphatase (G-6-Pase) and D: acetyl-coenzyme A carboxylase alpha (ACC1). None of the other measured mRNA expression profiles of interest were significantly altered by the interventions above. Data were analysed by one way ANOVA with Dunnett's post-hoc test. N=7-10 per group

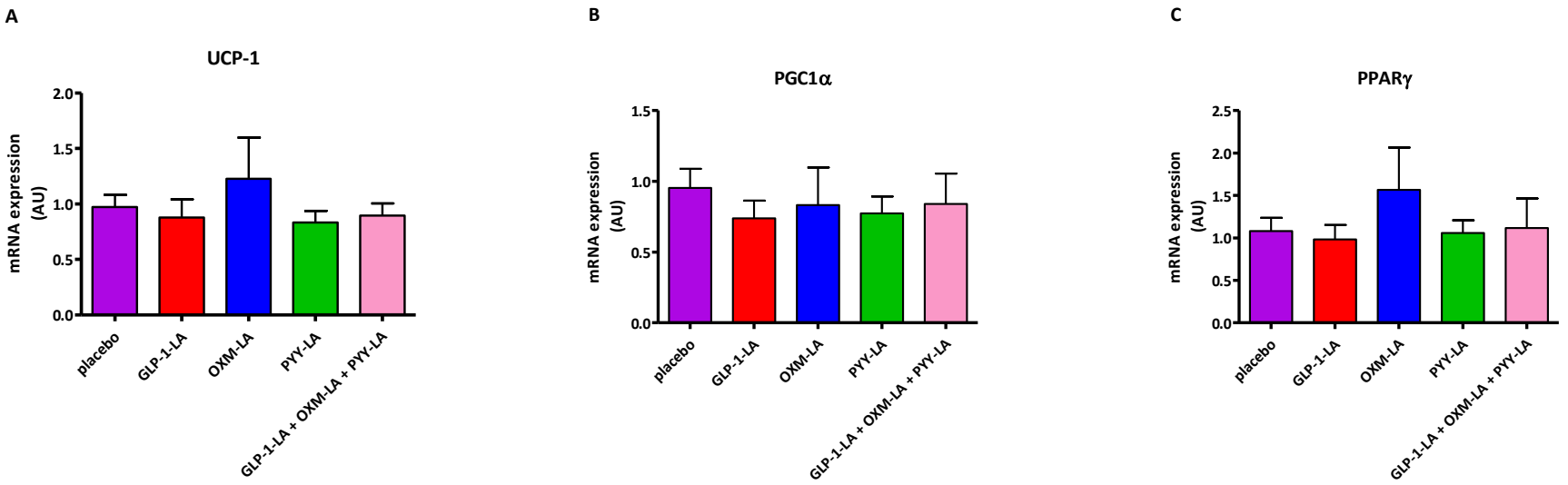


Figure 2.7 mRNA expression in BAT in DIO mice following a chronic feeding study. DIO mice received either placebo, GLP-1-LA, OXM-LA, or PYY-LA alone or in combination. A: uncoupling protein-1 (UCP-1), B: peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC1α) C: peroxisome proliferative activated receptor gamma (PPARγ). None of the measured mRNA expression profiles of interest were significantly altered by the interventions above. Data were analysed by one way ANOVA with Dunnett's post-hoc test. N=7-10 per group

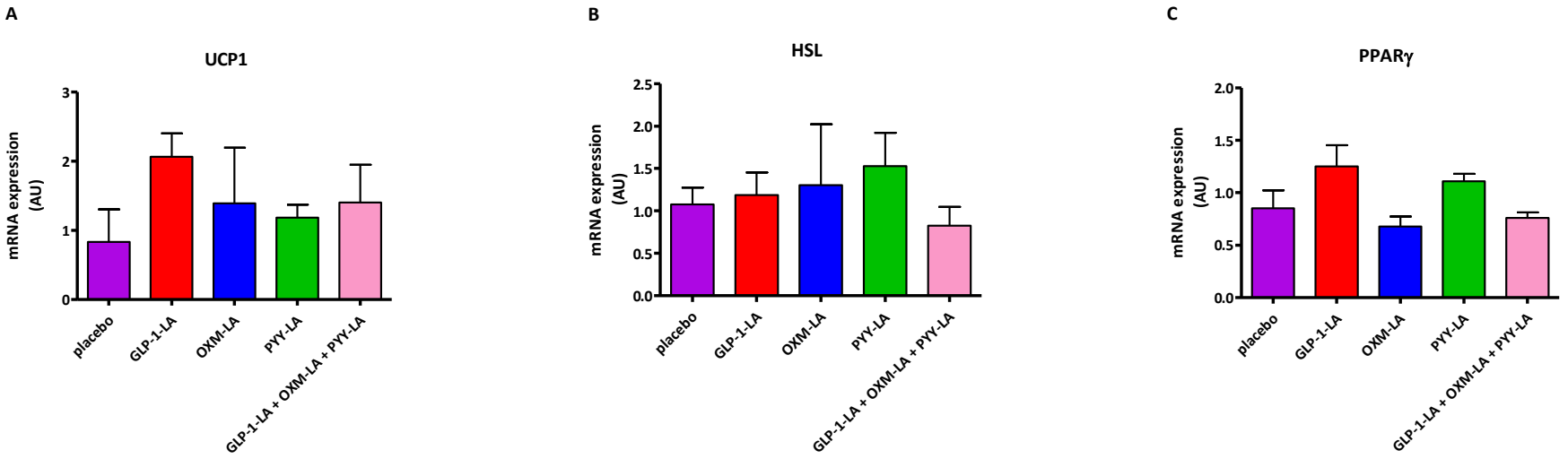


Figure 2.8 mRNA expression in WAT in DIO mice following a chronic feeding study. DIO mice received either placebo, GLP-1-LA, OXM-LA, or PYY-LA alone or in combination. A: uncoupling protein-1 (UCP-1), B: Hormone sensitive lipase (HSL), C: peroxisome proliferative activated receptor gamma (PPARγ). None of the measured mRNA expression profiles of interest were significantly altered by the interventions above. Data were analysed by one way ANOVA with Dunnett's post-hoc test. N=2-6 per group

Markers of Energy Homeostasis in Plasma

Plasma from the DIO mice was obtained at the conclusion of the feeding study by cardiocentesis. Plasma was analysed for Leptin, an adipokine with effects on mediating food intake which exhibits levels proportional to body fat mass (see section 1.3.2). Free T3 was also measured, with thyroid hormones being directly involved in mediating EE in BAT (see section1.4.1.3). β-hydroxybutyrate and triglycerides were measured as markers of lipolysis and β oxidation, and finally, I measured Adiponectin, an insulin-sensitising adipokine with levels inversely correlated to body fat mass, which is a downstream effector of both PPARγ and FGF-21.

Circulating leptin concentration was significantly reduced in the group who received triple analogue therapy compared to all other groups (21.9 ± 2.9 ng/ml vs Placebo 34.6 ± 0.43 ng/ml (***$p<0.001$); GLP-1-LA 32.7 ± 1.0 (***$p<0.001$); OXM-LA 29.2 ± 2.1 ng/ml (*$p<0.05$); PYY-LA 33.3 ± 0.72 ng/ml (***$p<0.001$)). This is not an unexpected finding given the significantly greater body weight reduction in the triple analogue group.

In addition, triglyceride levels were significantly elevated in the PYY-LA group vs the GLP-1-LA group (2.94 ± 0.34 mg/dl vs 1.71 ± 0.29 mg/dl (*$p<0.05$)).

All data were analysed by a one-way ANOVA with Tukey's post-hoc test. N=10-12

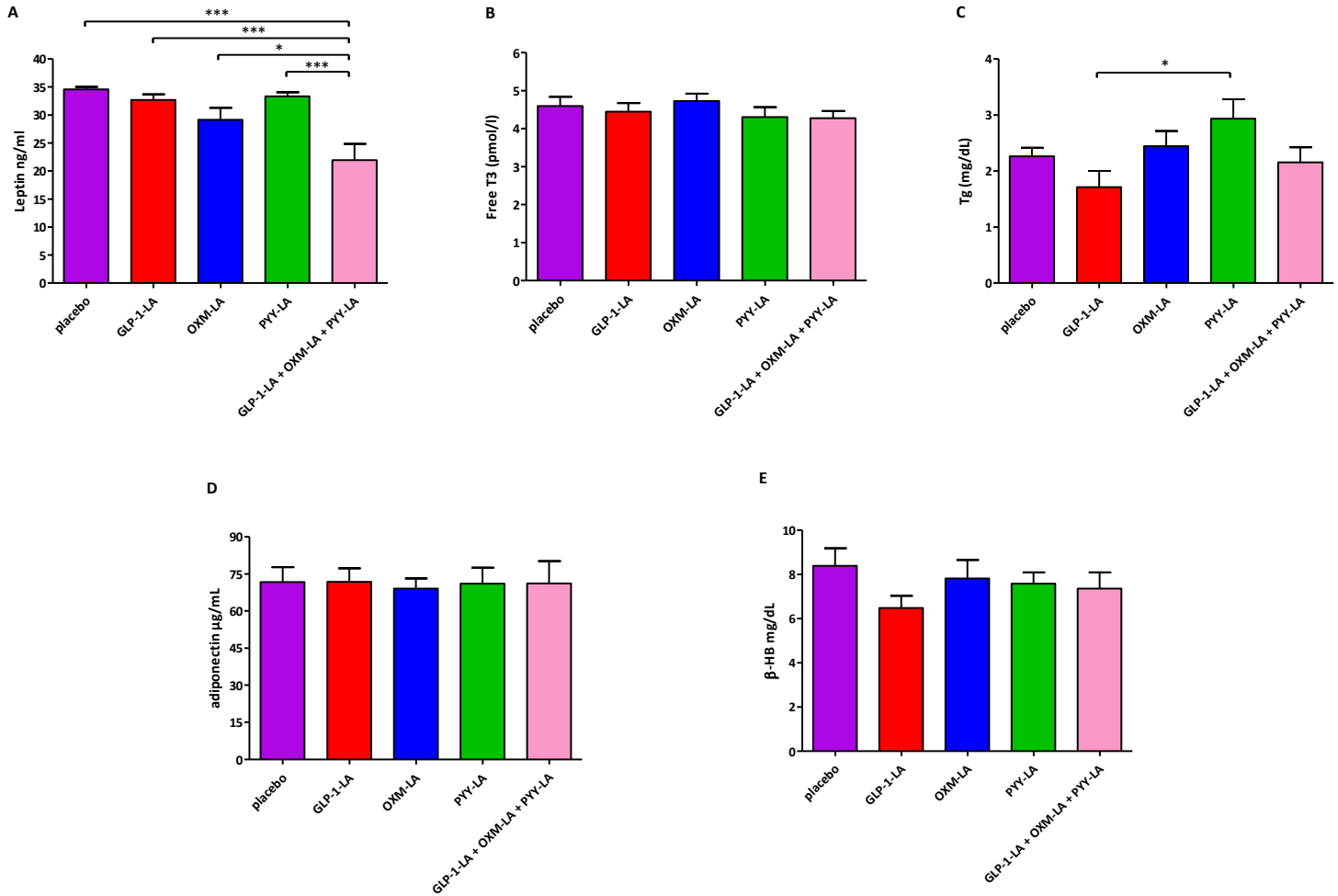


Figure 2.9 Harvested plasma from DIO mice following a chronic feeding study was analysed for markers of energy homeostasis. DIO mice received either placebo, GLP-1-LA, OXM-LA, or PYY-LA alone or in combination. A: Leptin, B Free Tri-iodothyronine (FT3), C: Triglycerides (Tg), D: Adiponectin and E: Beta-hydroxybutyrate (β-HB) were measured in the plasma samples. The triple combination of analogues reduced Leptin compared to placebo and each analogue alone (*p<0.05, ***p<0.001). Triglyceride levels were significantly elevated in the group receiving PYY-LA vs GLP-1-LA (*p<0.05). All data were analysed with a one-way ANOVA and Tukey's post-hoc test. N=10-12 per group

2.3.5 Discussion

In this study I have shown that a combination of long-acting analogues of the hormones GLP-1, OXM and PYY cause a significant reduction of body weight above that seen with any of the analogues given alone. Whilst there is no significant reduction in cumulative food intake between the groups, there is a reduction in food intake, in particular in the PYY-LA and triple combination groups, compared to placebo. This would suggest that a reduction in food intake plays a part in body weight loss but is not the only mechanism. Therefore, the mechanism of body weight loss must include an increase in energy expenditure. However, on investigation of selective markers of increased energy expenditure, there was no significant increase seen. UCP-1 and FT3 are intrinsic in the facilitation of adaptive thermogenesis, and therefore increase EE in BAT, but neither of these were seen to be increased or upregulated in this study. This would suggest that any increase in EE must be generated via an alternative mechanism. 'Britening' of WAT cells, wherein white adipose tissue begins to show characteristics of brown adipose tissue, expressing UCP-1 and generating heat upon stimulation by catecholamines (Petrovic et al., 2010, Wu et al., 2012) could be an explanation for the increase in EE seen in this study. However, experimental support for this theory is lacking. I have not shown an increase in UCP-1 expression in WAT, and there was no increase in WAT weight in the group receiving the triple analogue combination, which could perhaps be expected if there was transformation of WAT to 'brite' cells. However, confirmation by histological examination would be necessary to refute this theory particularly as there was substantial technical difficulty in mRNA extraction from WAT.

An increase in EE could be due to futile cycling or an alternative tissue source of adaptive thermogenesis as discussed in section 1.4.1, but investigation of these possibilities is beyond the scope of the current study. A calorimetric study to assess the effect of these analogues on oxygen consumption would be a next step, as this would confirm an increase in EE, and thus more extensive mechanistic studies could follow.

The initial fall in body weight in this study was not sustained for the analogues administered singly (figure 2.2 A&B). After around day 35 of the study, a slow but steady increase in body weight was seen – both in absolute terms and compared to placebo. This effect was not seen to such an extent in the triple analogue combination group. This escape from the effect of peptides or their analogues, also known as tachyphylaxis, has been described for PYY(3-36) when assessing inhibition of food intake(Van den Hoek et al., 2007). It has also been recently reported that after an initial improvement with the use of Liraglutide, beta cell function (albeit in an experimental model) deteriorated and there was an associated escape of glycaemic control (Abdulreda et al., 2016). Escape from the effects of an administered peptide could be due to receptor down regulation, or potentially decreased sensitivity to the peptide, as described in the case of glucagon (Premont and Iyengar, 1988). In addition, it is well documented that the administration of certain peptides in clinical use such as insulin (Wredling et al., 1990) and GLP-1 (Aroda and Ratner, 2011) is associated with the development of antibodies that are described as non-neutralising. In one study, up to 40% of type 2 diabetic patients using insulin had detectable insulin antibodies, and interestingly those patients were found to have much higher serum insulin levels than those without antibodies (Hattori et al., 2014). A further report documents resistant hyperglycaemia in type 2 diabetic patients on insulin with documented insulin antibodies (Hu et al., 2015), and in a study of patients with exenatide (a GLP-1 mimetic) antibodies, a HbA1c reduction was seen in 87% of antibody negative compared to just 62% of antibody positive subjects (Faludi et al., 2009). It can therefore be speculated that the presence of antibody, although not completely neutralising to the effect of peptide, could affect potency and mediate an escape from treatment effects. Antibody formation was not measured in this study but would be an important addition if this approach was to be taken forward as a potential obesity therapy.

As a measure of glucose homeostasis, an intraperitoneal glucose tolerance test was performed (IPGTT) at the conclusion of the food intake study. An IPGTT was chosen over an oral glucose tolerance test

(OGTT) as the animals were used to handling and injections, thus stress levels would be minimised. Although OGTT may be a more physiological test, the mice would need to receive oral glucose via gavage which would cause stress in unacclimatised animals, and potentially result in a confounding effect on glucose levels. As discussed by Andrikopoulos et al, a glucose dose of 2g/kg was used for maximal sensitivity of the test (Andrikopoulos et al., 2008) The IPGTT demonstrated that at baseline, the groups which had received OXM-LA and the triple combination had significantly lower basal insulin concentrations than the other two analogue treated groups and placebo. This is in keeping with the lower body weight in the triple therapy group. A lower basal insulin in the OXM-LA treated group is likely to reflect the lower overall glucose levels seen in that group compared to the other treatment arms, despite a lack of significant reduction in baseline glucose values per se. There was no difference in basal glucose levels between the groups. The groups receiving GLP-1-LA and the triple combination had a significant improvement in glucose control compared to the other treatment groups, thus an increased insulin response might have been expected to account for this improvement. However, no significant increase in insulin secretion was seen, suggesting that weight loss and the subsequent fall in insulin resistance is a more important mediator of the observed improvement in glucose homeostasis. Weight loss cannot, however, be the only determining factor, as the PYY-LA group had comparable body weights to the GLP-1-LA and OXM-LA group, but significantly worse glucose homeostasis on IPGTT. PYY is not known to adversely affect insulin sensitivity, and indeed as shown in chapter 5, seems to have little effect on glucose disposal after a glucose load. It is possible that there is an improvement in hepatic glucose handling in the groups who received a GLP-1-LA either alone or in combination, and this is further discussed below.

Markers of gluconeogenesis in harvested liver tissue were analysed to assess if there was any effect of the analogues on hepatic glucose handling. Any up-regulation of enzymes integral to gluconeogenesis such as glucose-6-phosphatase (G-6-Pase), phosphoenolpyruvate carboxykinase 1 (PEPCK1), and peroxisome proliferative activator receptor gamma coactivator 1 alpha (PGC1α) could result in raised blood glucose levels, but there was no evidence of this experimentally (figure 2.6).

There was however a significant increase in Fibroblast growth factor-21 (FGF-21) expression in the OXM-LA and triple combination groups. Glucagon is known to increase FGF-21 expression (Habegger et al., 2013), and OXM-LA acts via both the GLP-1 and glucagon receptors. It is therefore likely that this increase in FGF-21 is mediated via the glucagon receptor. The downstream effects of FGF-21 include an improvement in glycaemic control, body weight loss, improvement in dyslipidaemia (Habegger et al., 2013) and possibly an improvement in insulin sensitivity (Mu et al., 2012) which could explain the favourable IPGTT results for the OXM-LA group. FGF-21 also increases EE and may therefore be responsible for the reduction in BAT mass seen in the OXM-LA and triple combination groups.

The reduction in liver weight in the group that received GLP-1-LA compared to placebo correlates with a recent paper by Trevaskis et al wherein they suggest that agonism at the GLP-1 receptor reduces features of non-alcoholic fatty liver disease (NAFLD), including liver weight, hepatic lipids and plasma triglycerides (Trevaskis et al., 2012). An assumed reduction in hepatic lipids may also confer a benefit on hepatic insulin resistance, and therefore may contribute to the improvement in glucose homeostasis. Furthermore, I also demonstrated a reduction in plasma triglycerides in the GLP-1-LA group, but only in comparison to the PYY-LA group. Whilst it is possible this is a true effect mediated through the GLP-1 receptor, (as GLP-1 agonism is known to improve the lipid profile (Davies et al., 2015, Ben-Shlomo et al., 2011, Patel et al., 2014)), the fact that it is not replicated in the triple analogue combination group makes it perhaps a less robust observation.

The reduced leptin levels observed in the triple analogue combination group compared to all the other interventions are an expected result, given the weight loss. Circulating leptin concentrations are directly correlated with body weight, and more specifically with body fat mass. The significant drop in levels in the triple group would suggest that the reduction in body weight is secondary to a reduction in body fat rather than a loss of lean weight. Body composition studies with techniques such as MRI

to confirm this would be a future step. In contrast, adiponectin concentrations are generally inversely correlated to body fat mass, but they were not altered in any of the treatment groups.

The contribution of PYY-LA to the observed results is likely to be through a reduction of food intake. Despite a lack of statistical significance at the end of the study, PYY-LA does appear to reduce food intake compared to placebo. This may therefore be contributing to the weight loss in this study. PYY is not known to have any effect on increasing EE, and in fact Sloth et al documented a tendency toward a reduction of EE with administration of PYY(3-36) (Sloth et al., 2007). In addition, PYY(3-36) does not have any direct effect on insulin secretion or insulin sensitivity (Tan et al., 2014), but may exert an indirect effect on glucose homeostasis by acting to increase GLP-1 levels in the hepatic circulation (Chandarana et al., 2013).

In summary, a combination of long acting analogues of GLP-1, OXM and PYY are able to significantly reduce body weight in DIO mice, an effect not sustained when any of the analogues were administered alone. This reduction in body weight occurred without a significant reduction in food intake, although there was a reduction in food intake which did not reach significance in the PYY-LA and triple combination groups. This suggests a multifactorial mechanism for weight loss including an increase in EE. The mechanism for the increase in EE remains unclear. The triple analogue group also exhibited an improvement in basal insulin levels, and a favourable glucose profile following an intraperitoneal glucose challenge. These effects may be mediated at least in part via the glucagon receptor with FGF-21 as a downstream effector. In addition, the action of GLP-1 in improving the hepatic lipid profile may play a role in reducing hepatic insulin resistance, and therefore have a further favourable effect on glucose homeostasis.

CHAPTER 3: THE ACUTE EFFECTS OF CO-INFUSION OF GLUCAGON AND GLP-1 ON ENERGY EXPENDITURE AND GLUCOSE HOMEOSTASIS IN HUMANS

3.1 Introduction

With the growing obesity crisis, new avenues for treatment are constantly being explored. More recently, it has become apparent that exploiting the properties of two or more agents in combination offers the potential for much more successful therapies. Indeed, in our department we have previously shown that OXM, a gut hormone derived from the same proglucagon precursor as GCG and GLP-1 (Fig 1.4) and known to act via both the GCG and GLP-1 receptors is able to increase EE and reduce food intake without any detrimental effect on glycaemic control (Wynne et al., 2006, Wynne et al., 2005). Further to this evidence that dual activation of the GCG and GLP-1 receptors may be beneficial, gut hormone analogues which are dual agonists at the GCG and GLP-1 receptors have achieved weight loss with no deterioration of glycaemic control and an improved lipid profile in diet-induced obese rodents (Day et al., 2009, Pocai et al., 2009). In this study I have therefore substituted the use of both glucagon and GLP-1 for the use of OXM, in order to study the relative contribution of each receptor to the measured parameters.

Weight loss can broadly be claimed to result from a reduction in food intake, combined with an increase in energy expenditure. Glucagon is well known to increase energy expenditure (Davidson et al., 1960, Nair, 1987) but has powerful effects on glucose homeostasis, causing hyperglycaemia via hepatic glycogenolysis and gluconeogenesis (Striffler et al., 1981, Studer et al., 1984, Stevenson et al., 1987, Wasserman et al., 1989). Generating hyperglycaemia as a side effect of any new therapy is undesirable, but for a weight loss therapy where a proportion of patients will have disordered glucose regulation or even frank diabetes it is unacceptable. In order to counteract the hyperglycaemic effect of glucagon whilst retaining its influence on EE, I investigated the combined administration of GCG with GLP-1. GLP-1 is an incretin hormone, increasing glucose-dependent insulin secretion (Kreymann et al., 1987), and therefore with the potential to ameliorate the hyperglycaemic effects of glucagon.

In this chapter I have investigated the use of a combination of the hormones GLP-1 and glucagon acutely in man, and examined their effect on energy expenditure, glucose homeostasis and food

intake. Using the two hormones in synergy will allow an assessment of the ideal ratio in which they should be used to achieve optimal desirable effects on, EE and blood glucose.

3.2 Hypothesis and Aims

3.2.1 Hypothesis

- Infusion of glucagon will increase EE
- Any rise in blood glucose which results from infusion of glucagon will be ameliorated by the co-infusion of GLP-1.

To test this hypothesis, I measured EE by indirect calorimetry during infusion of glucagon and GLP-1, both singly and in combination, in healthy overweight human volunteers.

3.2.2 Aims

- To investigate the acute effects of co-infusion of glucagon and GLP-1 on EE and blood glucose homeostasis in healthy overweight volunteers

3.3 Materials and Methods

3.3.1 Subjects

Ten healthy, overweight/obese participants were recruited by advertisement in local media. The acceptable age range for recruitment was 18-65 years, with a body mass index (BMI) of 25-35kg/m^2. The demographics of participants may be seen in table 3.1. Body weight was stable for at least three months prior to the start of the study. All participants were rigorously screened for inclusion through medical history, physical examination, 12 lead electrocardiogram, and biochemical/haematological testing. Both men and women were recruited, but female participants had to be pre-menopausal with regular menstrual cycles. The use of oestrogen-containing contraception was an exclusion criterion (Martínez de Morentin et al., 2014), but progesterone-only contraception was permitted as this has been shown to exert no effect on EE (Pelkman et al., 2001). Acceptability of the calorimeter canopy was also assessed at screening (Fig 3.1). Volunteers with any medical history or condition, or anyone using medication whether prescribed or over-the-counter, were excluded if the condition or medication was felt to interfere with the study or if enrolling in the study could potentially cause harm to the volunteer. Women were excluded if pregnant or breastfeeding. Anyone having donated blood in the previous three months, or having participated in another study involving an investigational drug within two months was also excluded. Participants were free to withdraw from the study at any time, and female volunteers were asked to maintain adequate contraception during the course of the study and for one month afterwards.

All volunteers gave informed written consent. The study was carried out in accordance with the principles of the Declaration of Helsinki, and was approved by the local West London Research Ethics committee (REC 09/H0707/76).

Volunteer	Weight (kg)	Height (m)	BMI (Kg/m^2)	Age	Sex
1	99.1	1.66	36.0	26	F
2	80.0	1.63	30.1	28	F
3	101.5	1.78	32.0	31	M
4	96.4	1.94	25.6	23	M
5	99.0	1.81	30.2	49	M
6	89.1	1.84	26.3	48	M
7	100.3	1.87	28.7	32	M
8	91.1	1.82	27.5	32	M
9	77.2	1.71	26.4	44	F
10	79.1	1.61	29.8	45	M
Mean	91.3	1.77	29.3	35.8	F3/7M

Table 3.1 Demographic details and means of all volunteers included in the study 'The acute effects of co-infusion of glucagon and GLP-1 on EE and glucose homeostasis'

3.3.2 Peptides

Glucagon (NovoNordisk, Crawley, UK) and GLP-1(7-36) amide (Clinalfa Basic, Bachem, Switzerland) were purchased for use in this study. GLP-1(7-36) amide was reconstituted in 1 ml of sterile 0.9% saline (Bayer, Haywards Heath, UK), and glucagon was reconstituted in the solvent provided by NovoNordisk (1ml of sterile water). The glucagon vial also contains lactose monohydrate 107mg.

3.3.3 Calorimetry

Measurement of energy expenditure can be performed in several ways. In this study I have used indirect calorimetry to measure resting energy expenditure (REE). In indirect calorimetry, the subject is placed under a clear Perspex hood (Fig 3.1). The calorimeter then measures how much oxygen they are breathing in (VO_2), and how much carbon dioxide they produce (VCO_2). From VO_2 and VCO_2 measurements, we can calculate REE. The amount of energy required to metabolise one unit of O_2 into one unit of CO_2 is known, and EE can therefore be calculated via the Weir equation (Weir, 1949). The Weir equation requires a measure of 24 hour urinary nitrogen excretion as a representation of protein metabolism as this is not reflected in the gas analysis. The modified Weir equation has been used in this study (see below).

In addition to EE, we can also calculate the respiratory quotient (RQ), as well as carbohydrate and fat oxidation rates. Respiratory quotient is the ratio of carbon dioxide produced to oxygen consumed during metabolism of various substrates. The RQ varies between 0.7 and 1 depending on the substrate being metabolised, with the RQ for carbohydrates usually measuring 1. The RQ for protein metabolism should be between around 0.8, and the RQ for fats is approximately 0.7 (Livesey and Elia, 1988).

Calculation of EE and oxidation rates from the modified Weir Equation(Weir, 1949, Frayn, 1983):

- **EE (Kcal/min)** = (3.94xVO2 (l/min)) + (1.11xVCO2(l/min)) – (2.17 x nitrogen excretion)

- **Nitrogen excretion (g/min**) = 0.028 x ((urine urea (mmol/l) x urine volume (litres))/time elapsed since last passed urine (mins))

- **RQ** = VCO2(l/min) /VO2(l/min)

- **Carbohydrate oxidation (g/min)** = (4.55 x VCO2(l/min)) – (3.21xVO2(l/min)) – (2.87 x nitrogen excretion)

- **Fat oxidation (g/min)** = 1.67 x (VO2(l/min) - VCO2(l/min)) – (1.92 x nitrogen excretion)

- **Protein oxidation (g/min)** = nitrogen excretion x 6.25

Prior to each use, the calorimeter is calibrated using 'span' (20.00% Oxygen, 1.00% Carbon Dioxide) and 'zero' (0.00% Oxygen, 0.00% Carbon Dioxide) gases (BOC gases, Surrey, UK).



Figure 3.1 The Indirect Calorimeter. Volunteers were asked to lie on a bed in a semi-recumbent position and the calorimeter hood was placed over their head. Expired air was collected via the tube exiting the hood and its composition analysed by the calorimeter. With kind permission from Dr E.S. Chambers

3.3.4 Protocol

Participants attended for five study visits, with at least 2 days between each session. The initial visit was a 'sham' or placebo visit (unblinded) to allow participants to become acclimatised to the calorimeter, cannulation, and any other procedures involved in the study. This was to prevent any anxiety regarding the conduct of study visits, as anxiety could increase heart rate, respiratory rate, and activity of the sympathetic nervous system and hence markedly affect EE. During this sham visit, participants received an infusion of placebo (gelofusine (B.Braun, Crawley, UK)). Following the acclimatisation visit, volunteers subsequently underwent four further study visits, conducted in a randomised, double blinded, crossover fashion. Each volunteer received: 1) gelofusine alone (control); 2) GLP-1 alone at 0.8 pmol/kg/min; 3) glucagon alone at 50ng/kg/min; and 4) Co-infusion of GLP-1 plus glucagon at the above doses. Infusions were prepared by an independent investigator according to a pre-determined treatment code. The dose of glucagon was chosen following an initial dose finding phase which determined a dose of glucagon that reliably increased EE without causing unacceptable side effects. GLP-1 was infused at a dose used in previous studies where GLP-1 was administered to humans (De Silva et al., 2011, Verdich et al., 2001). All infusions were made up using gelofusine as the vehicle to prevent peptide adsorption to the inside of the infusion apparatus (Kraegen et al., 1975).
In order to avoid depletion of hepatic glycogen stores, participants were asked to refrain from alcohol consumption and strenuous exercise for the 24 hours preceding a study visit. They were also asked to consume a high energy snack (an 'Alpen' bar containing 3.8g fat, 20.4g carbohydrate and 1.9g protein (Weetabix, UK)) at 2200h evening prior to their study visit.

At 0700h on the morning of the study visit, participants were asked to consume two McVitie's Rich Tea biscuits (per biscuit: 1.3g fat, 5.9g carbohydrate, 0.6g protein (United Biscuits)). They arrived at the clinical research facility at 0830h.

On arrival, volunteers were asked to empty their bladder, and urine volumes were recorded. They were placed in a separate room with thermostatically controlled temperature, which was measured

at the beginning of each visit. A cannula was then sited in each arm – one for blood sampling, and one for administration of the hormone infusions. Volunteers reclined on a bed, and were allowed to watch television or listen to music through headphones. At this point, they were also asked to swallow a small temperature capsule (Equivital; Hidalgo, Swavesey, UK) to allow measurement of core body temperature during the infusions. Women of childbearing age underwent a urinary pregnancy test prior to each study visit.

At t=0, volunteers were placed under the calorimeter hood (Gas Exchange Monitor; GEMNutrition, Daresbury, UK) and measurements of EE commenced. Participants were asked to lie as still as possible in order that resting, rather than activity related EE could be measured. Calorimetry measurements were allowed to stabilise for 30 minutes, and a subsequent 15 minutes of measurement was taken as a true representation of resting EE (REE). At t=45, the hormone infusion was commenced. In order to rapidly achieve stable plasma levels of hormone, the infusion was 'ramped'. The rate of infusion was at 4x the basal rate for the first five minutes, then 2x the basal rate for the next five minutes after which it was reduced to the basal rate. The infusion then continued for a further 35 minutes. Calorimetry measurements continued during this time, and once again the last 15 minutes of measurement after plasma hormone levels had stabilised were taken as the representation of REE.

At t=90, both the calorimetry and infusion were stopped. Subjects were given a snack to compensate any potential hypoglycaemic effects from the hormone infusions. After a 25 minute period of observation to ensure participant safety, the study visit was terminated.

The two sets of 15 minute calorimetry measurements taken prior to, and at the end of the infusion were used to calculate REE, substrate oxidation rates and RQ. Vital signs for each subject (pulse and blood pressure) were taken at t=0, 45 and 90 minutes. At the end of the study visit, a sample of urine was collected for urine nitrogen measurements. This was used to assess protein oxidation rates.

Pre- and post-infusate samples of the hormone solutions were taken to ensure that there was no significant breakdown of peptide during the study which could render it inactive.

Blood samples were taken during the study at t=30, 45, 60, 75, 90 and 105 minutes. These were drawn for glucose and insulin levels, GLP-1, and glucagon. Additional blood tests were taken at 30 minutes and 90 minutes for thyroid hormones, nonesterified fatty acids (NEFAs), cortisol, and total/acyl ghrelin levels. Collection details are specified in table 3.2. Samples were immediately placed on ice, (except insulin samples which were allowed to clot first), centrifuged at 4^{o}C at 2590g 10 minutes, and separated. They were subsequently stored at -20^{o}C until analysis.

Sample	Collection media
Glucose	Fluoride Oxalate Vacutainer tube *(BD Diagnostics, Oxford UK)*
Insulin	Plain Serum Vacutainer tube containing clot activator *(BD Diagnostics, Oxford UK)*
GLP-1, Glucagon	Lithium-Heparin Vacutainer tube *(BD Diagnostics, Oxford UK)* Containing 1000 kallikrein inhibitor units (0.1ml) *(Trasylol, Bayer Scherin Pharma, Berlin, Germany)*
Ghrelin	Cryogenic Vials *(Alpha Laboratories, Eastleigh, UK)* Containing 0.8M Hydrochloric acid (20µl)
NEFAs	Lithium-Heparin Vacutainer tube *(BD Diagnostics, Oxford UK)*

Table 3.2 Collection details of samples obtained during study visit

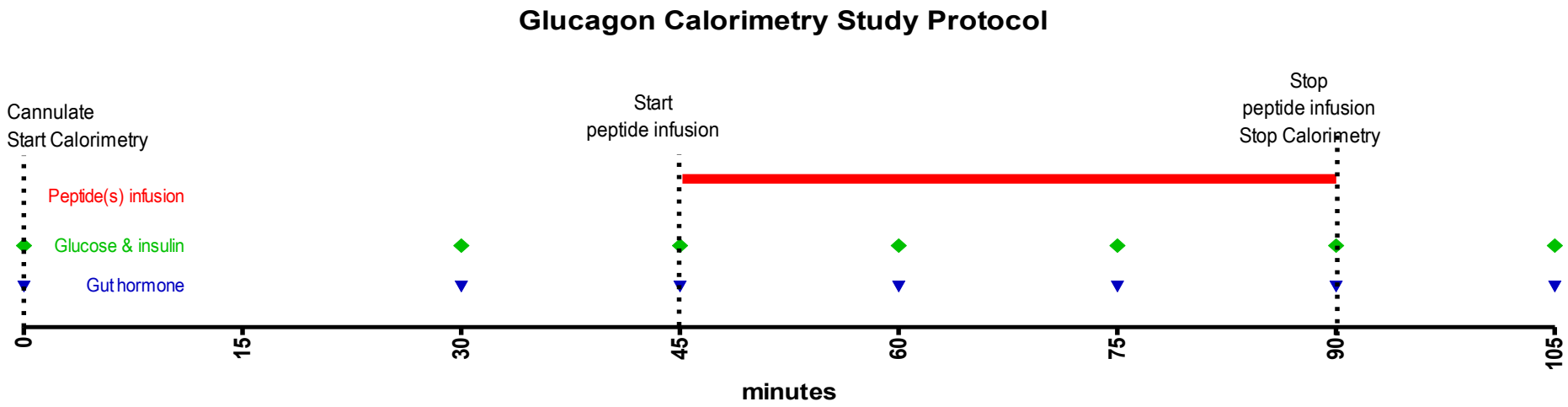


Figure 3.2 The acute effect of co-infusion of glucagon and GLP-1 on EE and blood glucose: Study Protocol. Participants arrived at t=0, when they were cannulated and calorimetry measurements commenced. The peptide hormone infusion was commenced at t=45 and continued until t=90. Blood samples were drawn as indicated by the blue (gut hormones) and green (glucose and insulin) markers

3.3.5 Assays

Glucagon

Glucagon immunoreactivity was measured using an established, specific and sensitive in-house radio-immunoassay with CV <10% (Ghatei et al., 1983). All samples were assayed in duplicate, in a single radioimmunoassay in order to eliminate inter-assay variation.

Glucagon antibody was raised in rabbits against the c-terminal of porcine glucagon coupled to bovine serum albumin (BSA). The antibody raised (RCS5) showed no cross-reaction with other gut hormones, demonstrating a high specificity for pancreatic glucagon.

^{125}I-labelled glucagon was prepared by Professor M. Ghatei by the iodogen method (Wood et al., 1981) and was purified by high performance liquid chromatography (HPLC).

The assay was performed in a total volume of 700µl, using veronal buffer (10.3g sodium barbitone plus 0.3g sodium azide in 1L of distilled water at pH 8 with 0.3% BSA), 50µl of antibody RCS5 (as above), and 50µl of label. The assay was then incubated at 4ºC for 96 hours before separation by the charcoal adsorption method. 4 mg of charcoal in 0.06M phosphate buffer plus dextran and gelatine was added to each sample. All samples were then centrifuged at 1317g, 4^{o}C for 20 minutes to create a pellet. The pellet consists of charcoal plus any free labelled hormone in the tube. The supernatant contains labelled hormone which has bound to antibody. Free and bound fractions were separated and both fractions counted for 180 seconds (γ-counter model NE1600, Thermo Electron Corporation).

The assay was able to measure changes between adjacent samples of 10 picograms per millilitre (pg/ml), with 95% confidence. Quality control samples (QCs) were run as standard.

GLP-1

Similarly to glucagon, the GLP-1 assay is an established, highly specific and sensitive in house RIA (Kreymann et al., 1987), CV <3.1%, developed and maintained by Prof M Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College). Once again, all samples were processed in duplicate within a single RIA.

GLP-1 antibodies were produced from inoculation of GLP-1 coupled to BSA into rabbits. They were raised against the N terminal of GLP-1, and the antibody cross reacted with all amidated forms of GLP-1 (such as GLP-1 1-36, 7-36 and 9-36). The antibodies do not react with extended molecules of GLP-1 (e.g. GLP-1 7-37) or any other gut hormones. The assay measures total GLP-1 concentrations.

^{125}I-GLP-1 was again produced by Professor Ghatei using the Iodogen method (Wood et al., 1981)

The assay was performed in duplicate in a total volume of 700µl, using veronal buffer (10.3g sodium barbitone plus 0.3g sodium azide in 1L of distilled water at pH 8 with 0.3% BSA plus 0.02% tween), 50µl of antibody, and 50µl of label. The assay was then incubated at 4^{o}C for 96 hours before separation by the charcoal adsorption method (as above). Quality control samples (QCs) were run as standard.

Ghrelin

Ghrelin was measured by sandwich enzyme-linked immunosorbent assay (ELISA), both for detection of total and acyl (active) Ghrelin levels (Millipore, Watford, UK). The principle is that ghrelin molecules are bound in the assay by anti-human ghrelin immunoglobulin G (IgG) ('capture' antibody'). The resulting antibody-ghrelin complex is bound to the wells of a 96 well microtitre plate via a known concentration of an 'anchoring antibody'. A further biotinylated antibody ('detection antibody') binds to ghrelin, which conjugates with horseradish peroxidase (HRP), an enzyme whose activity can be measured in the presence of the substrate 3,3'5,5'-tetra-methylbenzidine. All unbound materials are washed away, and following addition of the substrate, the activity of HRP (and therefore the amount of captured ghrelin) is measured spectrophotometrically at an absorbency of 450 nanometers (nm), corrected from absorbency at 590nm. The absorbency is directly proportional to the amount of captured ghrelin and a standard curve can therefore be constructed using reference values with known ghrelin concentrations.

The manufacturer's protocol was followed for both assays:

All reagents were used at room temperature. Each well was initially filled with 300µl of diluted wash buffer. Wash buffer was decanted and removed from the plate three times. At this point, assay buffer

was added to the wells. Lyophilised ghrelin standards at 20µl were reconstituted and added in duplicate in ascending concentrations to create a standard curve. Quality control samples (QC's) were also then added in duplicate to the appropriate wells. Plasma samples (20µl) were then added to the remaining wells, again in duplicate. 50µl of a 1:1 mixture of the capture and detection antibodies was then added to each well. The plate was incubated at room temperature for two hours on a plate shaker at 400 revolutions per minute (rpm). The plate was then washed a further three times to remove any unbound materials. 100µl of enzyme solution (HRP) was subsequently added to each well and incubated for a further 30 minutes at room temperature on the plate shaker.

Following this, the plate was washed six times, and 100µl of substrate (3,3'5,5'-tetra-methylbenzidine) added for a further 5-20 minutes. Incubation time at this step was determined by the development of blue colour of increasing intensity in the wells of the standard curve. Once the blue colour developed, 100µl of a stop solution (0.3 M HCl) was added to terminate the peroxidase reaction at which point the solutions in the wells turned from blue to yellow. Absorbance was read on a plate reader at 5 minutes (Multiskan RC microplate reader, Labsystems, Vienna, VA, USA) at both 450nm and 590nm, and the former subtracted from the latter. A standard curve was then constructed to enable calculation of ghrelin concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

NEFAs

Samples for NEFAs were sent to the Department of Chemical Pathology at Great Ormond Street Hospital NHS Trust. They were assayed using a Wako NEFA assay kit (Alpha laboratories, UK) on an ILab 650 analyser (Instrumentation Laboratory, Massachusetts, US).

Other Samples

All other samples were analysed by the Department of Chemical Pathology, Imperial College Healthcare NHS Trust. Commercially available assays were used to analyse plasma glucose [Abbott Architect, Coefficient of Variation (CV) <5%], insulin (Abbott Axsym platform using microparticle enzyme-immunoassay, CV <10%), thyroid stimulating hormone, free T3 and free T4 (Abbott

Diagnostics, Architect platform), cortisol (Abbott Architect) and urine urea (Abbott Architect enzyme kinetic assay with spectrophotometric detection).

3.3.6 Statistics

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). A two-way repeated measures ANOVA with Bonferroni post-hoc test was used to assess differences in insulin, glucose, NEFA, thyroid hormones, cortisol and ghrelin levels.

Energy expenditure and substrate oxidation change from baseline was assessed using a one-way ANOVA with Tukey's post hoc test.

Blood pressure and pulse were assessed as change from baseline and analysed by a one-way ANOVA with Tukey's post-hoc test.

All results are described as mean ±SEM, and statistical significance taken at $p<0.05$ unless otherwise stated.

3.4 Results

3.4.1 The acute effect of co-infusion of glucagon and GLP-1 on energy expenditure

Resting EE was measured both pre-infusion and 30 minutes into the infusion once a steady state plasma concentration of peptide had been achieved. REE was measured for infusion of each of placebo (gelofusine), GLP-1, glucagon, and GLP-1 plus glucagon (Figure 3.3).

Infusion of glucagon alone significantly increased resting EE in Kcal/day compared to both placebo and GLP-1. The change from baseline in REE following infusion of glucagon was 141 ±18.4kcal/day vs

55 ±21.9kcal/day $p<0.001$ (placebo) and -11 ±22.4kcal/day $p<0.001$ (GLP-1). Infusion of glucagon plus GLP-1 also significantly increased REE compared to both placebo and GLP-1 alone. The change from baseline in REE following the combined infusion of glucagon plus GLP-1 was 135 ± 35.1kcal/day vs 55 ±21.9kcal/day $p<0.001$ (placebo) and -11 ± 22.4kcal/day $p<0.001$ (GLP-1). There was no significant difference in change in REE from baseline between glucagon alone vs glucagon plus GLP-1. Values are expressed in kcal/day, extrapolated from measured values. N=10

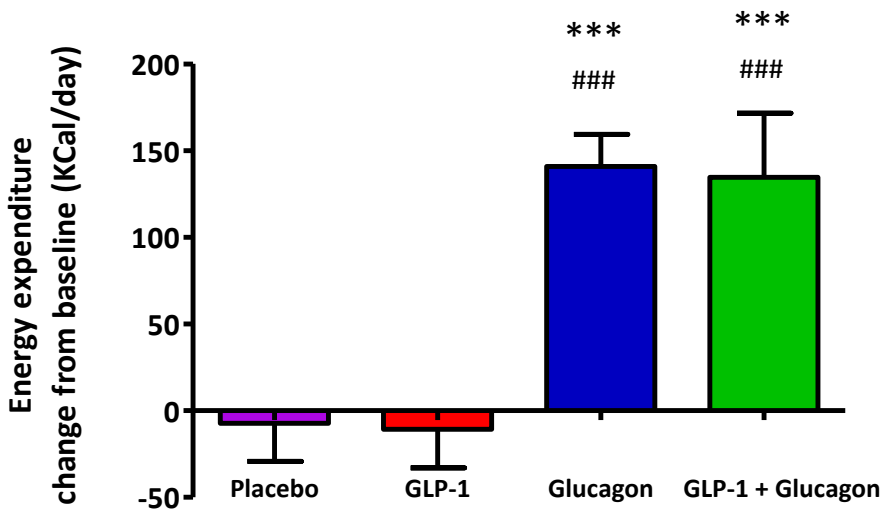


Figure 3.3 Energy expenditure change from baseline kcal/day. Participants were administered infusion of placebo (gelofusine), GLP-1 at 0.8pmol/kg/min, glucagon at 50ng/kg/min and GLP-1 plus glucagon in combination at the above doses. Measurement of REE was performed prior to the start of the infusion, and 30 minutes into the infusion once plasma peptide levels had attained steady state. Statistical analysis was performed using GraphPad Prism, using a one-way ANOVA with Tukey's post hoc test. N=10. ***p<0.001 vs placebo ### p<0.001 vs GLP-1

3.4.2 The acute effect of co-infusion of glucagon and GLP-1 on substrate oxidation

Substrate oxidation rates during the infusions for fat, carbohydrate and protein can be calculated using the equations in section 3.3.3. For these calculations we require the VO_2 and VCO_2, generated from the indirect calorimeter measurements. Also required are the urine nitrogen excretion rates which are derived from urine volume and urine urea content. Measurements for fat and carbohydrate (CHO) oxidation were taken both before and during the peptide infusion. Protein oxidation is measured for the whole experimental time and cannot therefore be compared before and after peptide infusion. Carbohydrate oxidation was significantly increased following infusion of glucagon + GLP-1 compared to placebo and GLP-1 alone (Figure 3.4A). The mean change from baseline in CHO oxidation following infusion of glucagon + GLP-1 was 0.071 ± 0.016 g/min vs -0.0053 ± 0.0089 g/min, $p<0.01$ (placebo) and 0.010 ± 0.005 g/min, $p<0.001$ (GLP-1). N=10. Fat oxidation was significantly decreased following infusion of glucagon + GLP-1 compared to placebo and glucagon alone (figure 3.4B). The mean change from baseline in fat oxidation following infusion of glucagon + GLP-1 was -0.018 ± 0.005 g/min vs 0.0016 ± 0.004 g/min, $p<0.05$ (placebo) and -0.0002 ± 0.005, $p<0.05$ (glucagon). N=10. There was no difference in protein oxidation detected across any of the groups following hormone infusion (figure 3.4C).

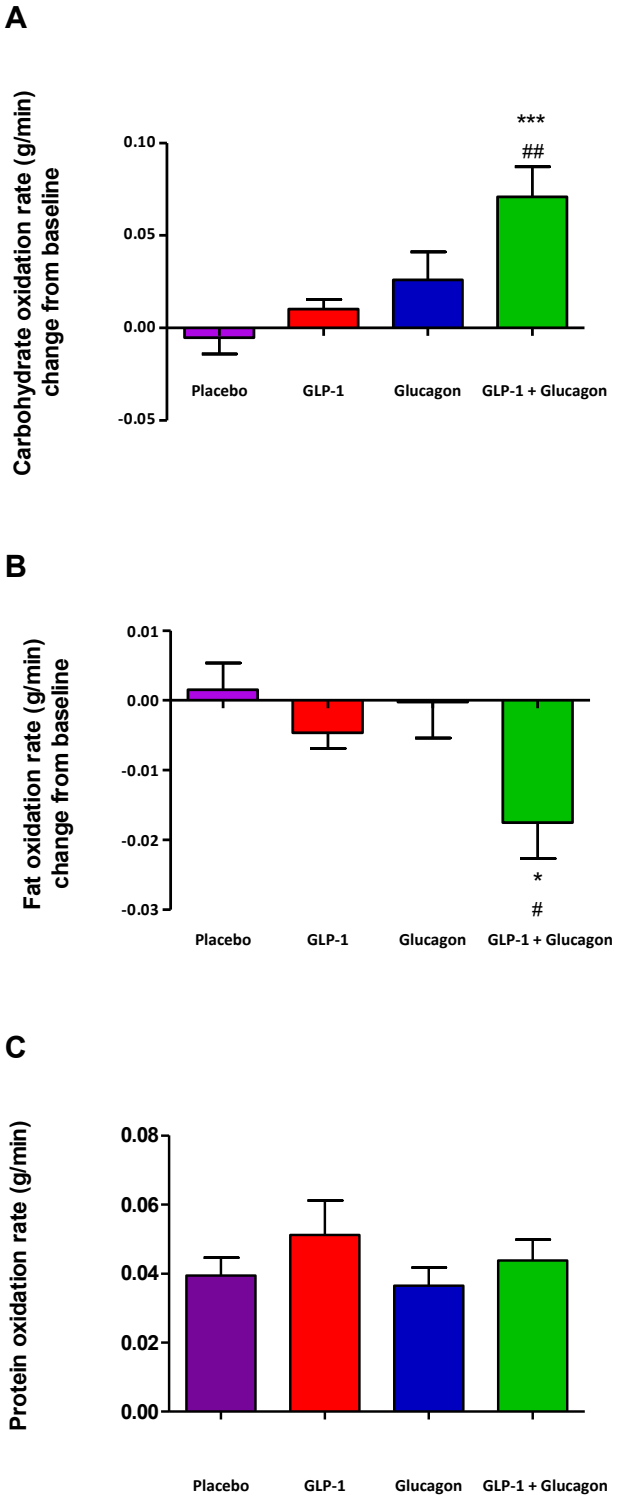


Figure 3.4 The acute effect of co-infusion of glucagon and GLP-1 on substrate oxidation rates. Participants were administered infusion of placebo (gelofusine), GLP-1 at 0.8pmol/kg/min, glucagon at 50ng/kg/min and GLP-1 plus glucagon in combination at the above doses. Fat and carbohydrate oxidation rates were calculated from VO_2 and VCO_2 measurements achieved during indirect calorimetry, and protein oxidation was calculated using urine urea and urine volume measurements. Data was analysed using GraphPad Prism, by a one-way ANOVA with Tukey's post hoc test. N=10. ***p<0.001 placebo vs glucagon + GLP-1. ## p<0.01 GLP-1 vs glucagon +GLP-1, *p<0.05 placebo vs glucagon + GLP-1, #p<0.05 glucagon vs glucagon + GLP-1.

3.4.3 The acute effect of co-infusion of glucagon and GLP-1 on plasma glucagon and GLP-1 concentrations

Participants received four infusions in a randomised double blinded crossover study design. The infusions were 1) gelofusine alone (control); 2) GLP-1 alone at 0.8 pmol/kg/min; 3) glucagon alone at 50ng/kg/min; and 4) Co-infusion of GLP-1 plus glucagon at the above doses.

Baseline GLP-1 concentration for the placebo group and glucagon group were 16.4 ± 1.9pmol/l (range 7.8 – 28.4 pmol/l) and 22.7 ± 3.6pmol/l (range 2.5 - 53.8pmol/l) respectively. Levels did not rise over the course of the infusion (figure 3.5A). Baseline GLP-1 concentrations in the GLP-1 and GLP-1 + glucagon groups were 15.8 ± 2.8 pmol/l (range 5.1 - 46.2pmol/l) and 16.7 ± 1.8 pmol/l (range 9-33.4 pmol/l) respectively. In contrast to the placebo and glucagon groups, plasma concentrations rose to peak levels at t=60 minutes with a peak concentration of 103.1 ± 26 pmol/l (range 31.3 – 239.1 pmol/l) in the GLP-1 group, and 89.7 ± 17.1 pmol/l (range 59.7 – 217.1 pmol/l) in the glucagon + GLP-1 group (figure 3.5A). Following the termination of the infusion at t=90 minutes, plasma hormone levels fell back to baseline within 15 minutes reflecting the relatively short half-lives of these peptides.

Baseline glucagon concentrations for the placebo and GLP-1 groups were 8.1 ± 0.96 pmol/l (range 2.5-15.3 pmol/l) and 11.0 - 1.3 pmol/l (range 2.8-25.3) respectively. Levels remained at baseline during the course of the infusion (figure 3.5B). Baseline glucagon concentrations for the glucagon and GLP-1 + glucagon groups were 9.2 ± 1.2 pmol/l (range 0.76-18.6 pmol/l) and 9.0 ± 2.1 pmol/l (range 2-31.6 pmol/l) respectively. Levels rose during the infusion to a peak at t=60 minutes of 260.9 ± 43.7 pmol/l (range 119.6 - 483.5pmol/l) in the glucagon group and 239.3 ± 42.1 pmol/l (range 91.7 - 470.4 pmol/l) in the GLP-1 + glucagon group (figure 3.5B). Peptide concentrations once again returned to baseline after termination of the infusions at t=90 minutes.

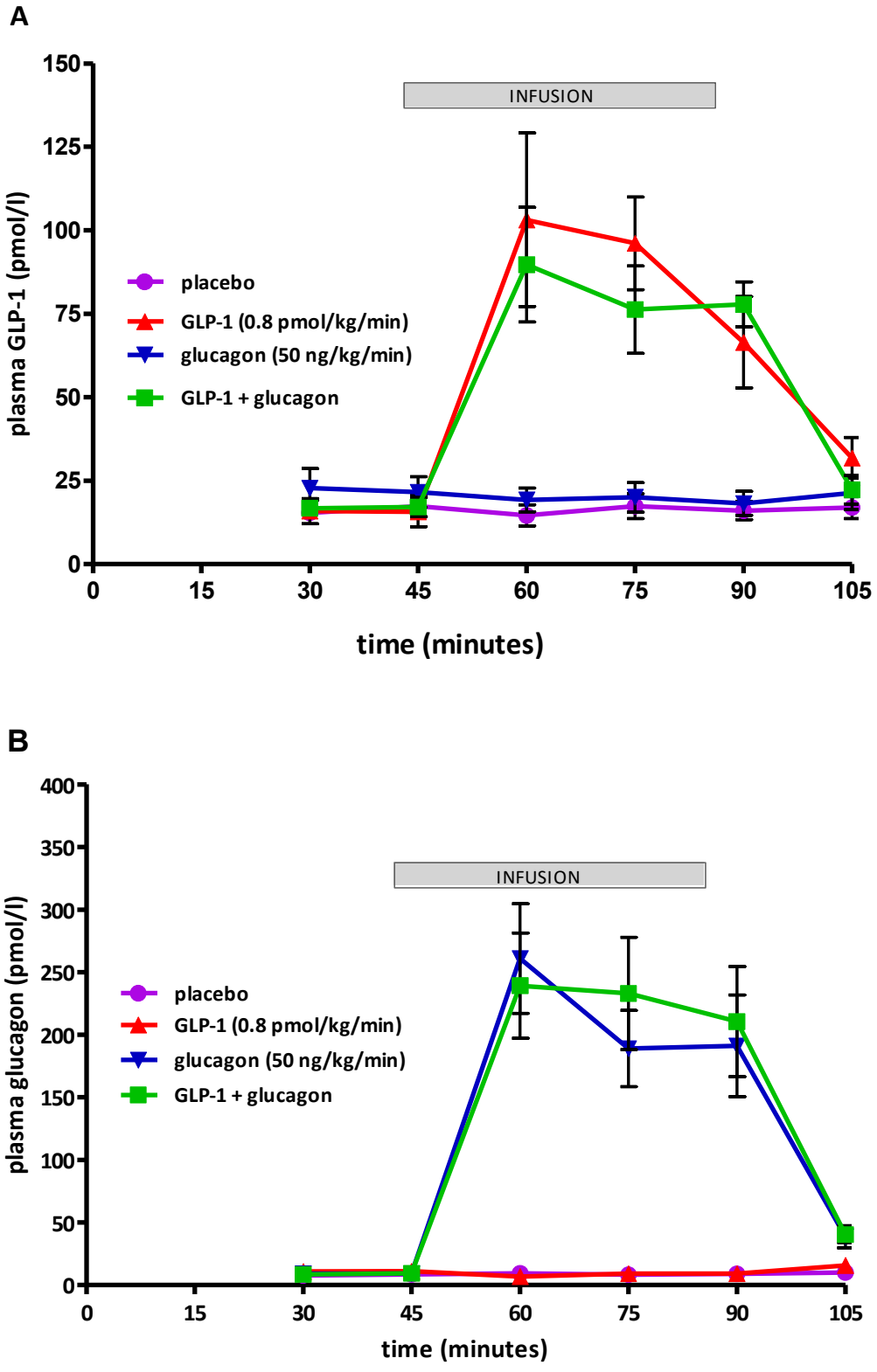


Figure 3.5 GLP-1 (A) and glucagon (B) plasma hormone concentrations following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. The infusion commenced at t=45 minute, and continued for 45 minutes (grey bar). Plasma samples were drawn at t=30, 45, 60, 75, 90 and 105 minutes for analysis of hormone profile.

3.4.4 The acute effect of co-infusion of glucagon and GLP-1 on plasma glucose and insulin concentrations

Insulin and glucose plasma levels were assessed during the course of each infusion. Blood was drawn at t=30, 45,60,75,90 and 120 minutes for analysis. The effect of each of the following infusions: 1) gelofusine alone; 2) GLP-1 alone at 0.8 pmol/kg/min; 3) glucagon alone at 50ng/kg/min; and 4) Co-infusion of GLP-1 plus glucagon at the above doses on plasma insulin and glucose was then analysed.

Baseline insulin concentration across all infusion groups and participants was 8.4 ± 0.83mU/l, with a range of 2.2-45mU/l. Compared to placebo, the combination of glucagon + GLP-1 caused a significant increase in serum insulin (***p<0.001). Compared to GLP-1, both glucagon alone (*p<0.05), and the combination of glucagon +GLP-1(### p<0.001) caused a significant rise in serum insulin. The combination infusion also caused a significant rise in insulin vs glucagon alone ($ p<0.05, $$ p<0.01, $$$ p<0.001) (Figure 3.6A).

Baseline glucose across all infusion groups and participants was 6.8 ± 0.06 mmol/l (range 3.9-6.6 mmol/l). GLP-1, glucagon and the combination of GLP-1/glucagon all caused a significant rise in plasma glucose concentration compared to placebo (*p<0.05, **p<0.01, ***p<0.001, # p<0.05). Glucagon and the combination of GLP-1/glucagon caused a rise in glucose compared to GLP-1 (### p<0.001, $$$ p<0.001), and glucagon alone caused a significant rise in glucose compared to the combination infusion (££ p<0.01, £££ p<0.001) (Figure 3.6B). The rise in plasma glucose levels seen with glucagon infusion is significantly ameliorated by the addition of GLP-1, as shown by the discrepancy between the glucagon and combination groups (Figure 3.6 A&B). This is likely due to the substantial increase in insulin mediated by the combination infusion compared to glucagon alone. Glucose levels in both the glucagon and the combination groups start to drop after the peak at t=75 minutes (8.3 ± 0.4 mmol/l) and (6.9 ± 0.7 mmol/l) respectively, with clearance of glucose seemingly occurring at a similar rate in both groups although the glucagon group did not achieve baseline glucose levels by the end of the study.

For both insulin and glucose, data was analysed by a two-way repeated measures ANOVA with Bonferroni post-hoc test. Further to this analysis, area under the curve (AUC) was calculated using the trapezoidal method. This allowed comparison of the cumulative measurement of insulin (Figure 3.7A) and glucose (Figure 3.7B) release with each of the four infusions. The combination infusion resulted in a significant rise in serum insulin compared to both placebo and GLP-1 ($p<0.001$, Figure 3.7A). Glucagon alone increased plasma glucose compared to both placebo and GLP-1 ($p<0.001$), and the combination infusion also increased plasma glucose compared to GLP-1 alone ($p<0.01$) (Figure 3.7B). Data was analysed by repeated measures one-way ANOVA with Tukey's post hoc test.

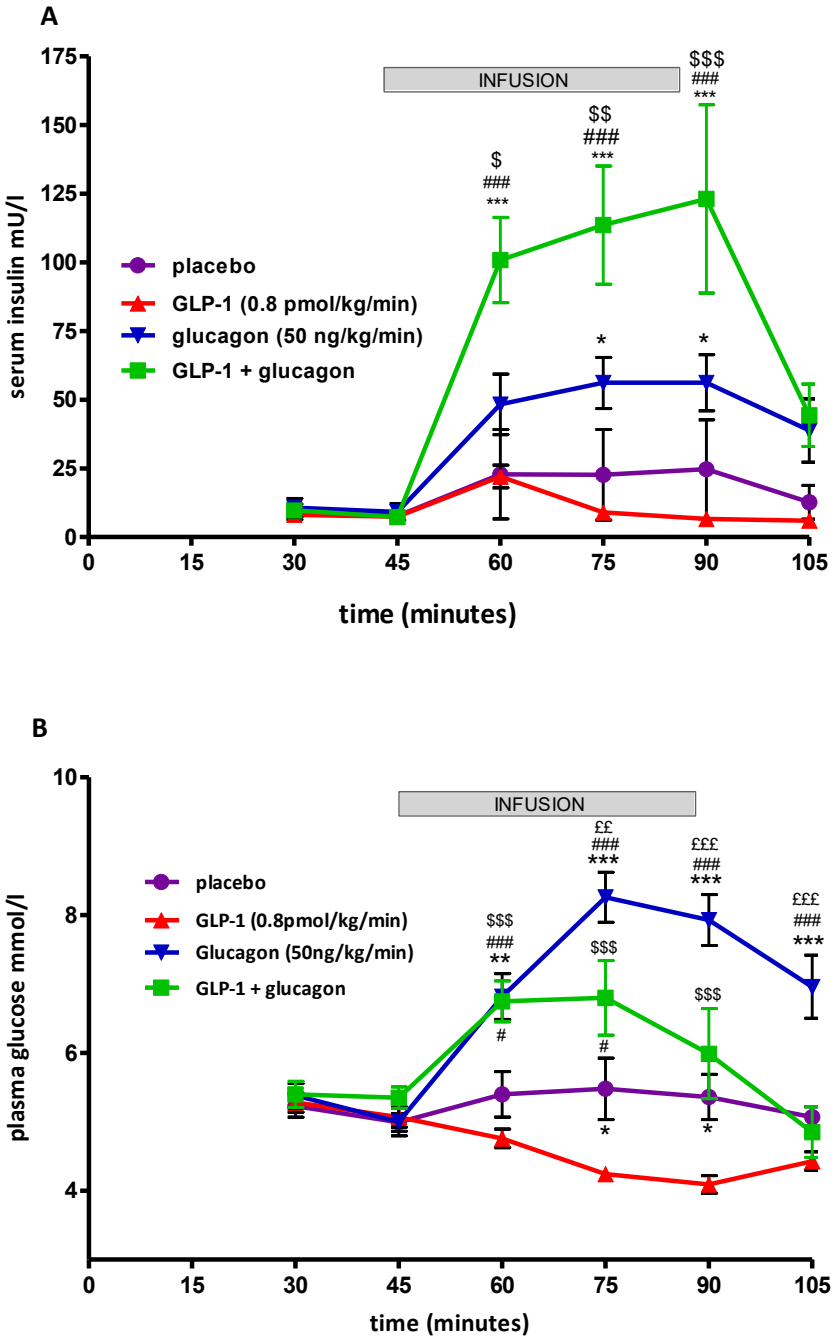


Figure 3.6 A & B The acute effect of co-infusion of glucagon and GLP-1 on plasma insulin (A) and glucose (B) concentrations following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. The infusion commenced at t=45 minutes, and continued for 45 minutes (grey bar). Plasma samples were drawn at t=30, 45,60,75,90 and 105 minutes for analysis. Compared to placebo, the combination of glucagon + GLP-1 caused a significant increase in serum insulin (*p<0.001). Compared to GLP-1, both glucagon alone (*p<0.05), and the combination of glucagon +GLP-1(### p<0.001) caused a significant rise in serum insulin. The combination infusion also caused a significant rise in insulin vs glucagon alone ($ p<0.05, $$ p<0.01, $$$ p<0.001). GLP-1, glucagon and the combination of GLP-1/glucagon all caused a significant rise in plasma glucose concentration compared to placebo (*p<0.05, **p<0.01, ***p<0.001, # p<0.05). Glucagon and the combination of GLP-1/glucagon caused a rise in glucose compared to GLP-1 (### p<0.001, $$$ p<0.001), and glucagon alone caused a rise in glucose compared to the combination infusion (££ p<0.01, £££ p<0.001). n=10. Data analysis was by a two-way repeated measures ANOVA with Bonferroni post-hoc test.**

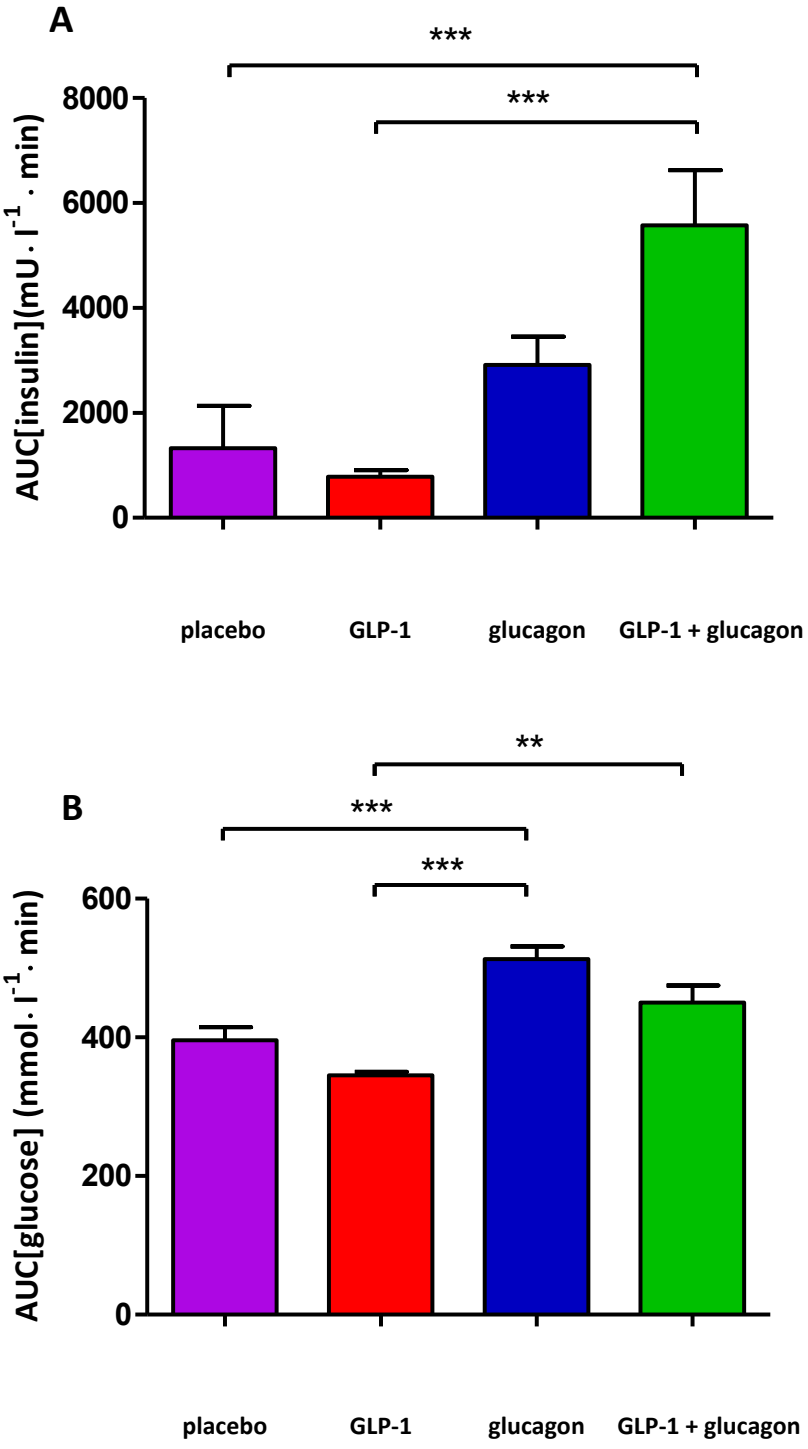


Figure 3.7 A & B Area under the curve for insulin (A) and glucose (B) following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. The combination infusion resulted in a significant rise in serum insulin compared to both placebo and GLP-1 ($p<0.001$). Glucagon alone increased plasma glucose compared to both placebo and GLP-1 ($p<0.001$), and the combination infusion also increased plasma glucose compared to GLP-1 alone ($p<0.01$). N=10. Analysis by one way ANOVA with Tukey's post-hoc test.

3.4.5 The acute effect of co-infusion of glucagon and GLP-1 on plasma levels of total and acyl ghrelin, thyroid hormones, non-esterified fatty acids, and cortisol

Ghrelin is an orexigenic hormone which increases food intake (see section 1.2.4). It has previously been shown that GLP-1 which is secreted post-prandially and reduces appetite can also suppress ghrelin levels, possibly through insulinotropic actions (Hagemann et al., 2007). Similarly, in at least one study, glucagon has also been shown to reduce ghrelin levels (Arafat et al., 2005). Samples were taken for ghrelin measurement prior to the start, and at the end of the infusions. Total and acyl ghrelin were both significantly reduced by infusion of the combination of GLP-1 + glucagon (*$p<0.05$, **$p<0.01$) (Figure 3.8 A & B). Total ghrelin reduced from a mean of 600.5 ± 119.8 pg/ml to a mean of 449.9 ± 75.0 pg/ml. Acyl ghrelin was reduced from a mean of 351.8 ± 122.3 pg/ml to a mean of 174.3 ± 54.4 pg/ml. However, in contrast to the studies above, neither hormone infused alone caused a significant reduction in circulating ghrelin levels.

It is well documented that glucagon stimulates ACTH and cortisol release – indeed the glucagon stimulation test for ACTH reserve is well established in clinical practice (Leong et al., 2001, Arvat et al., 2000). However, most data is for intramuscularly (IM) administered glucagon and intravenous (IV) administration may reveal alternative results. The response of cortisol has not previously been assessed following intravenous co-administration of glucagon and GLP-1. Samples were taken for cortisol measurement prior to the start, and at the end of the infusions. There was no significant difference in plasma cortisol levels post-infusion compared to baseline.

Glucagon is traditionally thought to increase lipolysis (Richter et al., 1989), but more recent studies showed no effect of either glucagon or GLP-1 on lipolysis when infused locally in adipose tissue or skeletal muscle(Bertin et al., 2001). Here, NEFA levels were determined as a measure of lipolysis. Both glucagon alone (0.46 ± 0.07 to 0.18 ± 0.03 mmol/l) and the combined infusion of glucagon and GLP-1 (0.4 ± 0.06 to 0.16 ± 0.03 mmol/l) caused a significant reduction in circulating NEFA levels (***$P<0.001$). When substrate oxidation rates were calculated there was no increase in the rate of

fat oxidation (indeed the opposite was observed) suggesting a reduction in lipolysis and a consequent fall in NEFAs (figure 3.4B).

Thyroid hormones are known to play an intrinsic role in energy homeostasis (see section 1.4.1.3), and should be considered in the context of any significant changes in EE as a result of the hormone infusions. Infusion of glucagon alone caused a reduction in TSH from 1.24 ± 0.23 to 1.05 ± 0.20 mU/l (**$p<0.01$) and FT3 from 4.67 ± 0.1 to 4.37 ± 0.14 pmol/l (*$P<0.05$) but no other infusion caused any significant changes in thyroid hormone levels (Figure 3.10).

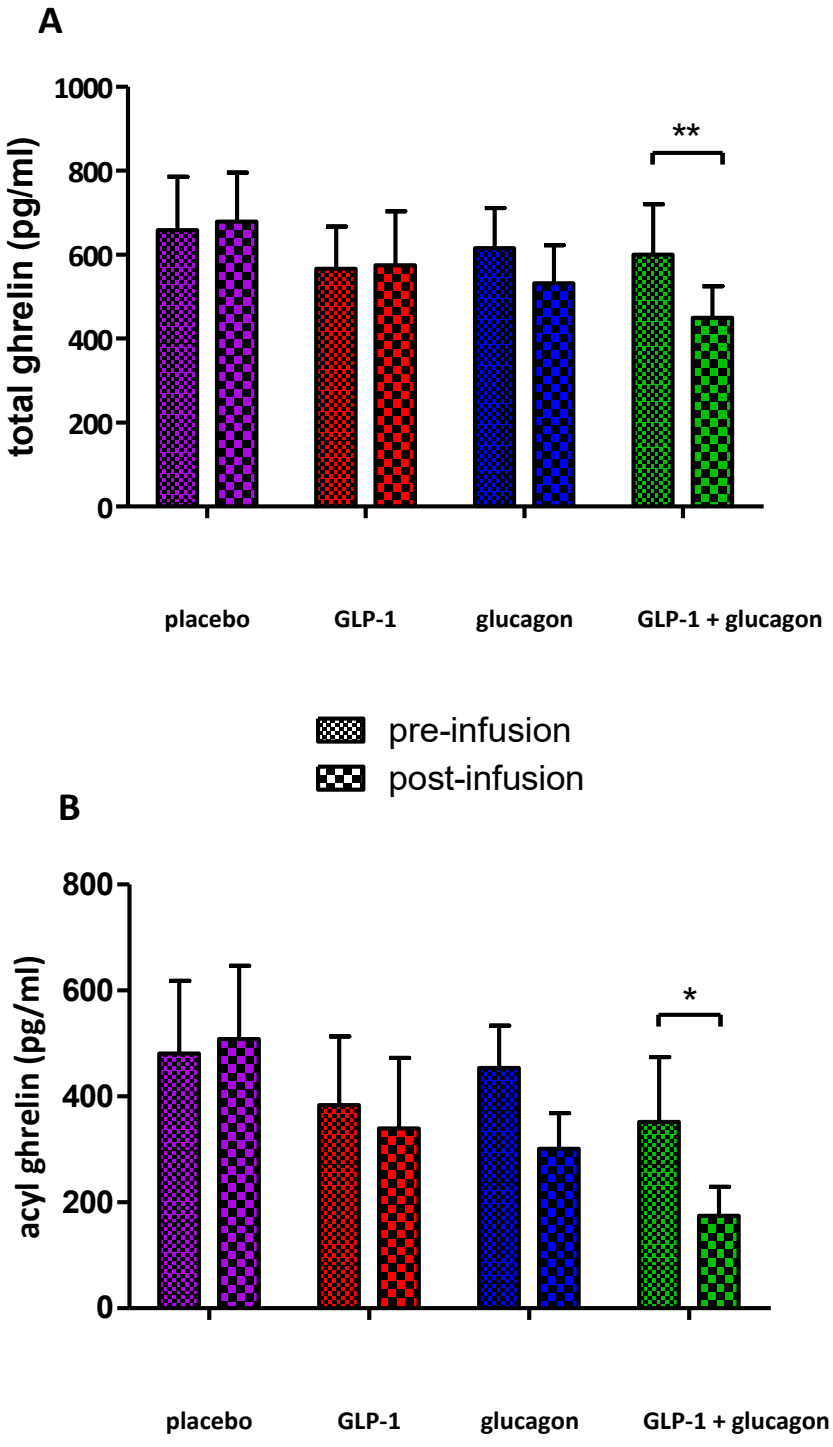


Figure 3.8 A & B Total and acyl ghrelin levels prior to and following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. The infusion of a combination of GLP-1 and glucagon significantly reduces both total and acyl ghrelin levels (*p<0.05, **p<0.01 respectively). N=10. Analysis was by two-way repeated measures ANOVA with Bonferroni post-hoc test.

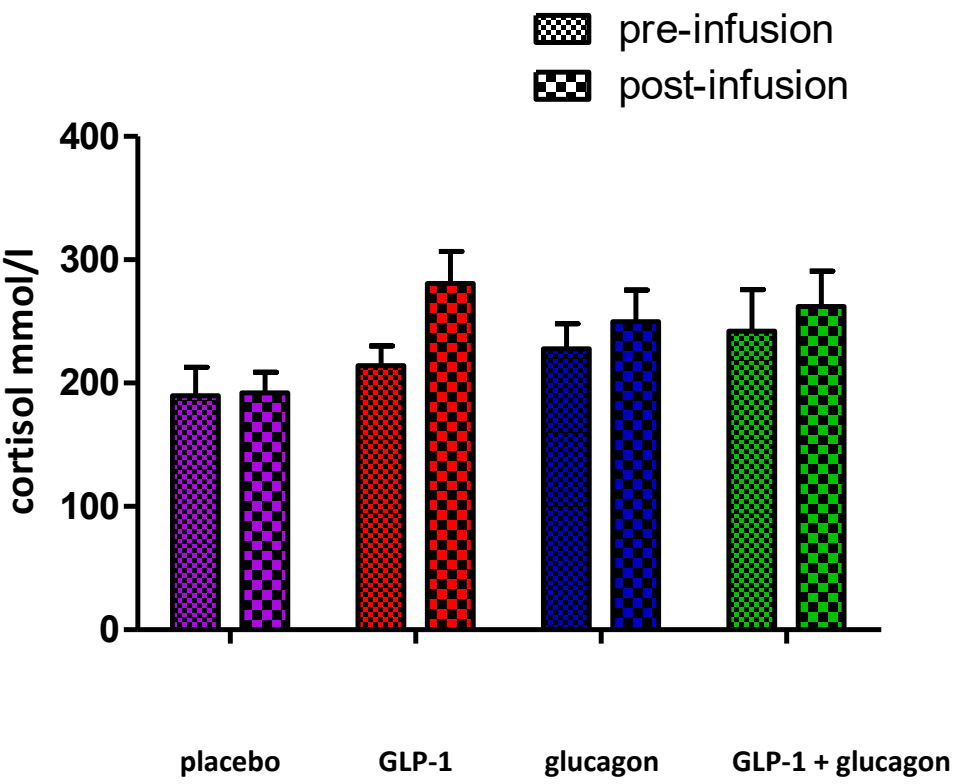


Figure 3.9 Cortisol level prior to and following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Data was analysed by a two-way repeated measures ANOVA with Bonferroni post-hoc test. N=10. There was no significant difference in plasma cortisol in any of the groups when post infusion levels were compared to baseline.

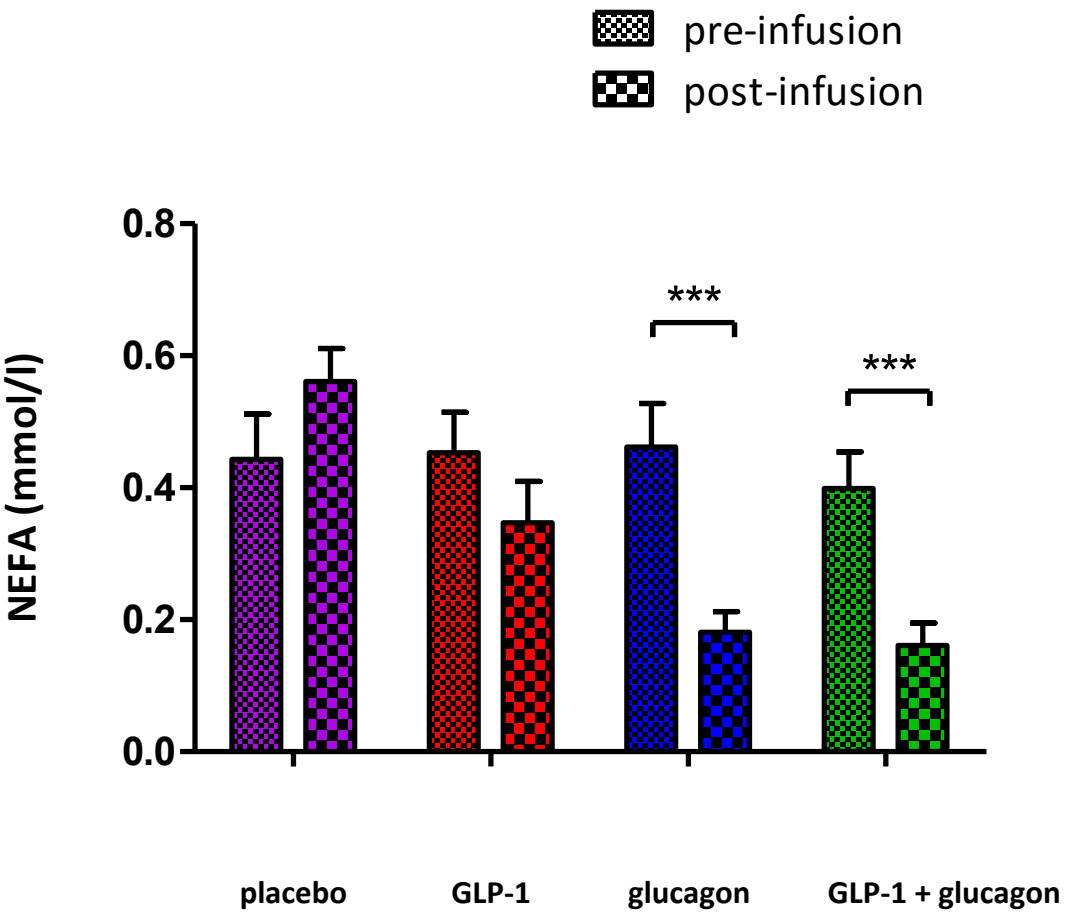


Figure 3.10 NEFA concentrations prior to and following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Data was analysed by a two-way repeated measures ANOVA with Bonferroni post-hoc test. N=10. Infusion of glucagon alone (*p<0.001) and the combination of GLP-1 + glucagon (***p<0.001) both caused a significant reduction in circulating levels of plasma NEFAs.**

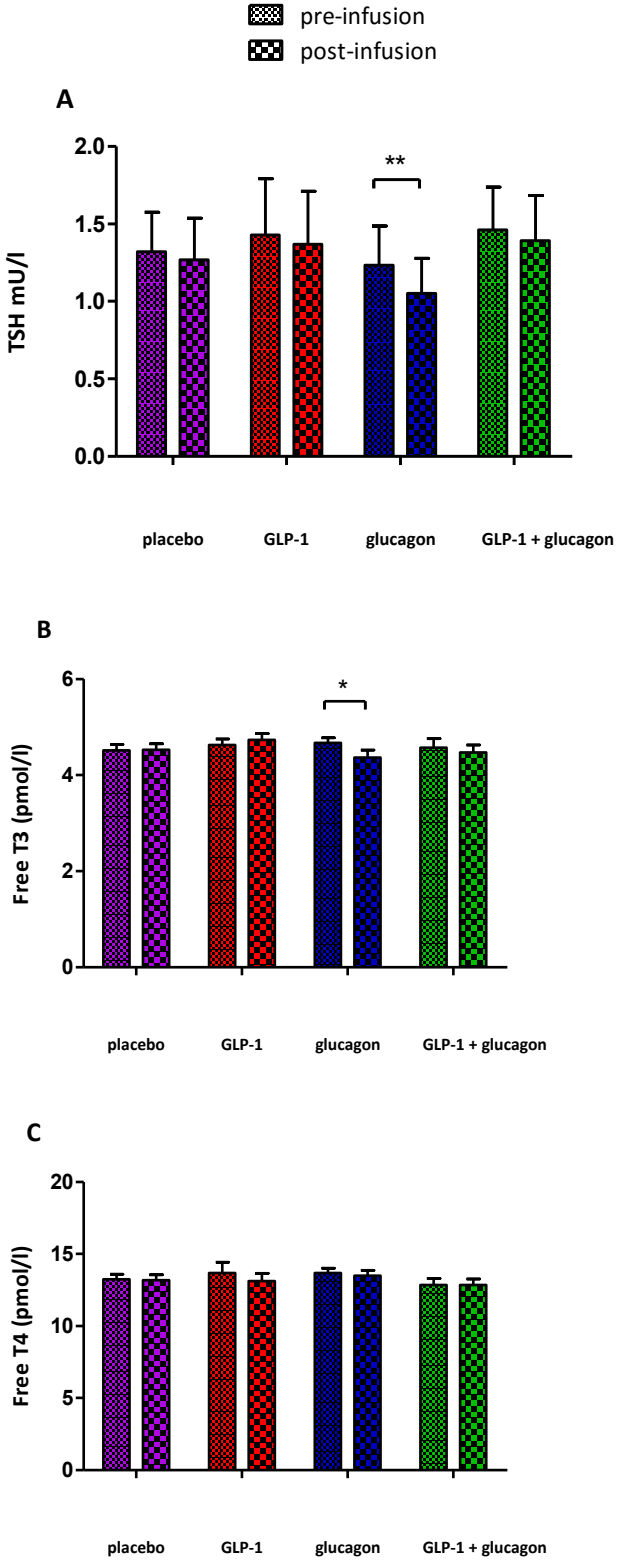


Figure 3.11 TSH, Free T4 and Free T3 plasma concentrations prior to and following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Data was analysed by a two-way repeated measures ANOVA with Bonferroni post-hoc test. N=10. Infusion of glucagon alone caused a reduction in TSH (p<0.01) and FT3 (*P<0.05) but no other infusion caused any significant changes in thyroid hormone levels.**

3.4.6 The acute effect of co-infusion of GLP-1 and glucagon on cardiovascular parameters and core body temperature

Pulse, blood pressure, and core body temperature were measured during the course of the study prior to the start of the infusion, and at the end of the infusion. The change in each of these parameters due to the infusion was calculated. None of the infusions caused any statistically significant difference in any of pulse, systolic blood pressure or diastolic blood pressure during the course of the study (Figure 3.12). However, the small increase in systolic BP in the order of 6-8mmHg seen in the groups receiving glucagon and the combination of glucagon + GLP-1 should be taken into consideration, as this may be of clinical significance. There was no effect of any of the infusions on core body temperature (data not shown).

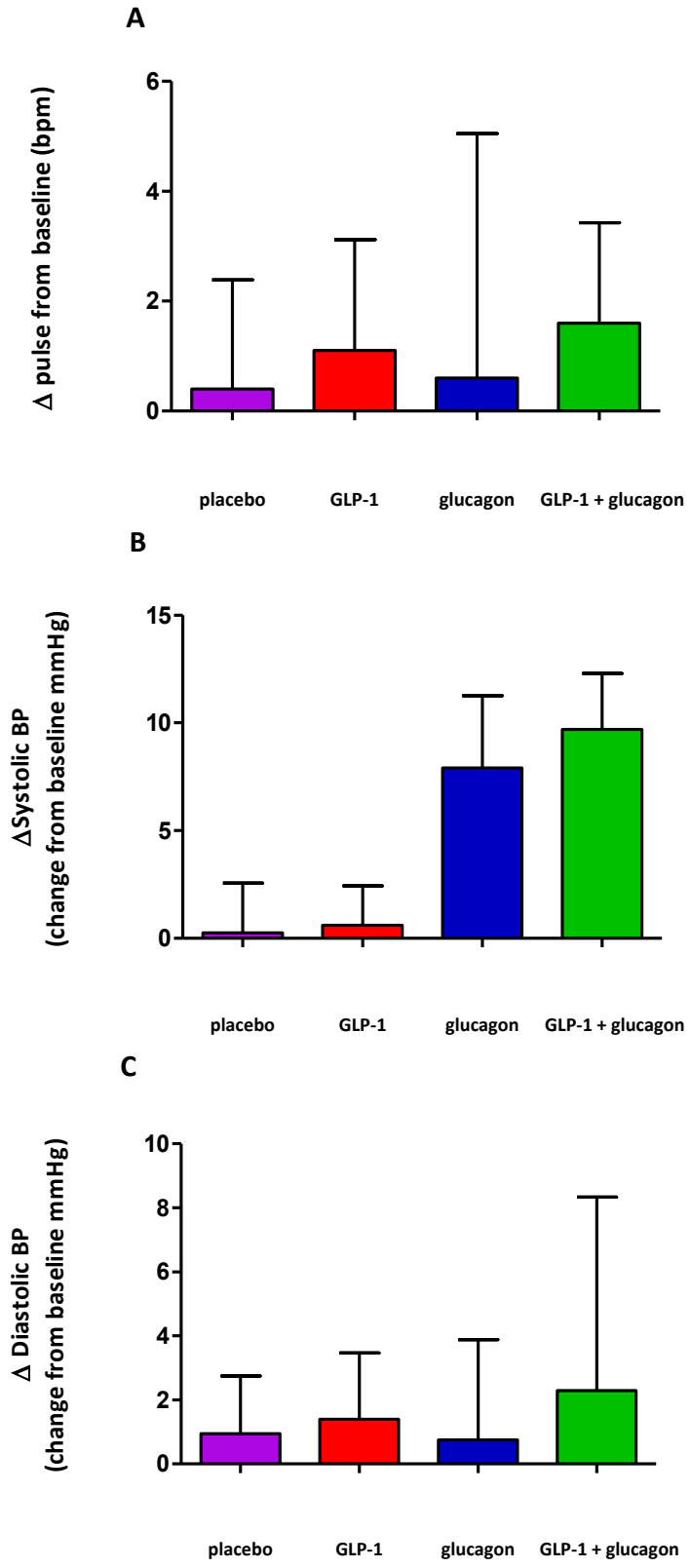


Figure 3.12 Change from baseline for pulse and blood pressure measurement following infusion of each of placebo (gelofusine (B. Braun, Crawley, UK)), GLP-1 alone at 0.8pmol/kg/min, glucagon alone at 50ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Data analysis was by a one-way ANOVA with Tukey's post hoc test. N=10. There was no significant change from baseline in cardiovascular parameters following any of the infusions.

3.5 Discussion

In this study, I have explored the hypothesis that co-infusion of glucagon and GLP-1 will cause an increase in energy expenditure via the action of glucagon, but any undesirable effects on glycaemic control conferred by the actions of glucagon will be ameliorated by the addition of GLP-1.

At a dose of 50ng/kg/minute of glucagon both alone and in combination with GLP-1, I have shown a highly significant rise in energy expenditure of around 150kcal per day compared to placebo, and to GLP-1 alone. Therefore, the rise in EE is attributable to glucagon, without any additional increase in EE due to GLP-1 as has been suggested in previous studies (Pannacciulli et al., 2006). Importantly, GLP-1 does not attenuate the effect of glucagon on EE. The mechanism of glucagon's effect on energy expenditure remains unclear. The rise in EE may be mediated via an increase in thermogenesis in BAT, as glucagon is known to play a role in the process of adaptive thermogenesis (Kinoshita et al., 2014, Billington et al., 1991, Billington et al., 1987). This effect may be indirect via an increase of catecholamines by glucagon (Lawrence, 1967) (see section 1.4.1), or possibly via glucagon receptors on BAT itself – the GCGR has been identified on adipocytes in the rat (Svoboda et al., 1994, Christophe, 1996). However, in rebuttal of the theory that BAT is intrinsic to the effect of glucagon on EE, a study was published very recently describing an increase of EE on administration of glucagon which was independent of BAT activation (Salem et al., 2016). In addition, if the rise in EE was catecholamine-mediated, one would expect to see a significant rise in pulse and blood pressure, which is not evident. A further theory on the mechanism of the glucagon – induced increase in EE is that of futile cycling – essentially two reactions occurring in a cyclical manner in opposite directions that are catalysed by different enzymes. There is a net consumption of ATP, but no product utilised due to the cyclic nature of the reaction(Vaitkus et al., 2015). A classic example is that of glycolysis/gluconeogenesis.

Thyroid hormones are known to have a direct contribution to BAT-mediated EE (see section 1.4.1), but I did not find any consistent effect of glucagon or GLP-1 on thyroid hormones in this study. Glucagon alone was associated with a reduction in thyroid stimulating hormone levels, but this was not replicated when I analysed the effect of the combination infusion. This lack of reproducibility when glucagon was given at the same dose, albeit in combination with GLP-1, calls into question the biological significance of this finding. Similarly, cortisol, a stress hormone which can activate catecholamine release and thus increase EE was not significantly raised by glucagon or GLP-1 infusion.

Substrate oxidation rates showed an increase in carbohydrate (CHO) oxidation during co-infusion of glucagon and GLP-1, and a trend towards an increase in CHO oxidation (not significant) with glucagon alone. In contrast, there was a fall in fat oxidation rates with the combination infusion, and no apparent effect on protein oxidation at all. The rise in CHO oxidation may be explained by the action of glucagon on hepatic glycogenolysis, which is well documented. The reduction in NEFAs seen with the combination infusion and reflected in a reduced rate of fat oxidation does not marry with the traditional thinking that glucagon increases lipolysis (Richter et al., 1989). Moreover, there is support for the theory that glucagon does not induce lipolysis (Ranganath et al., 2001) (Bertin et al., 2001)but that the insulinotropic effect of glucagon is responsible for the inhibition of the enzyme HSL (see fig 1.6) and therefore a fall in NEFAs. It is possible that this effect on HSL is an acute one, as a study in rodents showed that chronic administration of a combined glucagon and GLP-1 agonist actually increased activation of HSL, albeit in the context of reduced circulating insulin levels (Day et al., 2009).

The second part of the hypothesis in this study is that GLP-1 will attenuate any undesirable effects on glycaemic control caused by the administration of glucagon, and maintain euglycaemia as might be seen with oxyntomodulin. One of the primary actions of glucagon is the release of glucose from

hepatic stores via glycogenolysis and gluconeogenesis. Indeed, on administration of the glucagon infusion there was a significant rise in blood glucose levels compared to placebo. On addition of GLP-1 to the glucagon infusion there was an attenuation of this glucose rise from a mean peak of 8.3 ± 0.4 mmol/l (glucagon alone) down to 6.9 ± 0.7 mmol/l in the combination group, although glucose did not normalise. Glucose levels in both the glucagon and combination groups initially increased at the same rate, but once the peptide hormone infusions achieved steady state, after about t=60 (15 minutes into the infusion), blood glucose concentrations in both groups appeared to stabilise and then begin to drop back to baseline. The glucose levels in both study groups were still decreasing by the end of the infusion, although both had yet to reach baseline levels. If the infusions had continued for longer it is possible that blood glucose may have normalised, or even overshot into the hypoglycaemic range. In parallel with the rise in glucose in the glucagon and combination groups, there was a corresponding rise in plasma insulin levels. The insulin response was significantly greater in the combination group compared to all the other groups, including glucagon alone. Glucagon is known to be insulinotropic with a direct effect on the pancreatic beta cell to stimulate insulin release(Huypens et al., 2000, Kawai et al., 1995). GLP-1 is an incretin, acting to cause beta cell insulin secretion in a glucose dependent manner (Kreymann et al., 1987). Glucagon and GLP-1 appear to work synergistically here to increase insulin secretion, and it is this synergistic rise in insulin that ameliorates the rise in glucose due to glucagon in the combination group. There is also likely to be an additional contribution of the normal physiological insulin response to glucose. This normal physiological increase of insulin in the presence of hyperglycaemia may have been even more marked in the group receiving glucagon alone, as glucose levels were highest in this group, GLP-1 alone does not cause an increase in insulin secretion compared to placebo, and this is likely because there is no associated glucose rise following GLP-1 infusion. As discussed above, the rise in insulin is likely to be the cause of the reduction in NEFAs and fat oxidation observed. Insulin itself may also contribute to the increase in EE, as there is some evidence in rodents that central insulin sensing in the hypothalamus plays a role in governing BAT thermogenesis (Sanchez-Alavez et al., 2010, Menendez and Atrens, 1991, McGowan et al., 1992).

It should be noted that although both GLP-1 and glucagon are known to cause a reduction in food intake (with or without nausea and vomiting) at higher doses, assessment of this was beyond the scope of this study as the participants were largely immobile and restricted by the calorimeter hood. There was, however, a significant decrease in both total and acyl ghrelin with the combined glucagon and GLP-1 infusions, which may contribute to a reduction in food intake. If this combination of hormones is to be given for a longer time then it will be important to take the possibility of inducing nausea into account, and adjusting the doses of peptide accordingly, as a reduction in food intake is desirable, whilst nausea is not. All observations in this study relate to the acute short term effects of glucagon and GLP-1 administration, and it will be necessary to conduct a longer term study to assess whether the favourable effects are sustainable. It will also be necessary in the future to assess the effect of these hormones on people with disordered blood glucose regulation, which often goes hand-in-hand with obesity. It seems unlikely that the physiological responses to glucagon and GLP-1 infusions will remain the same in someone with impaired glucose tolerance who is already hyperglycaemic, and potentially hyperinsulinaemic/hyperglucagonaemic (Cryer, 2012).

CHAPTER 4: THE ACUTE EFFECT OF CO-INFUSION OF GLUCAGON AND GLP-1 ON FOOD INTAKE

4.1 Introduction

Following the increase in energy expenditure and improvement in glucose levels demonstrated in the previous study (Chapter 3), I went on to investigate the acute effect of co-infusion of GLP-1 and glucagon on food intake to assess whether the combination of both hormones could enhance the reduction of food intake over that attributable to each hormone alone. Each peptide infusion was longer than in the previous study (Chapter 3), and thus also presented an opportunity to assess a slightly extended infusion length on glycaemic control and EE.

Both GLP-1 and glucagon have independently been shown to reduce food intake and increase satiety (Verdich et al., 2001, Le Sauter and Geary, 1991, Schulman et al., 1957, Geary et al., 1993, Le Sauter et al., 1991). A combination of the two hormones has the potential therefore to synergistically reduce food intake, whilst increasing EE and maintaining euglycaemia.

Rodent studies suggest that at least some of the food intake reduction seen with GLP-1 mimetics is attributable to nausea. Whilst rodents do not vomit, surrogate measures of nausea were employed to demonstrate that satiety and reduction of food intake are not independent of nausea that is likely to be caused by CNS GLP-1 receptor activation (Kanoski et al., 2012). The effect of GLP-1 on food intake appears to be dose-dependent, with increasing doses ultimately causing nausea and vomiting. Furthermore, in man, incretin mimetics such as liraglutide and exenatide which are in clinical use for the treatment of type 2 diabetes and obesity are well documented to cause nausea – not only at high doses but also on initial administration, an effect which is ameliorated with time (Pi-Sunyer et al., 2015, Sun et al., 2015, Astrup et al., 2012, Lean et al., 2014). Glucagon has also been shown to cause nausea (Waldhausl et al., 1976) at least when given intramuscularly. This study offers the opportunity to subjectively measure and correlate satiety and nausea in participants receiving combined infusions of glucagon and GLP-1.

4.2 Hypothesis and Aims

4.2.1 Hypothesis

- The combination of glucagon and GLP-1 at sub-anorectic doses will give a greater reduction of food intake over that observed when each hormone is given individually, and will not cause excessive nausea.
- The combination of glucagon and GLP-1 at sub-anorectic doses and over a longer duration will maintain the favourable effects on EE and glucose homeostasis seen in the previous study (Chapter 3)

4.2.2 Aims

- To investigate the acute effects of co-infusion of sub-anorectic doses of glucagon and GLP-1 on food intake and satiety, REE, and blood glucose homeostasis in healthy overweight volunteers

4.3 Materials and Methods

4.3.1 Subjects

Sixteen healthy, non-diabetic volunteers were recruited by advertisement in local media. Inclusion criteria were age between 18-65 with a body mass index between 20 and 35 kg/m^2. Participants' body weight should have been stable for at least 3 months prior to commencing the study. Participants were not recruited if they had any past medical history or were on any medication which was felt to interfere with the study, or if enrolling in the study could potentially cause harm to the volunteer. Women were excluded if pregnant or breastfeeding. Anyone having donated blood in the previous three months, or having participated in another study involving an investigational drug within two months was also excluded. Participants were free to withdraw from the study at any time, and female volunteers were asked to maintain adequate contraception during the course of the study and for one month afterwards. Smokers were not recruited.

At the screening visit, all potential participants underwent a medical history and physical examination including 12-lead electrocardiography, and comprehensive haematological and biochemical screening. Abnormal eating behaviours were screened for using the Dutch Eating Behaviour Questionnaire (DEBQ) for restrained eating patterns, (van Strien et al., 1986) and the SCOFF questionnaire as a screen for eating disorders (Morgan et al., 1999). Participants also tasted various options for the meal they would consume during the study, and were allocated an option they neither 'disliked extremely' nor 'liked extremely' (Appendix B) as palatability could significantly affect the amount consumed.

Three participants were excluded from the final analysis due to abnormal eating behaviours not picked up in the original screening. One demonstrated restrained eating, consuming less than the minimum requirement of 300Kcal at the acclimatisation visit, one did not finish eating during the maximum allotted time (20 minutes), and the third demonstrated a progressive aversion to his chosen meal, as

assessed by visual analogue scores (VAS). Four women and nine men were recruited, with a mean BMI of 31.6 ± 0.7 kg/m^2, and mean age of 31.6 years (range 21-41years). Participant demographics can be seen in table 4.1.

All volunteers gave informed written consent. The study was carried out in accordance with the principles of the Declaration of Helsinki, and was approved by the local West London Research Ethics committee (REC 10/H0707/80).

Volunteer	Weight (kg)	Height (m)	BMI (kg/m^2)	Age	Sex
1	87.1	1.82	26.3	35	M
2	74.8	1.68	26.5	40	F
3	66.1	1.56	27.2	21	F
4	81.4	1.84	24	25	M
5	66.2	1.59	26.2	40	F
6	83.9	1.83	25.1	32	M
7	78.7	1.80	24.3	41	M
8	89.2	1.82	26.9	33	M
9	99.2	1.83	29.6	32	M
10	94.9	1.80	29.2	39	M
11	82.1	1.57	33.3	29	F
12	77.1	1.62	29.4	23	M
13	90.2	1.83	26.9	22	M
Mean	82.4	1.74	27.3	31.6	F4/9M

Table 4.1 Demographic details and means of all volunteers included in the study 'The acute effects of co-infusion of glucagon and GLP-1 on food intake'

4.3.2 Peptides

Glucagon and GLP-1 were obtained and reconstituted as described in section 3.3.2

4.3.3 Calorimetry

Indirect Calorimetry was performed as described in section 3.3.3

4.3.4 Protocol

An initial dose finding phase was undertaken to establish a dose of glucagon which was sub-anorectic, with doses between 1.5 ng/kg/min and 50 ng/kg/min assessed. The lowest sub-anorectic dose of glucagon alone was 15 ng/kg/min. Following this phase, glucagon at 3 different doses (10, 15 and 20 ng/kg/min) was then combined with GLP-1 to find the lowest effective anorectic dose without causing nausea (Data not shown). A known sub-anorectic dose of 0.4 pmol/kg/min of GLP-1 was chosen (Verdich et al., 2001, Neary et al., 2005). The doses employed in the subsequent study were glucagon 10 ng/kg/min and GLP-1 0.4 pmol/kg/min.

The study was then conducted in a randomised controlled, double-blind four-way cross over manner. All study visits were at least two days apart. Participants underwent a 'sham' or placebo visit which was unblinded wherein they received an infusion of vehicle alone (Gelofusine, B. Braun, Crawley, UK) to allow acclimatisation to the study procedures. Following the acclimatisation visit, participants underwent four further study visits as described. The four visits consisted of 1) placebo (gelofusine), 2) GLP-1(7-36 amide) (0.4pmol/kg/min; Clinalfa Basic, Bachem, Switzerland) 3) glucagon (10 ng/kg/min; NovoNordisk, Crawley, UK) and 4) glucagon and GLP-1 combined at the above doses. Gelofusine was used as the vehicle for all infusions to minimise peptide adsorption to the inside of the infusion apparatus (Kraegen et al., 1975).

Volunteers attended the clinical research facility at 0830h, having fasted from 2200h the night before, and refrained from alcohol and strenuous exercise for the preceding 24 hours. The study room

temperature was centrally controlled at 21^{o}C. On arrival, participants emptied their bladder and all female volunteers underwent a urinary βhCG pregnancy test. Two intravenous cannulae were then inserted, one for peptide infusion and one for blood sampling. An initial blood sample was taken at t=-60 minutes, and volunteers were then encouraged to relax in a reclining chair to watch television or listen to music. 30 minutes later at t=-30, volunteers were placed under the calorimeter hood and measurements taken for REE and substrate oxidation over the next 30 minutes. Indirect calorimetry (Gas Exchange Monitor; GEMNutrition, Daresbury, UK) was performed as described in section 3.3.3. After 30 minutes of calorimetry reading, the hood was removed and the peptide hormone infusion commenced at t=0 minutes. The infusion was initially 'ramped' in order to rapidly achieve stable plasma levels of hormone. The rate of infusion was at 4x the basal rate for the first five minutes, then 2x the basal rate for the next five minutes after which it was reduced to the basal rate. The infusion then continued for a further 120 minutes. At t=40 minutes a further 30 minutes of calorimetry was performed to assess REE and substrate oxidation. At t=90 minutes an ad-libitum meal of known calorific value was served (Spaghetti Bolognese 188kcal/100g; chicken tikka masala 178kcal/100g; macaroni cheese 194kcal/100g; Sainsbury's Supermarkets Ltd, London, UK). The meal was chosen by each participant at the screening visit, and the same meal was served at all five study visits. Participants had 20 minutes to eat, and were given instruction to eat until they were comfortably full then stop. The peptide hormone infusion continued for 120 minutes in total and was terminated 10 minutes after the end of the meal. Participants remained in the study room for 60 minutes following the end of the infusion. After this, at 180 minutes, both cannulae were removed and participants provided a further urine sample for urine urea measurement (see section 3.3.3 and 3.3.5). Participants were then discharged home.

Blood samples were taken at t=-60, -30, 0, 40, 70, 90, 120, 150 and 180 minutes for measurement of glucagon, active GLP-1, glucose and insulin. For sample collection details see table 3.2. At each of the specified time points, participants also completed a visual analogue scale (VAS) to subjectively assess nausea and satiety (Appendix A). Samples were also taken at t=0 and 90 minutes for measurement of

total and acyl ghrelin. Samples were immediately placed on ice, (except insulin samples which were allowed to clot first), centrifuged at 4 ^{o}C at 4000 revolutions per minute (rpm) for 10 minutes, and separated. They were subsequently stored at -20 ^{o}C until analysis.

Vital signs for each subject (pulse and blood pressure) were taken at t=-60, -30. 0, 40, 70, 90, 120, 150 and 180 minutes.

Pre- and post-infusate samples of the hormone solutions were taken to ensure that there was no significant breakdown of peptide during the study which could render it inactive.

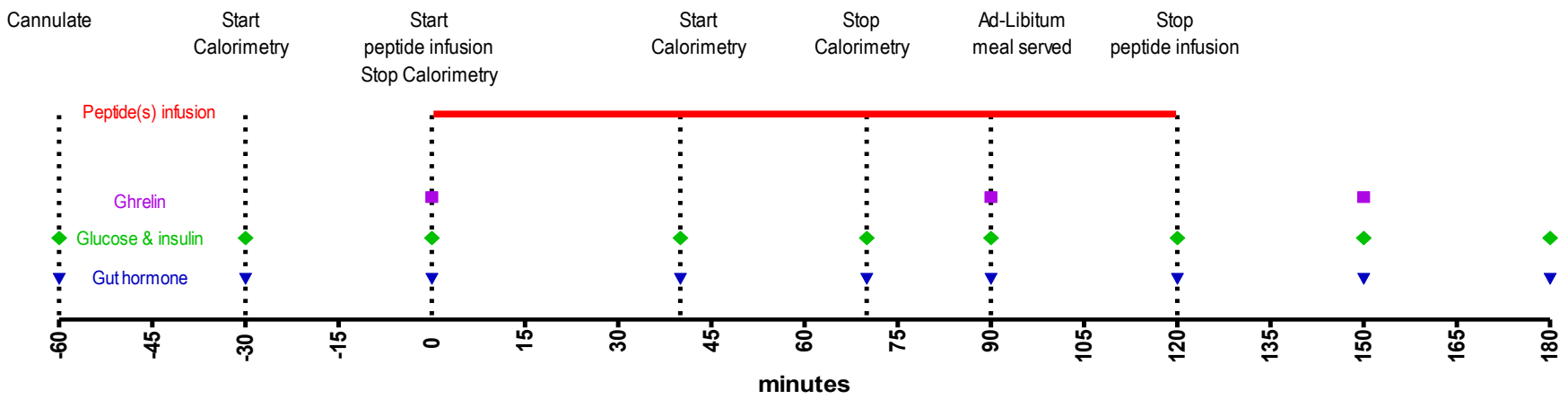


Figure 4.1 'The acute effect of co-infusion of glucagon and GLP-1 on food intake': Study Protocol. Participants arrived at t=-60, when they were cannulated. Calorimetry measurements were commenced at t=-30 until t=0. The peptide hormone infusion was commenced at t=0 and continued until t=120. Further calorimetry took place at t=40 to t=70. An Ad-libitum meal was served at t=90. The infusion was stopped at t=120 and participants discharged 1 hour later. Blood samples were drawn as indicated by the blue (gut hormones), green (glucose and insulin) and purple (ghrelin) markers

4.3.5 Assays

Glucose, Insulin, Ghrelin

Glucose and insulin samples were analysed by the Department of Chemical Pathology, Imperial College Healthcare NHS Trust (see section 3.3.5). Ghrelin was analysed by the method outlined in section 3.3.4.

Glucagon

Glucagon was analysed by an established sensitive and specific in-house radioimmunoassay (RIA) (See section 3.3.5).

Active GLP-1

Active GLP-1, i.e. the fragments GLP-1(7-36 amide) and GLP-1(7-37), was measured using a commercially available ELISA kit (Millipore, Livingston, UK) with a CV <7%. It was analysed according to the manufacturer's instructions. The principle is that active GLP-1 molecules are bound in the assay by anti-human GLP-1 immunoglobulin G (IgG) ('capture' antibody'). The resulting antibody-GLP-1 complex is bound to the wells of a 96 well microtitre plate via a known concentration of an 'anchoring antibody' which is specific for the N terminal region of the GLP-1 molecules. Next, an anti GLP-1-alkaline phosphatase detection conjugate is bound to the immobilized GLP-1, and any unbound conjugate removed by washing. Finally, the amount of bound detection conjugate is quantified by adding the substrate 'MUP' (methyl umbelliferyl phosphate), which forms the fluorescent product umbelliferone in the presence of alkaline phosphatase. The amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the sample, and therefore GLP-1 measurements can be read from a standard reference curve generated in the same assay using known concentrations of GLP-1 ('reference standards'). The assay can then be read on a fluorescence plate reader with an excitation/emission wavelength of 355 nm/460 nm.

The manufacturer's protocol was followed for the active GLP-1 ELISA. The assay was run in duplicate at a volume of 200µl over 2 days.

Day 1:

All reagents were used at room temperature. Wash buffer was decanted and then removed from a 96 well plate. Subsequently 200µl of assay buffer was added to the wells which would measure non-specific binding, with 100µl added to all other wells. 100µl of GLP-1 standard was added to the top row of wells in ascending concentrations, and followed by the addition of quality controls (QCs) to the appropriate wells. 100µl of sample was then added in duplicate to the remaining wells. The plate was shaken gently, covered and incubated overnight at 4ºC.

Day 2:

Liquid was decanted from the plate with the excess then tapped out. The plate was then washed five times with wash buffer with five minutes' incubation at the fourth wash. 200µl of detection conjugate (Anti GLP-1-Alkaline Phosphate Conjugate) was then added to each well and incubated for 2 hours at room temperature. It was then decanted and the plate washed three times. This was followed by the addition of 200µl of diluted substrate (MUP – see above), and incubated for 20 minutes at room temperature and in the dark. 50µl of 'Stop solution' was then added to each well to arrest the phosphatase activity. There was a further five minute incubation, and the plate was read on a fluorescence plate reader with an excitation/emission wavelength of 355 nm/460 nm. A standard curve was then constructed to enable calculation of active GLP-1 concentrations in the plasma samples (GraphPad Prism 5.0d, GraphPad Software, San Diego, USA).

4.3.6 Statistics

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA).

A two-way repeated measures ANOVA with Bonferroni post-hoc test was used to assess differences in insulin and glucose.

A repeated measures (non-parametric) Friedman's test with Dunn's multiple comparison post-hoc test was used to assess differences in VAS scores

Energy expenditure/substrate oxidation and food intake were assessed as change from baseline and were analysed using a repeated measures one-way ANOVA with Tukey's post hoc test.

Blood pressure and pulse rate were assessed via repeated measures two-way ANOVA with Tukey's post-hoc test.

All results are described as mean ± SEM, and statistical significance taken at $p<0.05$ unless otherwise stated.

4.4 Results

4.4.1 The acute effect of low-dose co-infusion of glucagon and GLP-1 on food intake

Food intake (FI) was measured by means of an ad-libitum meal of known calorie content and known quantity served at t=90, 90 minutes after the start of the infusion. The meal was chosen by the participant at screening, and deemed to be palatable but not excessively so. All study meal options contained roughly the same calorie content per 100g (mean 186.7 ± 4.7 kcal/100g). Participants were given 20 minutes to eat to satiety. The food was weighed before and after the meal, and the calories consumed calculated.

The combination of GLP-1 and glucagon caused a significant reduction in food intake compared to placebo and glucagon alone (mean FI 878 ± 94 kcal vs 1086 ± 110 kcal *$p<0.05$ and 1086 ± 97kcal $^{\$}p<0.05$ respectively) but not GLP-1 alone. Absolute food intake was averaged across the group, but relative food intake reduction was calculated per participant and subsequently averaged to take into account intra-individual food intake variability. This accounts for the positive deflection of the graph columns in figure 4.2B, rather than an overall negative deflection which might be expected at least in the GLP-1 group. Overall, this analysis gave a 13% reduction in food intake in the combination group compared to placebo. Analysis was performed via a repeated measures one way ANOVA with Tukey's post-hoc test.

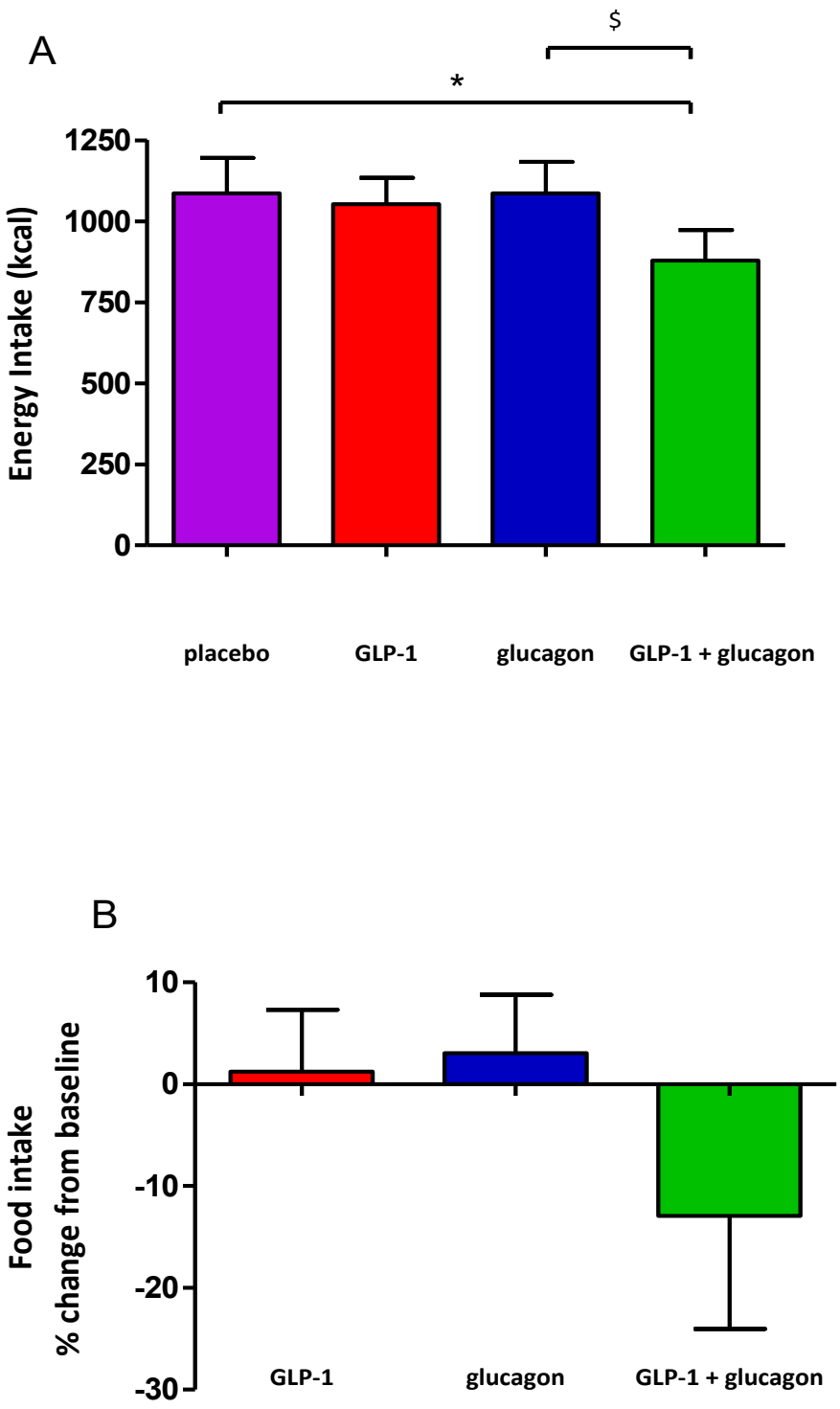


Figure 4.2 A: The acute effect of low-dose co-infusion of glucagon and GLP-1 on food intake following infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Analysis was by a repeated measures ANOVA with Tukey's Post hoc test. The combination of GLP-1 and glucagon significantly reduced FI vs placebo (*p<0.05), and glucagon alone ($ p<0.05) but not GLP-1. N=13 B: Energy intake as a percentage change of the placebo visit N=13.

4.4.2 The acute effect of low dose co-infusion of glucagon and GLP-1 on hunger, satiety, nausea, and palatability

Visual analogue scales (VAS) were completed at t=-60, -30, 0, 40, 70, 90, 120, 150 and 180 minutes. (See Appendix A). As the primary outcome of this study is to investigate the effect of the infusion on food intake, the VAS scores for hunger ('how hungry do you feel right now'), nausea ('how sick do you feel right now') and satiety ('how full do you feel right now') were analysed, along with that for palatability of the meal ('how tasty was the meal'). Participants placed a vertical line on the VAS scale and the distance along the scale from the left hand edge was measured in millimetres (mm). In the analysis, VAS score is calculated as change from baseline except for palatability score which is given as an absolute value in mm as this is a one-off subjective measurement (figure 4.3D).

As expected, hunger scores fell and satiety scores rose following the ad-libitum meal. There was a small but non-significant peak of nausea following the meal in the combination infusion group (figure 4.3C). This represents three subjects who experienced post-prandial nausea on receipt of the combination glucagon + GLP-1 infusion. In addition, two participants vomited post-prandially during the infusion of glucagon alone. It should be noted that all nausea and vomiting events occurred post-prandially between 120 and 150 minutes. There was no statistically significant difference between the groups in terms of nausea, hunger or satiety scores.

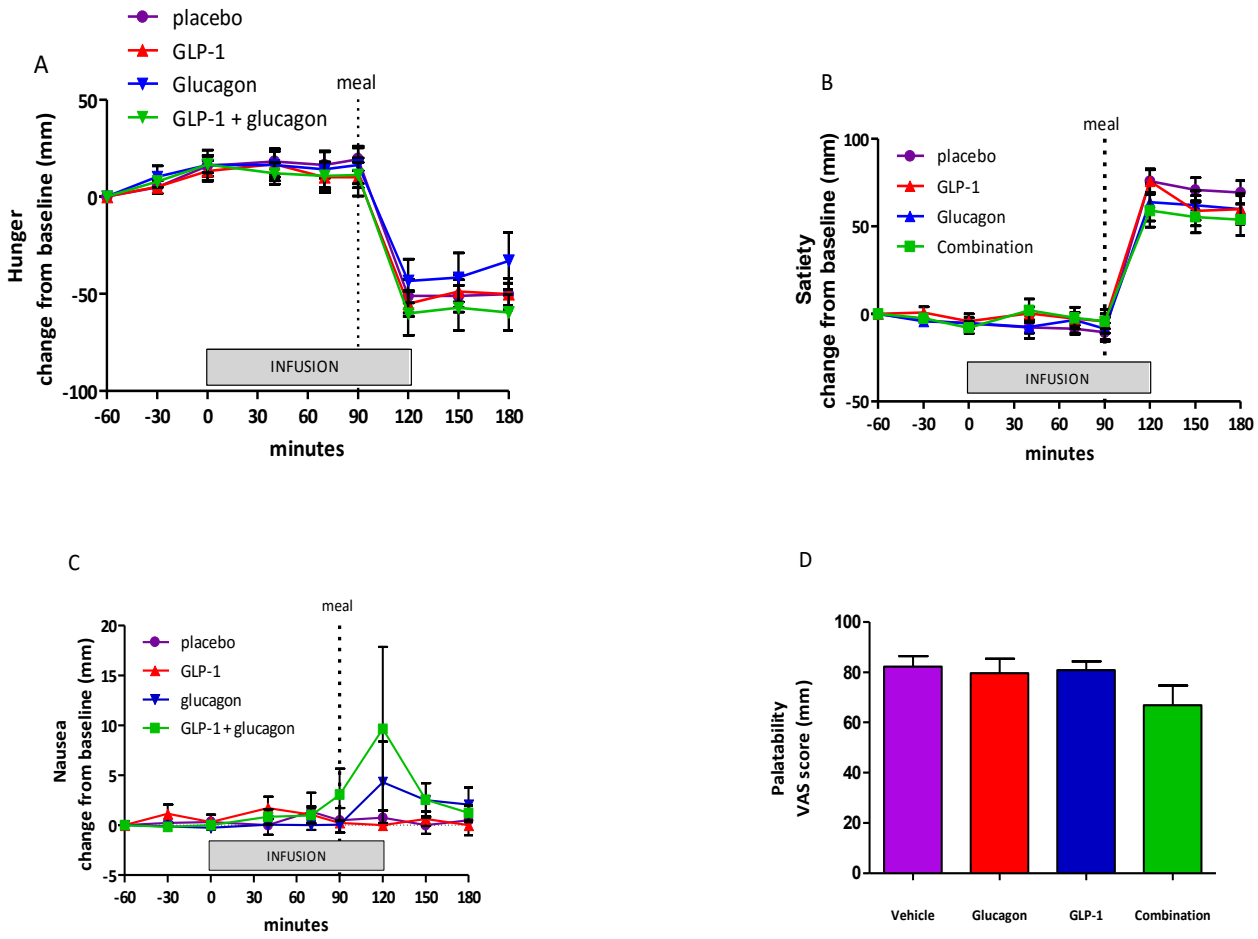


Figure 4.3 The acute effect of low dose co-infusion of glucagon and GLP-1 on A: hunger, B: satiety, C: nausea and D: palatability of an ad-libitum study meal. VAS scores were recorded during infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Nausea was increased at t=120 minutes (postprandially) with the combination infusion, but this was not significant. A, B & C analysed with a repeated measures non-parametric Friedman's test with Dunn's multiple comparison post-hoc test, and D analysed with a repeated measures one-way ANOVA with Tukey's post-hoc test. N=13

4.4.3 The acute effect of low dose co-infusion of glucagon and GLP-1 on plasma glucagon and active GLP-1 concentrations

Participants received four infusions in a randomised double blinded crossover study design. The infusions were 1) gelofusine alone (control); 2) GLP-1 alone at 0.4 pmol/kg/min; 3) glucagon alone at 10 ng/kg/min; and 4) Co-infusion of GLP-1 plus glucagon at the above doses.

Baseline GLP-1 concentration for the placebo group and glucagon group were 4.8 ± 0.7 pmol/l (range 0.5 - 17.5 pmol/l) and 5.1 ± 0.7 pmol/l (range 0.8 - 14.3 pmol/l) respectively. (Figure 4.3A). Baseline GLP-1 concentrations in the GLP-1 and GLP-1 + glucagon groups were 3.3 ± 0.4 pmol/l (range 0.3 - 13 pmol/l) and 4.8 ± 0.7 pmol/l (range 0.9-17.4 pmol/l) respectively. In contrast to the placebo and glucagon groups, plasma concentrations rose to peak levels at t=90 minutes with a peak concentration of 16.7 ± 2.8 pmol/l (range 3.0-30.1 pmol/l) in the active GLP-1 group, and 13.0 ± 2.1 pmol/l (range 3.8 – 29.8 pmol/l) in the glucagon + GLP-1 group (figure 4.3A). Following the termination of the infusion at t=120 minutes, plasma hormone levels fell back to baseline by t-180, the end of the study. Subsequent to eating the ad-libitum meal at t=90, there is a small rise in active plasma GLP-1 seen in the glucagon and placebo groups. This is likely to represent the physiological post-prandial rise in endogenous GLP-1 stimulated by oral ingestion of a nutrient load.

Baseline glucagon concentrations for the placebo and GLP-1 groups were 20.6 ± 3.2 pmol/l (range 6.0-98.5 pmol/l) and 14.6 – 2.4 pmol/l (range 7.5 - 59.4 pmol/l) respectively. Levels remained at baseline during the course of the infusion apart from a slight increase in glucagon in the placebo group just before the meal – again a predictable physiological response as glucagon is highest before meals and in the fasted state (figure 3.5B). Baseline glucagon concentrations for the glucagon and GLP-1 + glucagon groups were 12.3 ± 1.6 pmol/l (range 6.0-36.1 pmol/l) and 26.5 ± 9.6 pmol/l (range 5.0-370 pmol/l) respectively. Levels rose rapidly to give a peak hormone concentration at t=40, and then remained at a steady state until the infusion was terminated at t=120. In the glucagon group the peak level was 169.1 ± 35.1 pmol/l (range 9.8 – 480.0 pmol/l) in the glucagon group and 159.0 ± 18.0 pmol/l

(range 58.7 – 292.0 pmol/l) in the GLP-1 + glucagon group (figure 3.5B). Plasma glucagon concentrations had returned to baseline by the end of the study at t=180.

The peak levels of glucagon and GLP-1 observed correlate to the peak in nausea reported in section 4.4.2 (n=3).

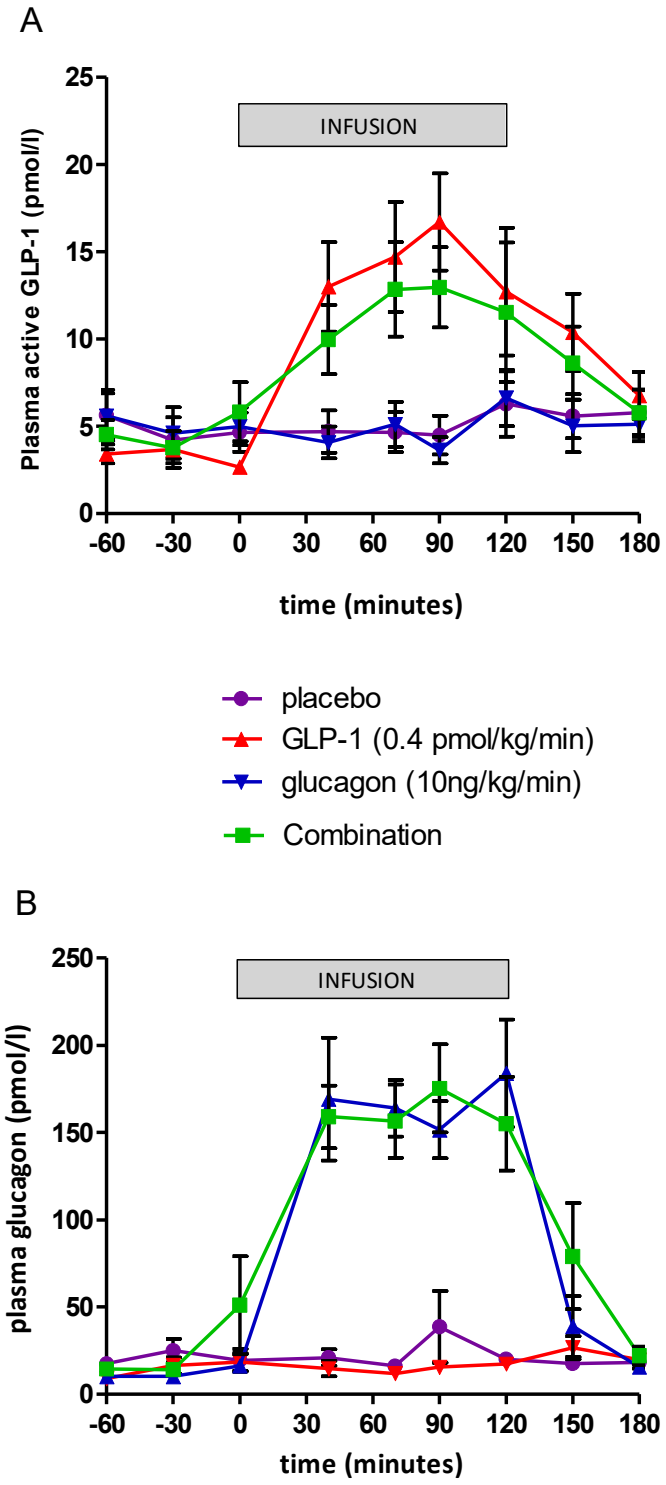


Figure 4.4 A & B The acute effect of low-dose co-infusion of glucagon and GLP-1 on A: plasma active GLP-1 and B: glucagon levels following infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. The infusion commenced at t=0 and lasted for 120 minutes. Blood samples were drawn at t=-60, -30, 0, 40, 70, 90, 120, 150 and 180 for analysis of plasma hormone profiles.

4.4.4 The acute effect of low dose co-infusion of glucagon and GLP-1 on energy expenditure

Resting EE was measured both pre-infusion and 40 minutes into the infusion once a steady state plasma concentration of peptide had been achieved. REE was measured for infusion of each of placebo (gelofusine), GLP-1, glucagon, and GLP-1 plus glucagon (Figure 4.4).

Infusion of glucagon alone, and GLP-1 plus glucagon increased resting EE in kcal/day compared to both placebo and GLP-1, but this increase did not reach significance. The change from baseline in REE following infusion of glucagon was 67 ± 28 kcal/day, and that of the combination infusion was 61 ± 29kcal/day compared to placebo (20 ±19 kcal/day).

Data were analysed via a repeated measures one way ANOVA with Tukey's post-hoc test. N=12 as data was missing for one participant.

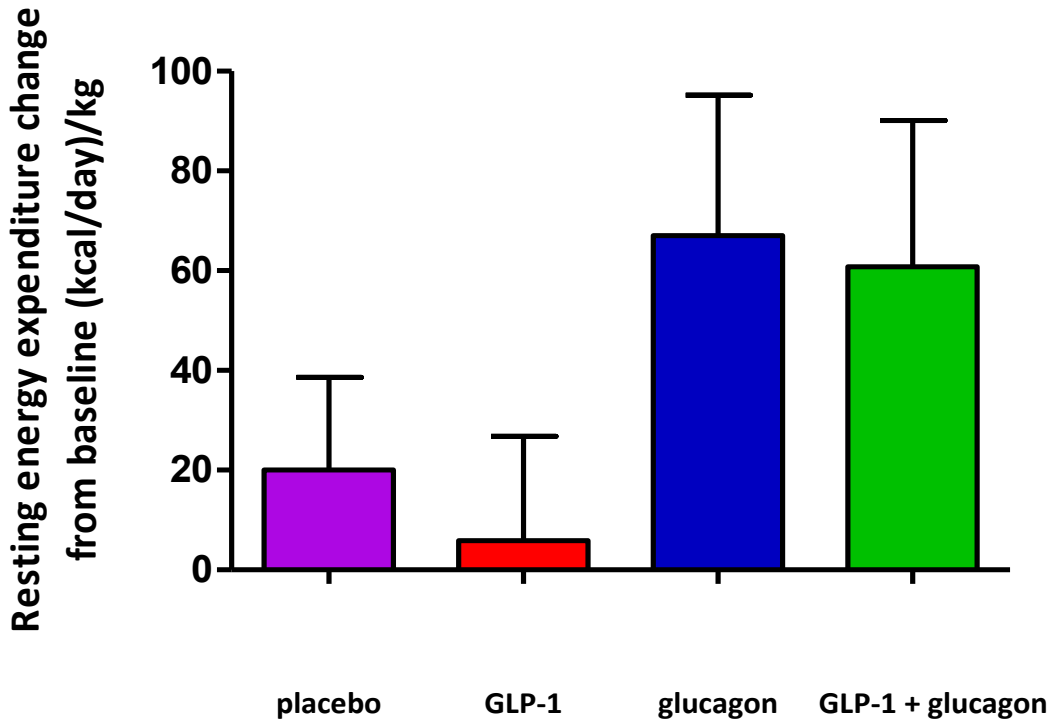


Figure 4.5 The acute effect of low-dose co-infusion of glucagon and GLP-1 on resting energy expenditure both prior to, and following infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. There was no statistically significant change in REE detected between the groups on analysis with repeated measures one way ANOVA and Tukey's post-hoc test. N=12.

4.4.5 The acute effect of low-dose co-infusion of glucagon and GLP-1 on substrate oxidation

Substrate oxidation rates during the infusions for fat, carbohydrate and protein can be calculated using the equations in section 3.3.3. For these calculations, we require the VO_2 and VCO_2, generated from the indirect calorimeter measurements. Also required are the urine nitrogen excretion rates which are derived from urine volume and urine urea content. Measurements for fat and carbohydrate (CHO) oxidation were taken both before and during the peptide infusion. Protein oxidation is measured for the whole experimental time and cannot therefore be compared before and after peptide infusion. Analysis was performed via a repeated measures one way ANOVA with Tukey's post-hoc test. N=12 (one data set missing).

Carbohydrate oxidation was significantly increased following infusion of glucagon alone, and glucagon + GLP-1 compared to placebo and GLP-1 alone (Figure 4.5A). The mean change from baseline in CHO oxidation following infusion of glucagon (0.052 ±0.013 g/min) and glucagon + GLP-1 (0.099 ± 0.012 g/min) vs placebo (-0.0052 ± 0.012 g/min, $p<0.001$) and GLP-1 (-0.015 ± 0.011 g/min, $p<0.001$). The combination infusion also significantly increased CHO oxidation compared to glucagon alone (0.099 ±0.012 g/min vs 0.052 ±0.013 g/min $p<0.01$).

Fat oxidation was significantly decreased following infusion of glucagon (-0.017 ± 0.005 g/min) and glucagon + GLP-1 (-0.03 ± 0.005 g/min) compared to placebo (0.001 ±0.005 g/min $p<0.01$ and $p<0.001$ respectively) and GLP-1 alone (0.006 ±0.005 g/min, $p<0.001$). Fat oxidation was also significantly lower in the combination group compared to glucagon alone (-0.03 ±0.005 g/min vs -0.017 ± 0.005 g/min, $p<0.05$) (figure 4.5B).

Protein oxidation was significantly increased in the glucagon alone group compared to GLP-1 alone (0.06 ± 0.007 g/min vs 0.04 ± 0.004 g/min, $p<0.05$)

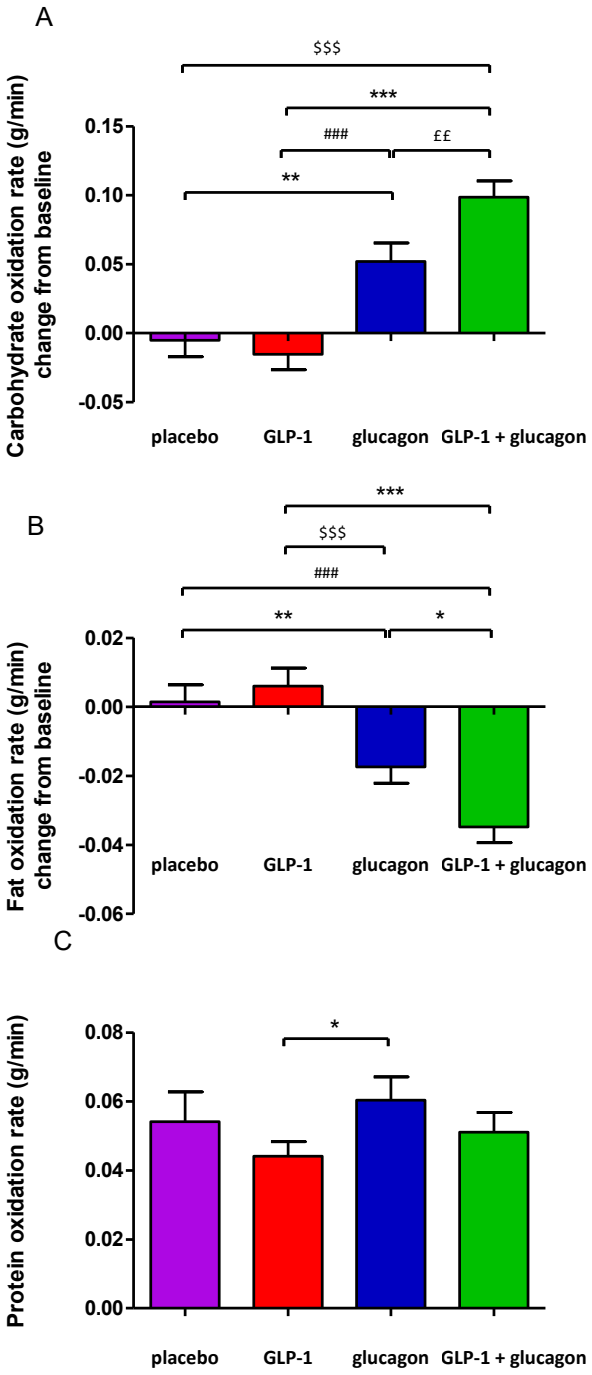


Figure 4.6 The acute effect of low dose co-infusion of glucagon and GLP-1 on A: Carbohydrate B: fat and C: protein oxidation. Oxidation rates were calculated both prior to, and following infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Fat and carbohydrate oxidation rates were calculated from VO_2 and VCO_2 measurements achieved during indirect calorimetry, and protein oxidation was calculated using urine urea and urine volume measurements. Data was analysed by a one-way repeated measures ANOVA with Tukey's post hoc test. N=12 (one data set missing). A: **p<0.05 placebo vs glucagon, ff p<0.05 glucagon vs glucagon + GLP-1, ### p<0.001 GLP-1 vs glucagon, ***p<0.001 GLP-1 vs glucagon + GLP-1, $$$p<0.001 placebo vs glucagon + GLP-1. B: *p<0.05 glucagon vs glucagon + GLP-1, **p<0.01 placebo vs glucagon, ###p<0.001 placebo vs glucagon + GLP-1, $$$ p<0.001 GLP-1 vs glucagon alone, ***p<0.001 GLP-1 alone vs glucagon + GLP-1. C: p<0.05 GLP-1 vs glucagon.

4.4.5 The acute effect of low dose co-infusion of glucagon and GLP-1 on plasma glucose and insulin concentrations

Insulin and glucose plasma levels were assessed during the course of each infusion. Blood was drawn at t=-60,-30, 0,40,70,90,120,150 and 180 minutes for analysis. The effect of each of the following infusions: 1) gelofusine alone; 2) GLP-1 alone at 0.4 pmol/kg/min; 3) glucagon alone at 10 ng/kg/min; and 4) Co-infusion of GLP-1 plus glucagon at the above doses on plasma insulin and glucose concentrations was then assessed.

Baseline insulin concentration across all infusion groups and participants was 7.2 ± 0.3 mU/l, with a range of 2-22.9mU/l. Compared to placebo, the combination of glucagon + GLP-1 caused a significant increase in serum insulin (**$p<0.01$). Compared to GLP-1, the combination of glucagon +GLP-1(* $p<0.05$) caused a significant rise in serum insulin. (Figure 4.7A).

Baseline glucose across all infusion groups and participants was 4.7 ± 0.04 mmol/l (range 2.9-6.0 mmol/l). Glucagon caused a significant rise in plasma glucose concentration compared to placebo (*$p<0.05$, ***$p<0.001$). Glucagon and the combination of GLP-1/glucagon caused a rise in glucose compared to GLP-1 (### $p<0.001$, &&& $p<0.001$), and glucagon alone caused a significant rise in glucose compared to the combination infusion (£ $p<0.05$) (Figure 4.7B). The rise in plasma glucose levels seen with glucagon infusion is significantly ameliorated by the addition of GLP-1, as shown by the discrepancy between the glucagon and combination groups (Figure 4.7 A&B), although not to the same degree as was observed in the previous study (Chapter 3). Again, this is likely to be secondary to the greater insulinotropic effect of the combination infusion compared to glucagon alone. Glucose levels in all groups achieved a reasonably steady state after t=40, but then rose as would be expected after the meal (t=90) in the placebo, GLP-1 and combination groups despite termination of the infusion at t=120. However, in the glucagon group, there was a fall in glucose levels post-prandially. Despite this trend, there was no significant difference in glucose or insulin levels between the groups after the meal. Pre-prandially, in the GLP-1 group, there was a reduction of blood glucose in the absence of a

corresponding rise in insulin. As in section 2.3.5, it is possible this could at least in part be attributable to alterations in hepatic glucose handing/hepatic insulin sensitivity mediated by GLP-1. Further work in this area is warranted.

For both insulin and glucose, data was analysed by a two-way repeated measures ANOVA with Bonferroni post-hoc test. Further to this analysis, area under the curve (AUC) was calculated using the trapezoidal method. This allows comparison of the cumulative measurement of insulin (Figure 4.8A) and glucose (Figure 4.8B) release with each of the four infusions. In this study, AUC was calculated from t=0, the start of the infusion, to t=90 when the ad-libitum meal was served. Thus the actual effect of the peptide hormone infusions on insulin and glucose can be analysed, rather than the effect of the infusion plus the meal. As the meal was ad-libitum and different caloric and nutrient contents were consumed by all participants, analysis of insulin and glucose post-prandially would not confer useful information. The combination infusion resulted in a significant rise in serum insulin compared to all other groups (placebo $$$ $p<0.001$; GLP-1 ###$p<0.001$; glucagon *$p<0.05$). Glucagon alone also significantly increased insulin secretion compared to placebo and GLP-1 alone (***$p<0.001$, £££$p<0.001$ respectively). (Figure 4.8A). Glucagon alone increased plasma glucose compared to both placebo and GLP-1 (***$p<0.001$ and $$$$p<0.001$ respectively), and the combination infusion also increased plasma glucose compared to GLP-1 alone (##$p<0.01$) and glucagon alone (*$p<0.05$) (Figure 4.8B). Data was analysed by repeated measures one-way ANOVA with Tukey's post hoc test. N=13

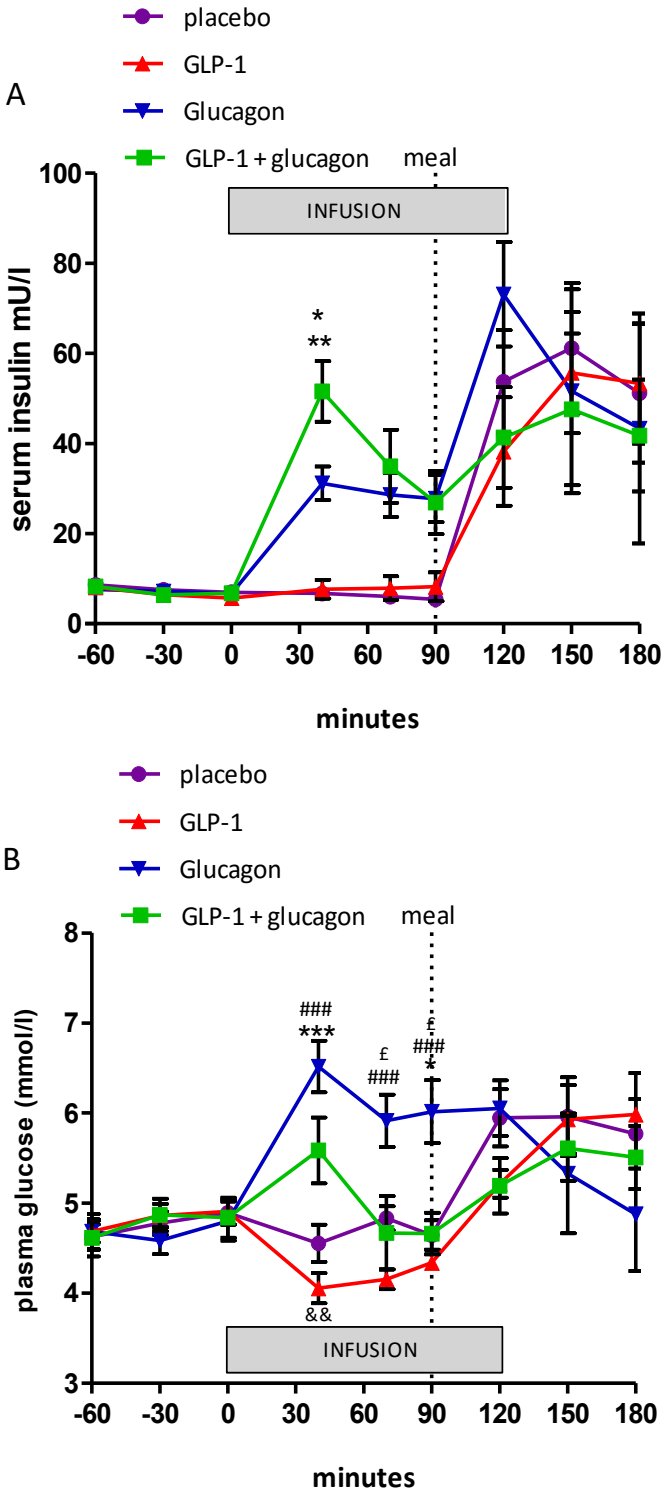


Figure 4.7 A & B. The acute effect of co-infusion of low dose glucagon and GLP-1 on A: plasma glucose and B: insulin concentrations. Samples were taken during infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Glucagon caused a significant rise in glucose levels compared to placebo (*p<0.001, *p<0.05), GLP-1 (###p<0.001), and the combination infusion (£p<0.05). In addition, the combination caused a significant rise in glucose compared to GLP-1 alone (&& p<0.01). The combination infusion of glucagon and GLP_1 caused a significant rise of insulin compared to placebo (**p<0.01), and GLP-1 alone (*p<0.05). All data were analysed with repeated measures two-way ANOVA with Bonferroni post-hoc test. N=13**

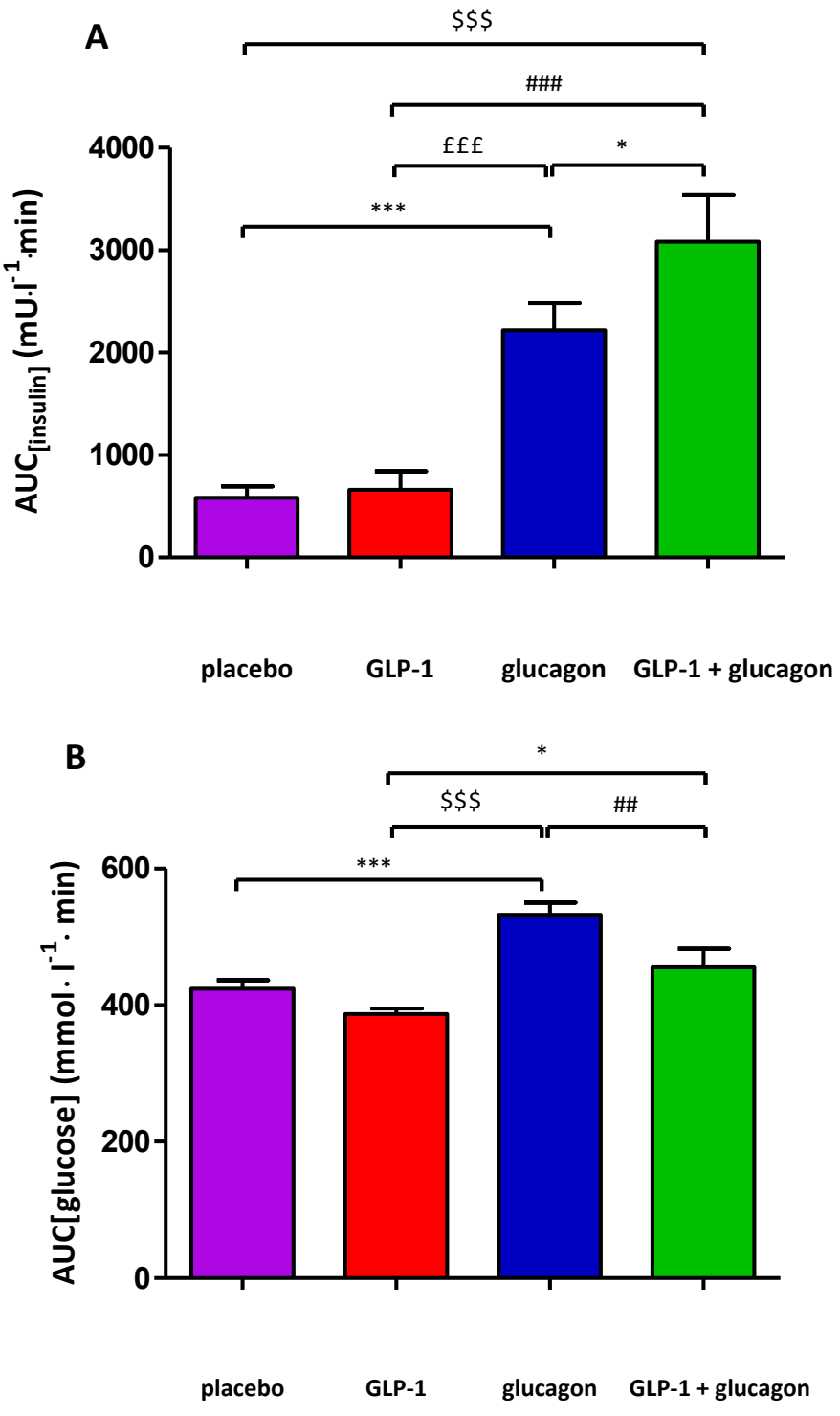


Figure 4.8 A & B Area under the curve for insulin (A) and glucose (B) following infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. A: The combination infusion resulted in a significant rise in serum insulin compared to all other groups (placebo $$$ p<0.001; GLP-1 ###p<0.001; glucagon *p<0.05). Glucagon alone also significantly increased insulin secretion compared to placebo and GLP-1 alone (*p<0.001, £££p<0.001 respectively). B: Glucagon alone increased plasma glucose compared to both placebo and GLP-1 (***p<0.001 and $$$p<0.001 respectively), and the combination infusion also increased plasma glucose compared to GLP-1 alone (##p<0.01) and glucagon alone (*p<0.05) N=10. Analysis by repeated measures one way ANOVA with Tukey's post-hoc test.**

4.4.6 The acute effect of low dose co-infusion of glucagon and GLP-1 on plasma levels of total and acyl ghrelin

Samples were taken for ghrelin measurement at t=0 and 90 minutes, 'pre' and 'post' infusion. Total and acyl ghrelin were not significantly affected by infusion of glucagon or GLP-1, either alone or in combination at the doses specified (Figure 4.9 A & B). Blood samples for ghrelin were taken prior to, and during the hormone infusion. The sample taken during the infusion was taken prior to the ad-libitum meal. Physiologically, ghrelin increases in the fasted state or prior to a meal, as it is orexigenic, thus a small rise might have been expected in the samples taken during infusion. However, there is no change compared to placebo so this lack of ghrelin increase is unlikely to be an effect of the hormone infusions.

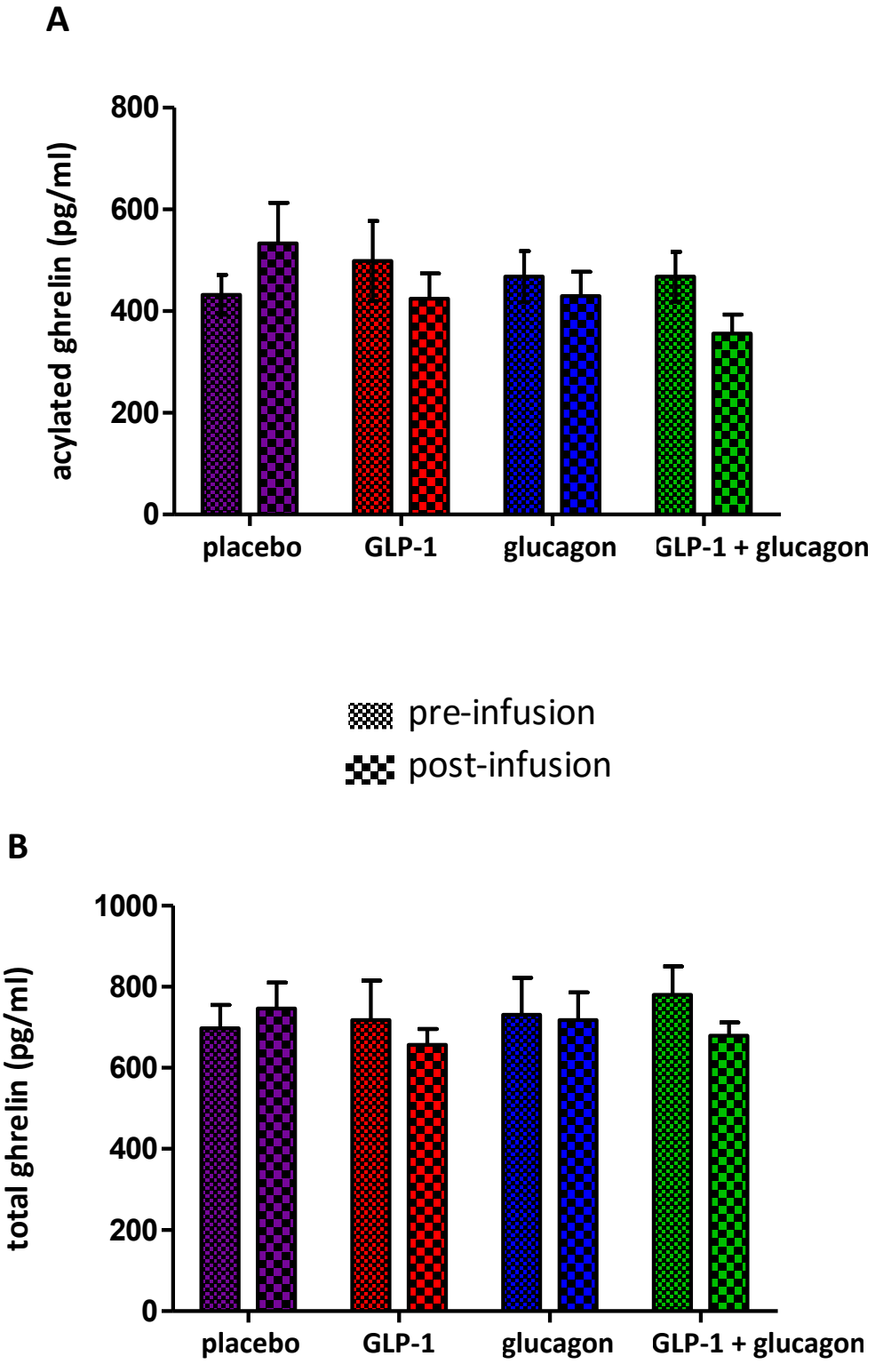


Figure 4.9 The acute effect of low dose co-infusion of glucagon and GLP-1 on A: acylated and B: total ghrelin levels. Samples were taken both before, and following infusion of each of placebo (gelofusine, B. Braun, Crawley, UK), GLP-1 alone at 0.4 pmol/kg/min, glucagon alone at 10 ng/kg/min, and a combination of GLP-1 and glucagon at the above doses. Infusion of the hormones either alone or in combination had no significant effect on ghrelin concentrations. Data were analysed using a repeated measures two-way ANOVA with Bonferroni post-hoc test. N=13

4.4.7 The acute effect of low dose co-infusion of glucagon and GLP-1 on cardiovascular parameters

Pulse and blood pressure were measured during the course of the study at t=-60, -30, 0, 40, 70, 90, 120, 150 and 180 minutes. None of the infusions caused any statistically significant difference in any of pulse, systolic blood pressure or diastolic blood pressure during the course of the study (Figure 4.10). Data was analysed with a two-way repeated measures ANOVA and Tukey's post-hoc test. N=13.

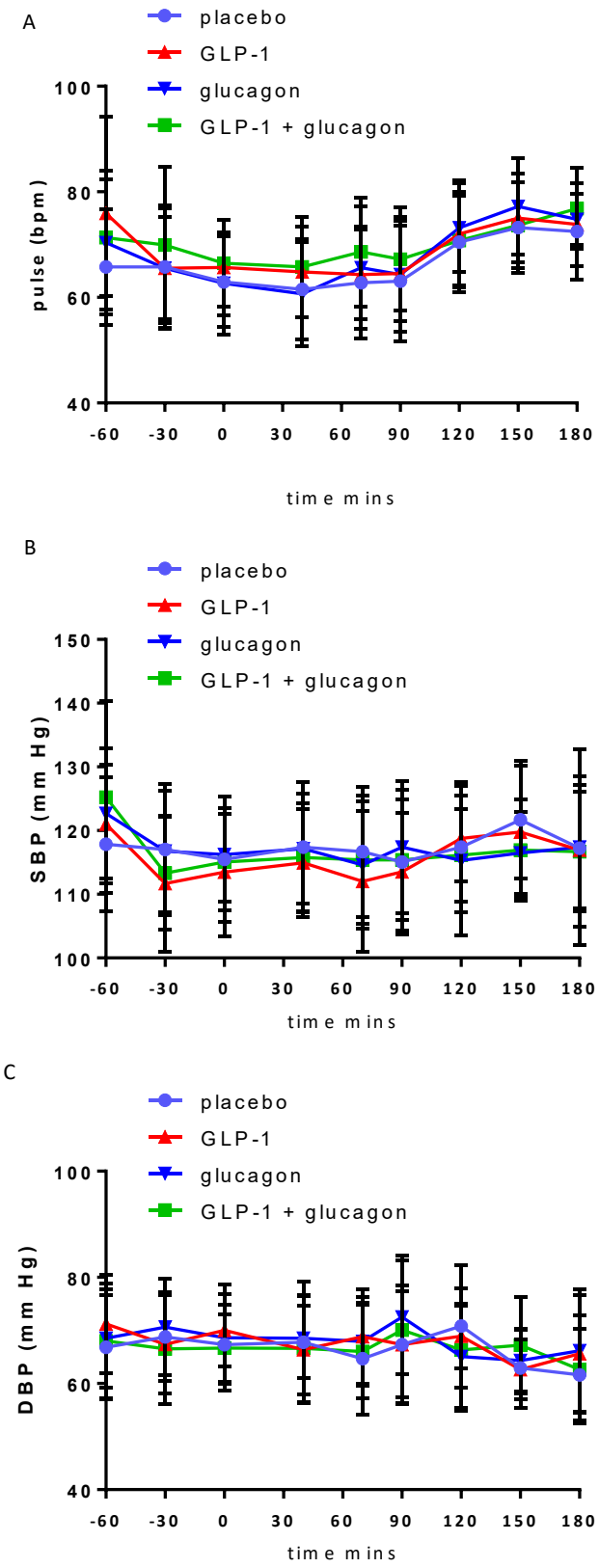


Figure 4.10 The acute effect of co-infusion of low dose glucagon + GLP-1 on cardiovascular parameters (A: pulse, B: systolic blood pressure and C: diastolic blood pressure). There was no statistical significance between the groups for any of these parameters. Analysis with two-way repeated measures ANOVA with Tukey's post hoc test n=13.

4.5 Discussion

In this study I have once again explored the effects of a combination of glucagon and GLP-1 in order to mimic the actions of oxyntomodulin, a dual agonist at the glucagon and GLP-1 receptors. However, in this instance both hormones were given at subanorectic doses to avoid nausea, and the subsequent reduction of food intake was assessed.

Glucagon and GLP-1 at doses of 10 ng/kg/min and 0.4 pmol/kg/min respectively caused a significant reduction in food intake when co-infused in healthy overweight volunteers. The combination of these two hormones at the low doses specified was able to confer a 13% reduction in food intake compared to placebo. Neither hormone when infused alone was able to replicate this anorectic effect. Any anxiety regarding the study which may have accounted for a reduction in food intake was addressed by the inclusion in the study design of an acclimatisation visit, the data for which was not included in the subsequent analysis. In addition, any reduction in food intake which could potentially have been attributed to the subjects tiring of their meal choice as the study progressed, the so-called 'order effect', was avoided by the randomised crossover design of the study, wherein the order of study visits was different between participants.

The nausea experienced by a small number of study participants in the group receiving the combination infusion was unlikely to have been responsible for any reduction in food intake, as all episodes of nausea were reported to be post-prandial, and therefore had a limited bearing on how much food these participants consumed. Two participants vomited after the ad-libitum meal, in the group who had received glucagon alone, thus it would appear that the post-prandial nausea and vomiting could be mediated more strongly via the glucagon receptor than that of GLP-1. Both of these peptide hormones are known to cause nausea when given for their clinical indications - GLP-1 for the treatment of obesity and diabetes (Pi-Sunyer et al., 2015, Sun et al., 2015, Astrup et al., 2012, Lean et al., 2014) and glucagon for amelioration of hypoglycaemic episodes and as a test of pituitary reserve (Leong et al., 2001), albeit at much higher doses than were used in this study. A potential explanation

for the post-prandial nature of the nausea and vomiting is a delay in gastric emptying, a known effect of both GLP-1 and glucagon (Flint et al., 1998, Chernish et al., 1978) The reduction in food intake itself may in addition be mediated via the direct action of glucagon and GLP-1 on the hypothalamus and brainstem via the median eminence, (see sections 1.1.2, 1.2.1 and 1.3.1) or by the action of glucagon and GLP-1 via vagal afferents. Indeed, following vagotomy, the anorectic effects of peripheral administration of glucagon and GLP-1 are attenuated (Abbott et al., 2005) (Geary and Smith, 1983). In the previous study (Chapter 3), I have demonstrated a fall in the levels of both total and acyl ghrelin after administration of the combination of glucagon and GLP-1, and moreover both GLP-1 and glucagon have independently been shown to reduce ghrelin levels (Hagemann et al., 2007, Arafat et al., 2005), albeit at high doses. Whilst the lower doses used in this study did not achieve a significant fall in ghrelin levels as was seen previously, it is possible that there is some effect on ghrelin suppression which may have contributed to the reduction in food intake.

The GLP-1 and glucagon levels achieved in this study cannot be compared to previous studies as different doses of peptide, different study protocols and different assays were employed – in this study I measured active rather than total GLP-1 levels, and this offers an explanation as to why I achieved peak plasma concentrations of 16.7 ± 2.8 pmol/l (range 3.0-30.1 pmol/l) in the GLP-1 group, and 13.0 ± 2.1 pmol/l (range 3.8 – 29.8 pmol/l) in the glucagon + GLP-1 group (figure 4.3A) compared to the peak levels of around 60 pmol/l seen in Neary et al's study (at the same time point, t=90) which infused GLP-1 at the same dose. After the ad-libitum meal at t=90, I have demonstrated a small rise in active plasma GLP-1 in the glucagon and placebo groups. This is likely to represent the physiological post-prandial rise in endogenous GLP-1 stimulated by oral ingestion of a nutrient load. This post prandial GLP-1 release is not evident in the groups receiving GLP-1 either alone or in combination with glucagon, and it is likely that in these two groups the physiological rise was either blunted or masked by the supraphysiological hormone levels conferred by the infusions.

The dose of glucagon in my previous study (Chapter 3) was 5-fold higher than the dose in this current study. The current much lower dose of glucagon was chosen to be subanorectic to avoid nausea, whereas the dose in the previous study was chosen to reliably and reproducibly increase energy expenditure. Nausea was not assessed in the previous study as it was impractical to obtain VAS scores in subjects who were under the calorimeter hood for the majority of the study. In addition, there was no ad-libitum study meal against which to compare in the previous study. Peak glucagon levels in the previous study were higher than in the current study but not five-fold higher. This non-linear kinetic behaviour suggests that the metabolic clearance mechanisms for glucagon, which are predominantly hepatic (Alford et al., 1979), were not saturated in either study. Of note, there was a small increase in plasma glucagon levels just prior to the ad-libitum meal in the placebo group. This is a predictable physiological phenomenon as glucagon is highest before meals and in the fasted state (figure 3.5B).

In the previous study (Chapter 3), the addition of GLP-1 to glucagon was able to ameliorate but not negate completely the hyperglycaemic effect of glucagon. In the current study, I have once again demonstrated a significant fall in plasma glucose attributable to the addition of GLP-1, as shown by the discrepancy between the glucagon and combination groups (Figure 4.7 A&B). Indeed, at the lower doses used in the current study the combination of glucagon and GLP-1, despite an initial peak in blood glucose levels at t=40, was able to normalise blood glucose to levels comparable with the placebo group by t=70. Once again, this is likely to be secondary to the greater insulinotropic effect of the combination infusion compared to glucagon alone (Figure 4.7A & 4.8A). Plasma glucose and insulin levels following t=90 when participants consumed an ad-libitum meal are very difficult to interpret, as by the nature of the ad-libitum meal, all participants had markedly different caloric intakes. Post-prandially there is a rise in serum insulin levels in all groups as would be expected, but interestingly there is a fall in plasma glucose in the group receiving glucagon. It is likely that this is due to the marked post-prandial rise in insulin levels in this group which could be attributable to a dual effect of both the infusion and the meal consumption. In this study with a slightly extended hormone infusion protocol and lower peptide hormone doses, the GLP-1 mediated amelioration of hyperglycaemia caused by

glucagon is much more evident than in my previous study. If this combination of hormones was employed in the longer term, it is possible that the result could be glucose-neutral, but in order for this to be of clinical use the combination must retain its benefit when tested in participants with impaired glucose tolerance or diabetes. Furthermore, the likely cause for the hyperglycaemia seen with glucagon infusion is liberation of hepatic glycogen stores. Whether hyperglycaemia would still be seen when these stores were depleted also remains to be investigated.

In addition to favourable effects on glycaemia and food intake, I also investigated the ability of this lower dose hormone combination to increase energy expenditure, as demonstrated in my previous study. Not unsurprisingly the lower doses of hormones, and specifically glucagon which appears to be responsible for the increase in EE seen in Chapter 3, did not increase EE to the same extent as the higher doses used previously. However, infusion of glucagon alone and GLP-1 plus glucagon did increase resting EE in Kcal/day compared to both placebo and GLP-1 despite not reaching statistical significance. The change from baseline in REE following infusion of glucagon was 67 ± 28 kcal/day, and that of the combination infusion was 61 ± 29 kcal/day compared to placebo (20 ±19 kcal/day). It could be hypothesised that the chronic effect of this small and seemingly insignificant increase in REE would in the long term confer weight loss, but this remains to be investigated.

The effect on carbohydrate oxidation and fat oxidation is reassuringly reproducible when compared to that seen in the previous study (Chapter 3), with an increase of carbohydrate oxidation in the glucagon alone and combination groups, and a fall in fat oxidation in the same groups. As previously discussed, these are likely to be secondary to the action of glucagon on hepatic glycogenolysis, and the action of insulin in the inhibition of hormone sensitive lipase (see section 3.5). The increase in protein oxidation in the glucagon group compared to the GLP-1 group is of questionable significance, as there is no effect compared to placebo, and the finding is not replicated compared to the combination group.

In summary, the combination of low dose infusion of glucagon and GLP-1 is able to additively reduce food intake by 13% compared to placebo. It is also able to ameliorate the hyperglycaemia caused by glucagon alone, to levels comparable with placebo. Although not significant, the combination of hormones is also able to increase EE compared to placebo which in the long term may confer weight loss. Further studies incorporating chronic administration of the two hormones (or analogues thereof) in combination will be necessary to investigate this. Furthermore, studies of this combination of hormones in participants with impaired glucose tolerance will be important to investigate if they confer a potential clinical benefit.

CHAPTER 5: THE ACUTE EFFECT OF CO-INFUSION OF PYY AND GLP-1 ON GLUCOSE HOMEOSTASIS

5.1 Introduction

Following gastric bypass surgery, there is a rapid and sustained improvement of glycaemic control – at least for the first few years post-operatively (Sjöström, 2013, Mingrone et al., 2012, Brethauer et al., 2013, Pournaras et al., 2010b). The improvement in glycaemic control becomes apparent within the first few days after surgery, and is seemingly independent of weight loss, although in the longer term, weight reduction almost certainly plays a role in improving insulin resistance and glycaemic handling. The mechanism remains unclear for this improvement in blood glucose that occurs rapidly after surgery, and it is felt that it may at least in part be mediated by the enhanced post-prandial gut hormone response post-operatively. The enhanced GLP-1 incretin effect acts to increase insulin secretion, but questions remain as to the contribution of other gut hormones to the improvement in glycaemic control. In this study, I have assessed the contribution of PYY(3-36) on the improved glucose homeostasis seen post bariatric surgery.

PYY(3-36) has a particular affinity for the Y2 receptor. PYY(1-36), the full-length peptide, is able to activate the Y1 receptor. Chandarana et al confirmed the presence of Y1 and Y4 receptor mRNA expression in pancreatic islet beta cells, but failed to show expression of Y2 or Y5 receptors, suggesting that any effect of PYY(3-36) on glucose homeostasis is not mediated directly by the islet (Chandarana et al., 2013). In contrast, it has been well documented that activation of the Y1 receptor on pancreatic islet cells by NPY inhibits insulin release (Wang et al., 1994, Schwetz et al., 2013, Chandarana et al., 2013), and that this effect is replicated by PYY(1-36) (Chandarana et al., 2013).

PYY(3-36), the active form of the hormone, has been shown in previous animal studies to acutely improve insulin sensitivity by enhancing insulin-mediated glucose disposal in the context of hyperinsulinaemia although it had no effect on endogenous glucose production (Van den Hoek et al., 2004). The same group went on to show that this effect on glucose disposal was not lost during chronic administration of PYY(3-36) although they did note a tachyphylaxis in terms of reduction of food intake by 7 days of PYY(3-36) administration (Van den Hoek et al., 2007). Further to this, Chandarana et al

suggested that PYY can enhance insulin secretion and improve glucose levels. They demonstrated that PYY(3-36) reduced glucose levels after an intraperitoneal glucose tolerance test (IPGTT) in mice, and they also observed an associated increase in initial insulin secretion, although this effect reduced with time. Interestingly, these effects were only achieved in the post-prandial state and were not reproducible in fasted animals (Chandarana et al., 2013). The group also confirmed that this effect was mediated via the Y2 receptor by reproducing their results with administration of a Y2 agonist, and suggested that whilst PYY(3-36) does not appear to have any direct effect on insulin secretion itself, it may exert its effects on insulin secretion by increasing hepato-portal GLP-1 concentrations (Chandarana et al., 2013)

In humans, Sloth et al showed an increased post-prandial insulin response with infusion of PYY(3-36) at a dose of 0.2pmol/kg/minute (Sloth et al., 2007). They also infused PYY at a higher dose of 0.8pmol/kg/minute which caused significant nausea.

As outlined in previous chapters, novel obesity therapies are increasingly incorporating two or more drugs at low doses to confer therapeutic benefits without debilitating side effects. Here, I have studied the effects of a combination of GLP-1 and PYY(3-36) on the acute insulin response to glucose to investigate the mechanisms behind the improvement in blood glucose seen so rapidly after bariatric surgery. Whilst both PYY(3-36) and GLP-1 are known to cause nausea at higher doses, the low doses used in combination in this study aim to negate this effect.

5.2 Hypothesis and Aims

5.2.1 Hypothesis

- In healthy overweight human volunteers an infusion of GLP-1 will enhance the acute insulin response to glucose and co-administration with PYY will not attenuate this response. The use of the two peptides in combination at low doses will not cause excessive nausea.

5.2.2 Aims

- To investigate the effect of co-infusion of PYY and GLP-1 on the acute insulin response to glucose in healthy overweight human volunteers

5.3 Materials and Methods

5.3.1 Subjects

Fourteen overweight and obese volunteers were recruited by advertisement in local media. The mean age was 34.5 ± 2.6 years (range 21-50), and the average BMI was 30.1 ±0.9 kg/m^2 (range 26.8-35.9). Volunteer demographics are outlined in table 5.1.

Inclusion criteria were that volunteers should be aged 18-65 years, with a BMI of 25-40 kg/m^2, non-smokers with a stable body weight for the preceding three months. All volunteers underwent screening with a full medical history and examination, 12-lead electrocardiography, haematological and biochemical assessment. Volunteers then underwent a standard 120 minute 75g oral glucose tolerance test (OGTT) to exclude diabetes or impaired glucose tolerance, and to standardise inter-participant variation as far as possible in terms of insulin secretion and sensitivity. Volunteers were excluded if they had diabetes or impaired glucose tolerance according to WHO criteria (WHO and IDF, 2006), if they were pregnant or breastfeeding, if they had a history of alcohol or substance abuse within the last five years, or if they had any past medical history or were on any medication which was felt to interfere with the study or if enrolling in the study could potentially cause harm to the volunteer. Anyone having donated blood in the previous three months, or having participated in another study involving an investigational drug within two months was also excluded. Participants were free to withdraw from the study at any time, and female volunteers were asked to maintain adequate contraception during the course of the study and for one month afterwards.

All volunteers gave informed written consent. The study was carried out in accordance with the principles of the Declaration of Helsinki, and was approved by the local West London Research Ethics committee (REC 09/H0707/77).

Volunteer	Weight (kg)	Height (m)	BMI (Kg/m^2)	Age	Sex
1	81	1.55	33.7	28	F
2	91.5	1.82	27.6	33	M
3	76.9	1.66	27.9	21	F
4	90.7	1.72	30.7	32	M
5	79	1.7	27.3	27	M
6	104.6	1.80	32.5	46	M
7	81.8	1.74	27.0	27	M
8	115	1.79	35.9	52	F
9	91.2	1.79	28.5	26	M
10	110.6	1.84	32.7	50	M
11	92.4	1.82	27.9	34	M
12	81.6	1.72	27.6	35	M
13	89.8	1.84	26.8	44	M
14	DM	DM	35	28	F
Mean			30.1	34.5	F3/11M

Table 5.2 Demographic details and means of all volunteers included in the study 'The acute effects of co-infusion of PYY and GLP-1 on glucose homeostasis' (DM = data missing)

5.3.2 Peptides

Peptides were obtained as described in the two previous studies. All safety tests and aliquoting in those studies had been performed by the supplying companies. In this study, a larger quantity of peptide was purchased from the supplier and subsequent measures outlined below were carried out in-house:

Peptides were synthesised by Bachem Ltd (St Helens, Merseyside). Following initial high fidelity synthesis, peptides were purified by high resolution high performance liquid chromatography (HPLC). Peptide purity and composition was verified by amino acid analysis.

In-house, peptides were then dissolved in sterile 0.9% saline (Bayer plc, Newbury, UK), aliquoted into sterile glass vials under strict aseptic conditions in a laminar flow cabinet, vacuum packed and freeze dried. A sample of vials was then sent to the Imperial College Healthcare NHS Trust microbiology department for culture to exclude common pathogenic micro-organisms. A further sample of vials was sent for amino acid analysis to verify the actual peptide content of the vials (Alta Bioscience, Birmingham, UK). Each batch of peptide also underwent Limulus Amoebocyte Lysate (LAL) testing, performed by Associates of Cape Cod International, Deacon Park, Moorgate Road, Liverpool, United Kingdom to check for endotoxins.

Toxicity studies were then carried out in mice. Greater than 10 x the dose to be given to man (in pmol/kg body weight) was administered to 20 mice and compared to a saline control group. Animals were observed for adverse events, and then one group was culled at 48 hours, and another at 14 days. All then underwent necropsies with histological examination of internal organs by an expert rodent pathologist, including lungs, heart and kidneys to exclude adverse effects of the peptide administration.

Peptide vials were then stored in a dedicated freezer at -20^{o}C.

5.3.3 Protocol

A preliminary dose finding phase was undertaken to find doses of peptide which gave a PYY plasma concentration of 80-120pmol/l, and a total GLP-1 plasma concentration of 100-140 pmol/l, and that were well tolerated by study participants. These plasma target levels were chosen because for infusion of PYY(3-36) and GLP-1, they have been shown to increase AUC for postprandial insulin secretion following an ad-libitum meal (Sloth et al., 2007) and increase insulin secretion in response to a graded glucose infusion (Kjems et al., 2003) respectively (Dose finding data not shown). The doses chosen for use in the study were PYY(3-36) 0.15pmol/kg/min, and GLP-1(7-37 amide) 0.2pmol/kg/min.

The study was then conducted in a randomised controlled, single-blind four-way cross over manner. Randomisation was performed by an independent investigator not otherwise involved in the study. All study visits were at least three days apart to allow for peptide washout. Volunteers underwent a 'sham' or placebo visit which was unblinded to them wherein they received an infusion of vehicle alone (Gelofusine, (B. Braun, Crawley, UK)) to allow acclimatisation to the study. Following this acclimatisation visit, participants then attended for four further study visits: 1) placebo (gelofusine), 2) GLP-1(7-36 amide) (0.2pmol/kg/min; (Bachem, St Helens, Merseyside) 3) PYY(3-36) (0.15pmol/kg/min; Bachem, St Helens, Merseyside) and 4) PYY(3-36) and GLP-1(7-36 amide) combined at the above doses. Gelofusine was used as the vehicle for all infusions to minimise peptide adsorption to the inside of the infusion apparatus(Kraegen et al., 1975).

Participants arrived at the clinical research facility at 0800h having fasted from 2200h the night before. They had refrained from alcohol and strenuous exercise for the preceding 24 hours. On arrival, female volunteers underwent a urinary βhCG test to exclude pregnancy. Two cannulae were then sited in peripheral veins, one for hormone infusion and one for blood sampling. The hormone infusion was commenced at t=0. At t=60 a frequently sampled intravenous glucose tolerance test (FSIVGTT) was performed. An IV bolus of 0.3g/kg of 20% dextrose was administered over two minutes (Pacini and Mari, 2003)and blood samples were drawn as shown in figure 5.2 for assessment of the acute insulin

response to glucose (AIRg) and the insulin sensitivity index (Si). The FSIVGTT was not augmented with insulin as in some protocols, as none of the participants had impaired glucose tolerance or diabetes. In addition, it was felt that infusion of GLP-1 may to some degree augment the insulin response. The infusions were continued until t=240 minutes and then terminated. Participants were then given a meal and discharged.

During the study, volunteers completed visual analogue scales (VAS) for subjective evaluation of hunger, nausea and satiety (Appendix A).

Blood samples were taken for glucose and insulin at t=-30, 0, 20, 40, 60, 62, 63, 64, 65, 66, 68, 70, 72, 74, 78, 80, 82, 85, 90, 100, 110, 130, 160, 200 and 240 minutes. In addition, samples were taken at t=0, 20, 40, 60, 80, 100, 160 and 240 minutes for analysis of plasma gut hormones (PYY and GLP-1). For blood collection details please see table 3.2. Sample collection for PYY is identical to that for GLP-1. All samples were placed immediately on ice, (except insulin samples which were allowed to clot first) then centrifuged at $4^{o}C$ at 4000 revolutions per minute (rpm) for 10 minutes, separated and stored at $-20^{o}C$ until analysis.

Vital signs for each subject (pulse and blood pressure) were taken at t=0, 20, 40, 100, 160, 240 minutes.

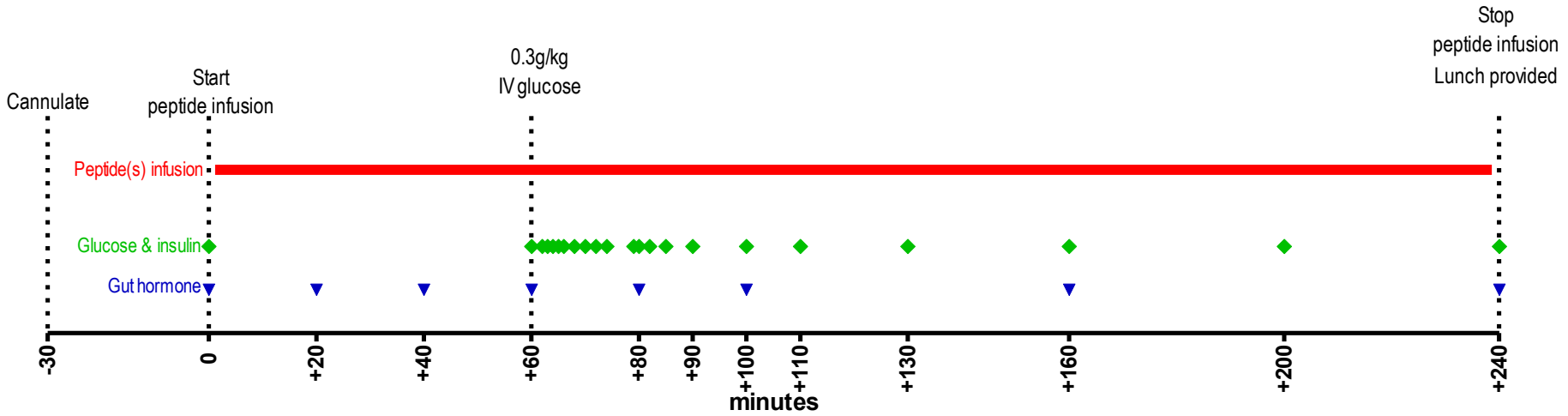


Figure 5.11 'The acute effect of co-infusion of PYY(3-36) and GLP-1 on glucose homeostasis': Study Protocol. Participants arrived at t=-30, when they were cannulated. The peptide hormone infusion was commenced at t=0 and continued until t=240. At t=60 a 0.3g/kg bolus of IV glucose was administered and a FSIVGTT was performed. Blood samples were drawn as indicated by the blue (gut hormones), and green (glucose and insulin) markers

5.3.4 Assays

Glucose and Insulin

Glucose and insulin samples were analysed by the Department of Chemical Pathology, Imperial College Healthcare NHS Trust (as described in section 3.3.5).

GLP-1

Total GLP-1 was measured as described in section 3.3.5 by established in-house radio-immunoassay.

PYY

Plasma total PYY was measured using an established, specific and sensitive in-house radio-immunoassay (Adrian et al., 1985), developed and maintained by Prof M Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College). Once again, all samples were processed in duplicate within a single RIA.

PYY antibodies (Y21) were produced from inoculation of synthetic porcine PYY coupled to BSA into rabbits. The antibodies were raised against the C terminal of PYY, and cross reacted fully with human PYY(1-36) and PYY(3-36). The antibodies do not react with the other 'PP fold' family members NPY or PP, and do not react with any other gut hormones

^{125}I-PYY was produced by Professor Ghatei using the Iodogen method (Wood et al., 1981)

The assay was performed in duplicate in a total volume of 700µl, using 0.06M phosphate buffer with 0.3% BSA, 100 µl of sample, 50µl of antibody, and 50µl of label. The assay was then incubated at 4^{o}C for 96 hours before separation by the secondary antibody method. Secondary antibody (sheep anti-rabbit antibody) was added to each sample. All samples were then centrifuged at 1500 rpm, 4^{o}C for 20 minutes to create a pellet. The pellet consists of secondary antibody plus any bound labelled hormone in the tube. The supernatant contains free labelled hormone which has been displaced from antibody by 'cold' sample. Free and bound fractions were separated and both fractions counted for 180 seconds (γ-counter model NE1600, Thermo Electron Corporation).

Quality control samples (QCs) were run as standard.

5.3.5 Statistics

Statistical analysis was carried out using GraphPad Prism 5.0d (GraphPad Software, San Diego, CA). All data are reported as mean ± SEM and significance taken at $p<0.05$ unless otherwise specified. Serum insulin and plasma glucose levels were analysed using a two-way repeated measures ANOVA with Bonferroni post-hoc test. AUC was calculated using the trapezoidal method for serum insulin concentration from t=0 to t=10 inclusive. This allows calculation of the acute insulin response to glucose (AIRg 0-10min), which is taken as an index of beta cell function, and the first phase insulin response (Kahn et al., 1993) .

VAS scores were analysed using a repeated measures (non-parametric) Friedman's test with Dunn's multiple comparison post-hoc test.

The 'Bergman Minimal Model' of glucose disappearance was used to determine SI, the insulin sensitivity index. This index describes the ability of insulin to enhance glucose disposal and is calculated using the insulin and glucose concentrations obtained during the intravenous glucose tolerance test (Pacini and Bergman, 1986). SI and AIRg were then used to calculate the disposition index (DI), which is SI x AIRg – this is a measure of beta cell function, representing the ability of the beta cell to adapt and compensate for changes in insulin sensitivity.

5.4 Results

5.4.1 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma glucose and insulin concentrations

Insulin and glucose plasma levels were assessed during the course of each infusion. Blood was drawn at t=-30, 0, 20, 40, 60, 62, 63, 64, 65, 66, 68, 70, 72, 74, 78, 80, 82, 85, 90, 100, 110, 130, 160, 200 and 240 minutes for analysis. The effect of each of the following infusions: 1) gelofusine alone, 2) GLP-1(7-36 amide) at 0.2pmol/kg/min 3) PYY(3-36) at 0.15pmol/kg/min and 4) PYY(3-36) and GLP-1(7-36 amide) combined at the above doses plasma insulin and glucose concentrations was then assessed.

Baseline insulin concentration across all infusion groups and participants was 10.9 ± 0.9mU/l (range 2.5 - 49.6 mU/l), with peak levels at t=66 minutes of A (placebo): 106.1 ± 18.1mU/l; B (GLP-1): 144.0 ± 23.6mU/l; C (PYY(3-36)): 112.9 ± 16.8mU/l and D (PYY(3-36) + GLP-1): 149.8 ±23.7mU/l (Figure 5.2).

Baseline glucose across all infusion groups and participants was 5.34. ± 0.06 mmol/l (range 4.3-6.5 mmol/l), with peak levels at t=66 minutes of A (placebo): 14.7 ± 0.6 mmol/l; B (GLP-1) 15.3 ± 0.6mmol/l); C (PYY(3-36)): 14.4 ± 0.7mmol/l and D (PYY(3-36) + GLP-1): 14.9 ± 0.6mmol/l (figure 5.3).

All groups were compared using a repeated measures two-way ANOVA with Bonferroni post-hoc test, but no significant difference between the groups was identified. N=14.

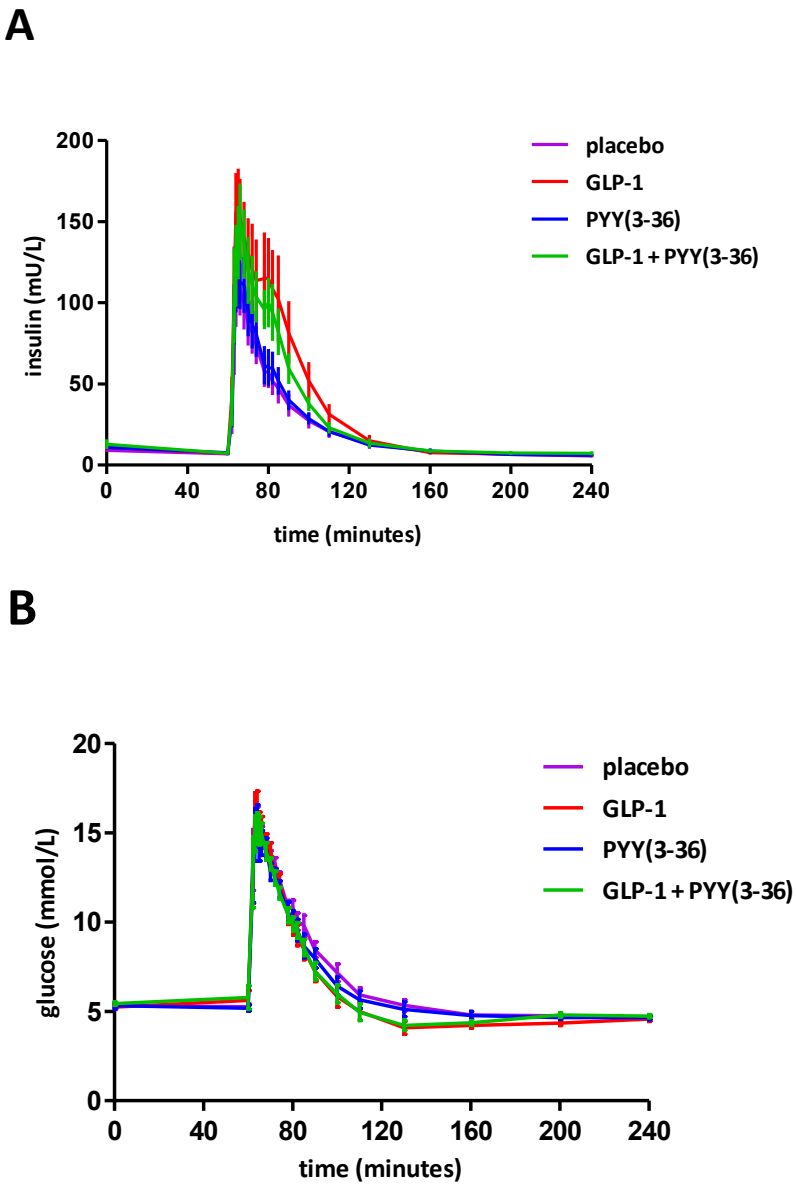


Figure 5.2A: The acute effect of co-infusion of PYY (3-36) and GLP-1 on serum insulin concentrations following infusion of gelofusine alone, GLP-1(7-36 amide) at 0.2pmol/kg/min, PYY(3-36) at 0.15pmol/kg/min and PYY(3-36) and GLP-1(7-36 amide) combined at the above doses. Data were analysed with a two-way repeated measures ANOVA and Bonferroni post-hoc test. There was no statistical significance between the groups. N=14. B: The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma glucose concentrations following infusion of gelofusine alone, GLP-1(7-36 amide) at 0.2pmol/kg/min, PYY(3-36) at 0.15pmol/kg/min and PYY(3-36) and GLP-1(7-36 amide) combined at the above doses. Data were analysed with a two-way repeated measures ANOVA and Bonferroni post-hoc test. There was no statistical significance between the groups. N=14

5.4.2 AUC for insulin and glucose for the first 10 minutes following the FSIVGTT glucose bolus during co-infusion of PYY(3-36) and GLP-1

The AUC for insulin in the first 10 minutes following the glucose bolus of the intravenous glucose tolerance test can also be termed the acute insulin response to glucose (AIRg). This can then be used as part of the minimal model tool to calculate the disposition index, DI, which is a dimensionless measure of how well beta cells respond to a glucose load, and therefore a surrogate for beta cell function.

AUC was calculated for both insulin and glucose for the first 10 minutes after the glucose bolus was administered (AUC insulin 0-10, and AUC glucose 0-10). AUC was calculated using the trapezoidal method, and was subsequently analysed using a one-way repeated measures ANOVA with Tukey's post-hoc test. GLP-1 alone was able to significantly increase the AUC insulin 0-10 compared to placebo (1136.1 ± 165.1 mU $\cdot L^{-1} \cdot$ min vs 799.2 ± 117.5 mU $\cdot L^{-1} \cdot$ min *$p<0.05$), but none of the other infusions had a significant effect on insulin release. There was no statistically significant difference in glucose levels between any of the groups.

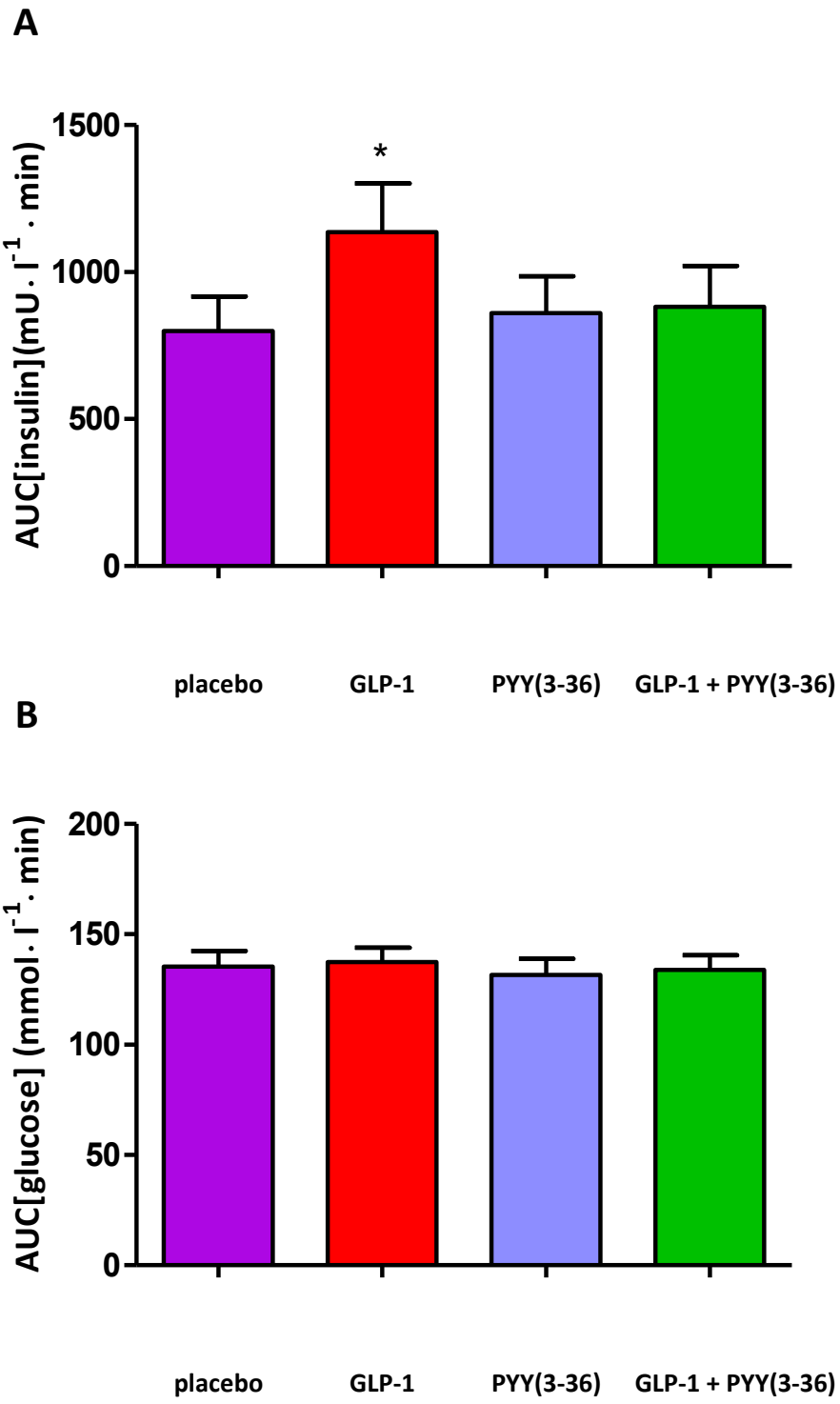


Figure 5.3 AUC for A: Insulin and B: glucose in the first 10 minutes following the intravenous glucose bolus in the FSIVGTT. There was no statistically significant change in plasma glucose levels during any of the infusions. There was a significant increase in insulin when participants received an infusion of GLP-1 alone (*p<0.05), but not the other infusions. Data were analysed using a one way repeated measures ANOVA with Tukey's post-hoc test. N=14

5.4.3 AIRg, S_i and DI response to IV glucose during co-infusion of PYY(3-36) and GLP-1

AIRg was achieved by calculating AUC insulin 0- 10 minutes. GLP-1 alone significantly increased the AIRg compared to placebo (1136.1 ± 165.1 mU · L^{-1} · min vs 799.2 ± 117.5 mU · L^{-1} · min *p<0.05), and there was no significant difference between any of the other groups.

S_i was calculated by Dr Ian Godsland, (Reader in Human Metabolism at Imperial College, London with an interest in insulin secretion and resistance), using a previously described method(Godsland et al., 2006). No statistical significance was found between the four groups for S_i.

DI is a product of AIRg and S_i. As S_i is effectively the same in all study groups, DI reflects the AIRg measurements. There was therefore a statistically significant change in DI in the GLP-1 groups vs placebo (5065.7 ± 1061.1 (S_i x AIRg) vs 2909.6 ± 373.0 (S_i x AIRg) *p<0.05), but not between any of the other groups.

All analysis was by repeated measures one way ANOVA with Tukey's post-hoc test. N=14

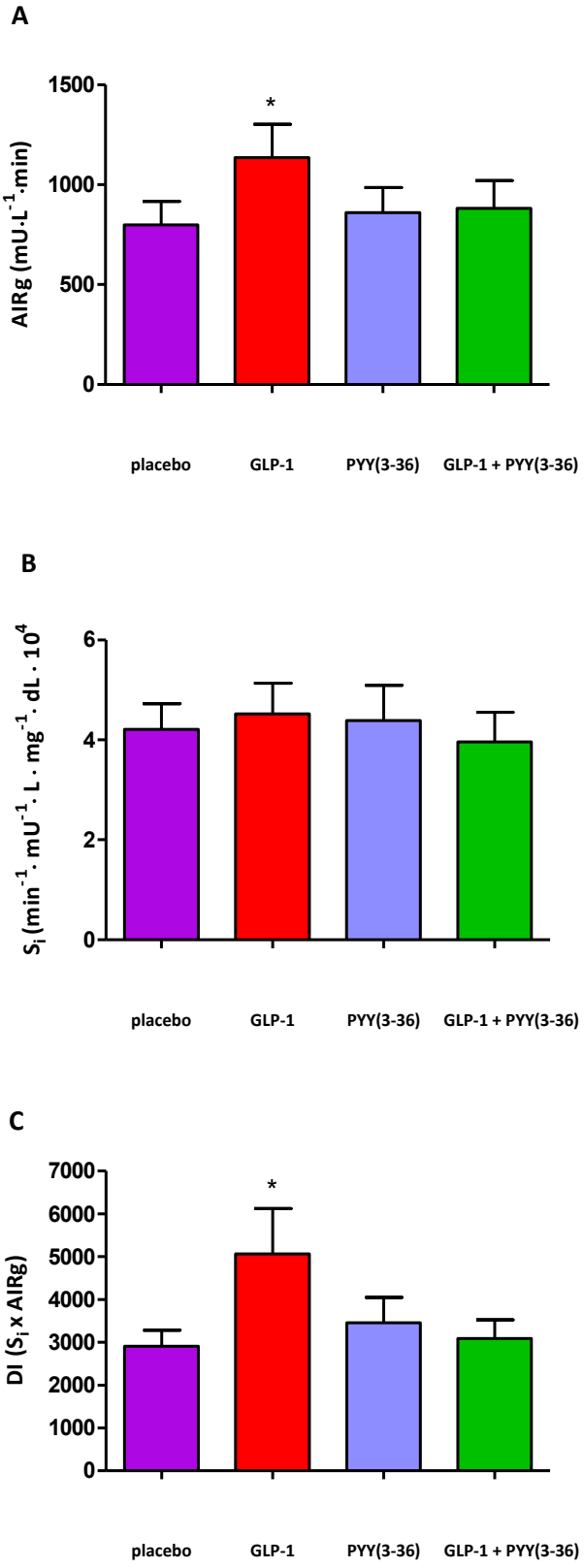


Figure 5.4 AIRg, S_i and DI following infusion of gelofusine alone, GLP-1(7-36 amide) at 0.2pmol/kg/min PYY(3-36) at 0.15pmol/kg/min and PYY(3-36) plus GLP-1(7-36 amide) combined at the above doses. GLP-1 causes a significant increase in A: AIRg vs placebo (*p<0.05), and for C: DI vs placebo (*p<0.05) but there was no statistical significance between any of the groups for S_i. Data were analysed by one-way ANOVA with Tukey's post hoc test N=14

5.4.4 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma PYY and GLP-1 concentrations

Participants received four infusions in a randomised single blinded crossover study design. The infusions were 1) gelofusine alone (control); 2) GLP-1 alone at 0.2 pmol/kg/min; 3) PYY(3-36) alone at 0.15pmol/kg/min; and 4) Co-infusion of GLP-1 plus PYY(3-36) at the above doses. Blood samples were drawn to t=0, 20, 40, 60, 80, 100, 160 and 240 minutes for analysis of PYY and GLP-1 levels.

Baseline GLP-1 concentrations for the placebo group and PYY group were 43.7 ± 6.2 pmol/l (range 5.7 – 85.2 pmol/l) and 43.8 ± 7.7pmol/l (range 16.3 – 111.0 pmol/l) respectively. (Figure 5.6A). Baseline GLP-1 concentrations in the GLP-1 and GLP-1 + PYY(3-36) groups were 55.6 ± 9.7 pmol/l (range 19.7 – 138.6 pmol/l) and 52.6 ± 15.2 pmol/l (range 5.9 – 227.3 pmol/l) respectively. Plasma GLP-1 concentrations rose to peak levels at around t=100 minutes and then plateaued, with a peak concentration of 109.6 ± 20.6 pmol/l (range 46.8-322.1 pmol/l) in the GLP-1 group, and 96.2 ± 10.3 pmol/l (range 58.2 – 190.5 pmol/l) in the PYY(3-36) + GLP-1 group (figure 5.6A).

Baseline PYY concentrations for the placebo and GLP-1 groups were 47.7 ± 8.7pmol/l (range 10.5-97.3 pmol/l) and 34.1 – 5.3 pmol/l (range 2.7 – 72.4 pmol/l) respectively. (Figure 5.6B). Baseline PYY concentrations for the PYY(3-36) and GLP-1 + PYY(3-36) groups were 45.8 ± 8.1 pmol/l (range 7.8 - 107.4 pmol/l) and 52.2 ± 10.9 pmol/l (range 7.0-126 pmol/l) respectively. Levels rose to give a peak hormone concentration at t=100, and then remained at a steady state until the infusion was terminated at t=240. In the PYY(3-36) group the peak level was 101.1 ± 14.8 pmol/l (range 37.8 – 206.0 pmol/l) and 99.2 ± 40.4 pmol/l (range 10.1 – 536.9 pmol/l) in the GLP-1 + PYY(3-36) group (figure 5.6B).

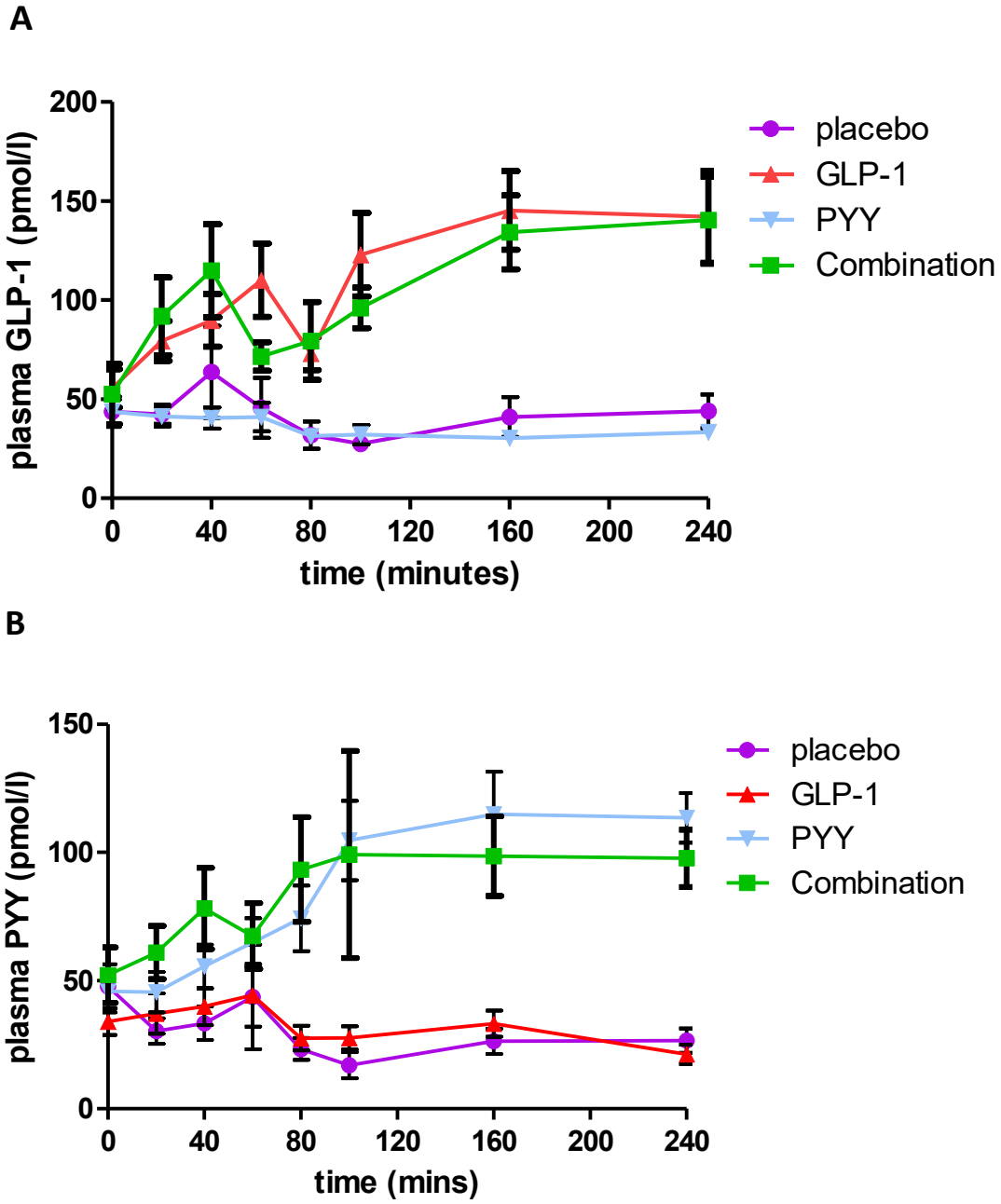


Figure 5.5 The acute effect of co-infusion of PYY(3-36) and GLP-1 on A: plasma GLP-1 concentrations and B: plasma PYY concentrations following infusion of gelofusine alone, GLP-1(7-36 amide) at 0.2 pmol/kg/min, PYY(3-36) at 0.15 pmol/kg/min and PYY(3-36) and GLP-1(7-36 amide) combined at the above doses. The infusion commenced at t=0 and lasted for 240 minutes. Blood samples were drawn at t=0, 20, 40, 60, 80, 100, 160 and 240 minutes for analysis of plasma hormone profiles. N=13

5.4.5 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma cortisol concentration

Blood samples were drawn for measurement of plasma cortisol at t= 60, 100, 130 and 160. Under conditions of stress, cortisol may be elevated and this can in turn cause an elevation of blood glucose levels, and insulin resistance(Baudrand et al., 2011, Kruyt et al., 2012).

Compared to placebo (210.8 ± 13.2; range 118 – 304 mmol/l and 201.9 ±16.3; range 83 – 305 mmol/l) GLP-1 + PYY(3-36) (284.7 ± 33.0; range 102.3 – 490 mmol/l *$p<0.05$ and 314.0 ±31.5; range 91 – 557 mmol/l **$p<0.01$) caused a significant increase in plasma cortisol levels at t=130 and t=160 respectively. This may be a consequence of nausea but it is not replicated in the PYY group as would be expected from nausea VAS scores (section 5.4.6). No other physiological stressors were identified to account for this rise in cortisol.

Given the supraphysiological glucose levels achieved by the glucose bolus in the FSIVGTT, any rise in glucose due to cortisol acting as a stress hormone may prove negligible.

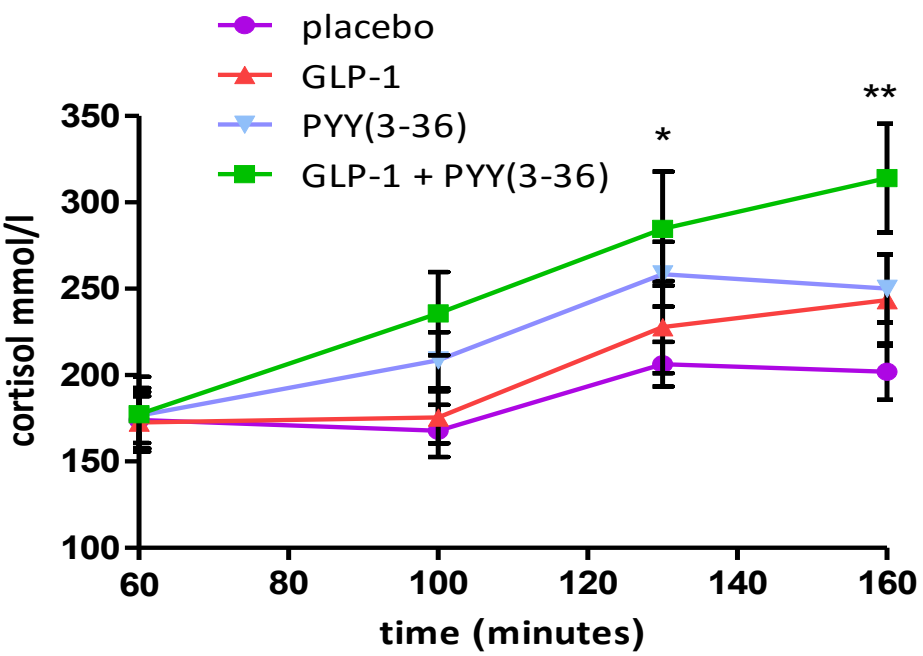


Figure 5.6 The acute effect of co-infusion of PYY(3-36) and GLP-1 on plasma cortisol levels following infusion of gelofusine alone, GLP-1(7-36 amide) at 0.2 pmol/kg/min, PYY(3-36) at 0.15 pmol/kg/min and PYY(3-36) and GLP-1(7-36 amide) combined at the above doses. GLP-1 alone (*p<0.05, **p<0.01) and the combination of PYY(3-36) + GLP-1 (#p<0.05, ##P<0.01) caused an elevation of plasma cortisol compared to placebo. Data analysed with a two way repeated measures ANOVA and Bonferroni post-hoc test. N=14

5.4.6 The acute effect of co-infusion of PYY(3-36) and GLP-1 on hunger, satiety and nausea

Visual analogue scores (VAS) were completed by participants at t=0, 20, 40, 100, 160 and 140 minutes. The VAS analysed the participants' change from baseline subjective feelings of A: Hunger, B: Nausea, and C: Satiety (figure 5.8). Data are presented as change from baseline in mm. There was a small but significant difference in nausea scores between the placebo and PYY(3-36) groups, but no significant difference between the other groups at any time point. Data were analysed using a repeated measures non-parametric Friedman's test with Dunn's multiple comparison post-hoc test. N=12

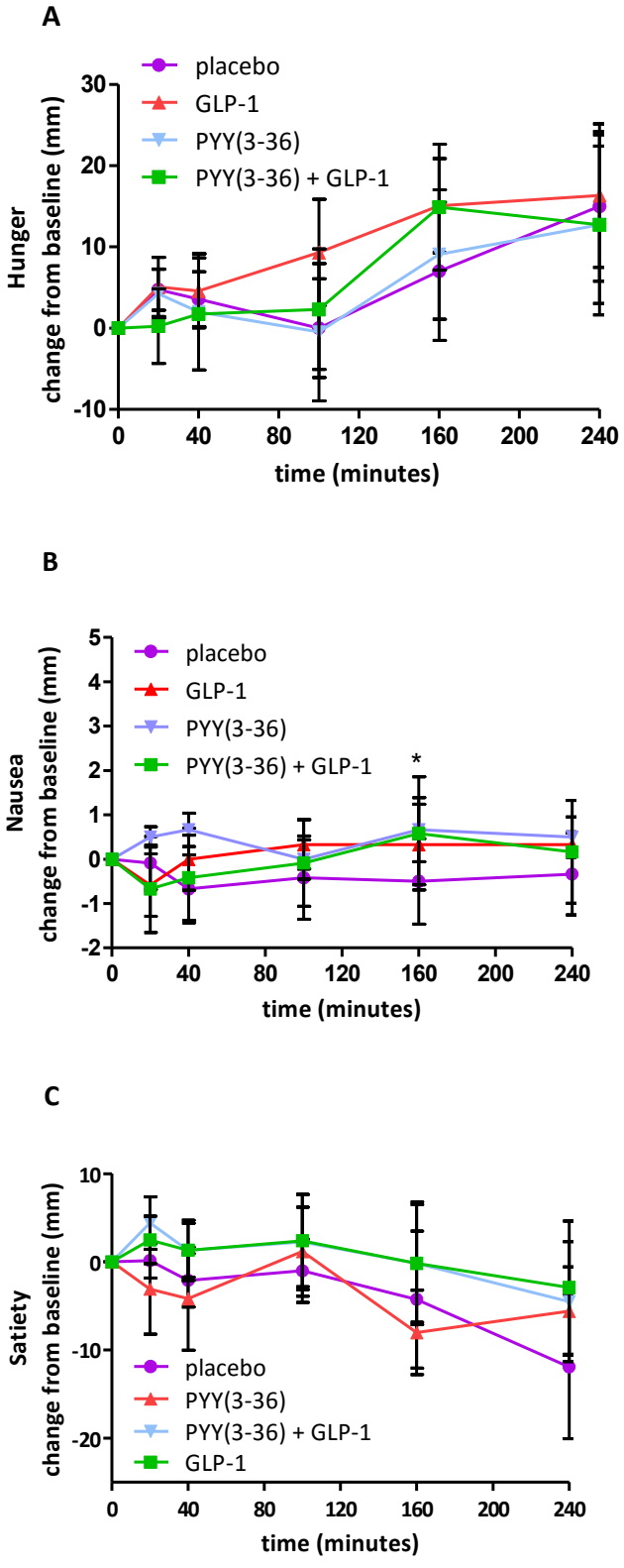


Figure 5.7 The acute effect of co-infusion of PYY(3-36) and GLP-1 on A: Hunger, B: Nausea, and C: Satiety. Data are presented as change from baseline in mm. There was a small but significant difference in nausea score between the placebo and PYY(3-36) groups ($p<0.05$), but no significant difference between the other groups at any time point. Data were analysed using a repeated measures non-parametric Friedman's test with Dunn's multiple comparison post-hoc test. N=12

5.4.7 The acute effect of co-infusion of PYY(3-36) and GLP-1 on cardiovascular parameters

The cardiovascular parameters pulse, systolic and diastolic blood pressure were measured at t=0, 20, 40, 100, 160 and 240 minutes. There was no statistically significant change in any of these parameters across any of the infusion groups (figure 5.9).

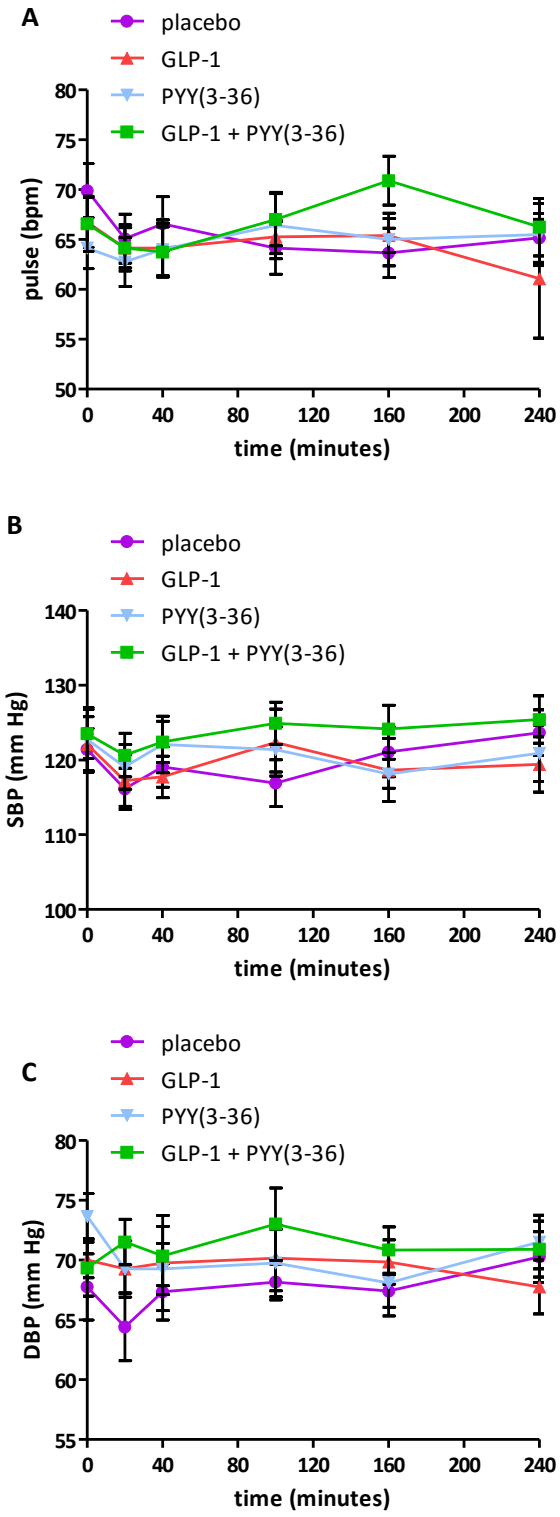


Figure 5.8 The acute effect on cardiovascular parameters A: Pulse, B: Systolic blood pressure (SBP), C: Diastolic blood pressure (DBP) following FSIVGTT and infusion of gelofusine alone, GLP-1(7-36 amide) at 0.2 pmol/kg/min, PYY(3-36) at 0.15 pmol/kg/min and PYY(3-36) and GLP-1(7-36 amide) combined at the above doses. There was no statistical significance between cardiovascular parameters in any of the infusion groups. Data were analysed using a repeated measures two-way ANOVA with Bonferroni post-hoc test. N=12

5.5 Discussion

There are multiple options when choosing a method to measure indices of insulin secretion and sensitivity/resistance. The FSIVGTT with minimal modelling is validated against the 'gold standard' hyperglycaemic clamp study for assessment of insulin sensitivity and AIRg (Korytkowski et al., 1995), but is unable to make any inferences on second phase insulin secretion, which is a limitation of the technique. Insulin sensitivity is widely measured using a euglycaemic-hyperinsulinaemic clamp, but this technique cannot assess beta cell function as insulin is infused as part of the protocol, and was therefore not a suitable choice for this study. In this study I employed a frequently sampled intravenous glucose tolerance test (FSIVGTT) in order to investigate the effects of the gut hormones GLP-1 and PYY alone and in combination on measures of insulin secretion and glucose disposal. The advantage of this method is that it gives indices for beta cell function as well as insulin sensitivity, but is less labour-intensive than a clamp study, which is widely considered as the gold standard. In reality, Korytkowski et al compared the FSIVGTT to a hyperglycaemic clamp protocol, and found good correlation between the two methods, suggesting that the FSIVGTT with minimal modelling is a valid alternative to a hyperglycaemic clamp study (Korytkowski et al., 1995).

Absolute insulin and glucose levels did not differ significantly between the participants (Figure 5.2 & 5.3), but when the AUC 0-10minutes was calculated, GLP-1 was able to significantly increase insulin secretion compared to the other experimental groups (figure 5.4). From the insulin and glucose measurements obtained during the FSIVGTT, and employing the minimal modelling method, I have calculated:

- AIRg - this is the AUC insulin 0-10, which represents first phase insulin secretion, and is a measure of how well the pancreatic beta cells respond to a glucose load

- S_i – this is the insulin sensitivity, and describes how well insulin can dispose of the glucose load
- DI – this is the product of AIRg and S_i which tells us how well the beta cell can respond to changes in insulin sensitivity.

As expected, given its role as an incretin hormone, GLP-1 was able to significantly augment the AIRg when given alone. However, the combination of GLP-1 + PYY(3-36) does not appear to have an additive or synergistic effect on insulin secretion, as the co-infusion of these two hormones did not cause a significant increase in AIRg. PYY (3-36) alone did not have any significant effect on either the acute insulin response to glucose, nor glucose disposal (S_i), therefore it is unclear why the combination of GLP-1 and PYY(3-36) did not replicate the increase in AIRg achieved by GLP-1 alone.

There was no difference in S_i between any of the groups, and thus DI, which is a product of AIRg and S_i simply reflects the AIRg in this study. This result is at odds with the previous paper published by Sloth et al (Sloth et al., 2007) which suggested that PYY was insulinotropic in its own right. In their study, PYY(3-36) appears to augment the post-prandial glucose and insulin responses after an ad-libitum meal, both at a dose of 0.8pmol/l and 0.2pmol/l. The mechanism behind this phenomenon remains unclear as it is associated with a rise in FFAs and therefore the authors postulate an increase in lipolysis causing increased insulin resistance and therefore a rise in glucose and insulin concentrations. I did not measure FFAs in this study and so am unable to clarify their relationship to PYY(3-36) infusion per se. In addition, my study was conducted in fasted participants, and so the two studies are not directly comparable.

Elevation of the stress hormone cortisol, as discussed in section 5.4.5, can cause a rise in glucose, and a subsequent increase in insulin resistance. However, in this study, despite a

significant rise in cortisol levels towards the end of the study visits for the combination infusion, it may be difficult to appreciate any rise in plasma glucose level which can be directly attributable to cortisol itself. Any subtle change in glucose due to cortisol could be masked by the large supraphysiological dose of glucose given as a part of the FSIVGTT. Kruyt et al suggest that an acute reduction in beta cell function can occur in situations of stress with elevated cortisol and an associated elevation of catecholamines (Kruyt et al., 2012), but if this was the case we would also see an elevation of blood pressure and pulse – which is absent in this study. In addition, a reduction in beta cell function would give a reduced AIRg, but I have shown the converse in the GLP-1 group which also had an elevated cortisol. Moreover, a rise in cortisol sufficient to confer an altered insulin resistance should have altered the S_i, which was not the case.

In this study there was only mild nausea reported by subjective recording on the visual analogue scale. I used a low dose of PYY, as previous studies had seen significant malaise and nausea (Sloth et al., 2007, Field et al., 2010b) and had been obliged to reduce the doses of PYY(3-36) that were given from 0.8pmol/kg/min down to 0.2pmol/kg/min, and from 0.5pmol/kg/min down to 0.25pmol/kg/min respectively. It is interesting that although PYY(3-36) caused significant nausea, this was not seen with infusion of PYY(1-36), and suggests that nausea (and possibly satiety as an extrapolation) is mediated via the Y2 receptor. In this study, satiety decreased and hunger increased toward the conclusion of study visits, as might be expected in subjects who were fasted from the night before and were receiving assumed subanorectic doses of PYY(3-36).

The work by Chandarana et al (Chandarana et al., 2013) demonstrating the lack of Y2 receptor on the beta cell supports my finding that PYY does not augment the AIRg. However, despite

no detectable effect on S_i attributable to PYY(3-36) on minimal modelling, it is possible that my sample size was insufficient to detect a true effect, or that an alternative method of assessing insulin sensitivity may yield different results. A labelled euglycaemic hyperinsulinaemic clamp study would be of interest, to further assess insulin sensitivity, and to examine the effect of PYY (3-36) on hepatic glucose production.

It is possible that higher doses of PYY(3-36) may have a small effect on AIRg or S_i, but use of higher doses is likely to be hampered by the malaise and nausea described by Sloth et al and also Field et al (Sloth et al., 2007, Field et al., 2010b). Longer term administration of PYY(3-36) at low doses to assess the chronic effect of this hormone on insulin secretion and glucose disposal would be interesting, and it is likely that at least some effect on insulin sensitivity would be seen after chronic PYY(3-36) administration if only due to an improvement in insulin sensitivity conferred by PYY(3-36) induced weight loss.

GENERAL DISCUSSION

The obesity crisis continues to grow, and has reached epidemic proportions. In the UK, 60% 0f men, 50% of women and 25% of children are predicted to be obese by 2050 (Foresight, 2007). Taking into account the associated health issues such as type 2 diabetes, ischaemic heart disease, stroke and cancer, this trend toward an obese population will result in significant cost, both financial and personal. To date, therapies for obesity have been limited. Until recently the only drug available on the UK market was Orlistat, a pancreatic lipase inhibitor which confers a modest weight loss at best and is limited by side effects that many people are unable to tolerate. A few other drugs have been developed and initially licenced but withdrawn due to side effects – nominally Sibutramine which caused cardiovascular side effects, and Rimonobant which raised concern regarding suicidality (James et al., 2010, Christensen et al., 2007). Encouragingly, several new weight loss therapies have recently been approved for clinical use in the US – Lorcaserin (Eisai), a 5HT-2C agonist conferring up to 3.6% weight loss compared to placebo (Fidler et al., 2011, Smith et al., 2010), and Qsymia® (Vivus), a fixed dose combination of phentermine and topiramate that results in a 6.6% weight loss compared to placebo (Gadde et al., 2011) have been approved in the US by the FDA but not by the EMA in Europe. The EMA have approved Mysimba® (Orexigen), a combination of bupropion and naltrexone which gives up to 5.2% weight loss at one year (Apovian et al., 2013, Greenway et al., Wadden et al., 2011). All of these new medications remain under scrutiny, with careful monitoring in place to identify any potential adverse effects. Whilst the development of these new treatments is encouraging, gastric bypass surgery, and specifically RYGB remains a gold standard for weight loss. In addition to weight loss, it has also gained approval as a treatment for type 2 diabetes in obese patients (NICE, 2014). Despite its superiority as a treatment for obesity and diabetes, gastric bypass surgery is taken up by a small proportion of those who are eligible, although this figure is increasing with time. Furthermore, gastric bypass is not without complications and indeed carries a mortality rate of between 0.15 and 0.5% depending on whether it is an open or laparoscopic procedure (Buchwald et al., 2004, Jones et al., 2006, Ma et al., 2006, Agaba et al., 2008). There is therefore an urgent need for non-surgical interventions which confer a sustained weight loss. The mechanism of the marked improvement in

body weight and glucose control following gastric bypass surgery remains unclear. It is purported that a rise in gut hormones seen post-operatively may play a role in mediating these improvements (Beckman et al., 2011, Le Roux et al., 2007, Pournaras et al., 2010a, Le Roux et al., 2006). No one hormone in isolation is likely to have the capacity to generate all of the favourable effects seen after surgery, and increasingly, investigators are looking towards the effect of administering a combination of peptides. In healthy, overweight volunteers, PYY(3-36) and GLP-1 in combination reduces food intake additively (Neary et al., 2005), as does PYY and OXM (Field et al., 2010b). OXM is a dual agonist at the GLP-1 and glucagon receptors, and in DIO mice, a synthetic dual agonist at these two receptors caused weight loss, and promoted favourable changes to lipid and glucose metabolism following chronic administration in two similar studies (Pocai et al., 2009, Day et al., 2009).

Work in this thesis investigates the contributions of the gut hormones GLP-1, PYY and OXM to the favourable glucose profile and reduction in body weight following gastric bypass surgery. PYY has effects on satiety, and reduces food intake (Batterham et al., 2002, Batterham et al., 2003). In rodents fed a high fat diet, PYY improves insulin sensitivity (Van den Hoek et al., 2007, Van den Hoek et al., 2004). GLP-1 is co-secreted with PYY from L-cells in the distal gut, and promotes insulin secretion in response to a glucose load (Kreymann et al., 1987) . Like PYY, it also increases satiety (Turton et al., 1996). OXM acts via the receptors for glucagon (Kosinski JR et al., 2012) and GLP-1(Pocai et al., 2009), and has been shown in both rodents and humans to reduce food intake
(Dakin et al., 2001, Dakin et al., 2004, Liu et al., 2010, Wynne et al., 2005, Wynne et al., 2006) In mice, chronic glucagon receptor agonism increases EE and spontaneous locomotor activity (Habegger et al., 2013) . Despite acting on the glucagon receptor, OXM does not cause the hyperglycaemia seen with glucagon itself (Maida et al., 2008). This is most likely due to simultaneous GLP-1 receptor agonism.
The overall hypothesis of this work is that weight loss and improved glycaemic control after gastric bypass surgery can, at least in part, be attributed to changes in the levels of these gut hormones. Combinations of the gut hormones PYY, GLP-1 and OXM (or GLP-1 plus glucagon as a surrogate for

OXM) or analogues thereof were used to investigate the effects of gut hormone changes seen after gastric bypass surgery.

Energy Expenditure

In Chapter 2, I investigated the chronic administration of long acting analogues of the gut hormones PYY, GLP-1 and OXM on food intake, body weight, glucose homeostasis and markers of energy homeostasis in DIO mice. Mice receiving the combination of all three analogues lost approximately 15% of their body weight compared to placebo, and significantly more than any of the animals receiving a single analogue. This occurred in the absence of any significant reduction in food intake suggesting an increase in EE. The increase in EE seems likely to be mediated by the long acting OXM analogue via the glucagon receptor, as neither GLP-1 nor PYY receptor agonism are associated with an increase in EE (Sloth et al., 2007, Pannacciulli et al., 2006). However, there was no significant effect on body weight of the OXM analogue alone, suggesting some degree of additive or synergistic effect on weight reduction with the other analogues. The mechanism of the increase in EE was investigated by examining plasma markers of energy homeostasis, and also by assessing mRNA expression in tissues of interest for genes which may contribute to an increase in EE.

Adaptive thermogenesis is a well-documented phenomenon occurring in BAT which increases EE through the generation of heat, and is mediated via UCP-1. Tri-iodothyronine (T3), the active form of thyroid hormone also plays a role in increasing thermogenesis via UCP-1. However, there was no up-regulation of UCP-1 in interscapular BAT in the DIO mice (in any treatment group) and no increase in plasma Free T3, suggesting an alternative mechanism for increased EE. Hormone sensitive lipase (HSL) expression in WAT was also examined. HSL increases lipolysis, and therefore production of FFAs, which can then be incorporated into thermogenic pathways as a substrate for β-oxidation. In addition, FFAs have a role in regulating UCP-1 itself. There was no alteration in HSL following any intervention in the study in Chapter 2. All of this evidence points away from adaptive thermogenesis in BAT being the cause of a glucagon-receptor mediated increase in EE. Additional support for this comes from a

recent study, whereby injection of glucagon in man failed to increase BAT activity, as measured by glucose uptake (Salem et al., 2016).

Further work examining glucagon-receptor mediated EE was performed in chapter 3. This work was in humans, and used a high dose of glucagon, but clearly demonstrated an acute increase in EE mediated via the glucagon receptor. In this study I did not measure tissue mRNA expression, but did measure plasma TSH and FT3, which both showed a fall in the glucagon treatment group, again pointing away from thyroid-axis mediated adaptive thermogenesis in BAT as a mechanistic explanation for the increase in EE. In addition, I measured FFA in plasma before and after each infusion, although HSL was not assessed. Interestingly, and against conventional dogma that glucagon increases lipolysis, I found a fall in FFAs. One reason could be that the hyperinsulinaemia seen in this study inhibits HSL (Holm et al., 2000) and therefore reduces FFA production. Whether this effect would be sustained in the context of chronic administration of glucagon (with or without GLP-1) remains to be seen. In contrast to the findings of chapter 2, Pocai et al found that a chronically administered dual agonist of the GCGR and GLP-1R resulted in a decrease in insulin levels, an up-regulation of HSL, and hence an increase in FFA availability for β-oxidation (Pocai et al., 2009).

A key finding in the chronic analogue study in chapter 2 is the increase in FGF-21 mRNA expression in liver tissue following administration of both OXM-LA alone, and also in the triple analogue group. This finding may be key to elucidating the mechanism of the increase in EE observed. Habegger et al found FGF-21 to be a downstream effector of the GCGR for many of its actions, but also specifically in the increase in EE mediated by GCGR activation – they showed that FGF-21 knockout mice (FGF-$21^{-/-}$) did not have the same increase in EE (as measured by indirect calorimetry) in response to GCGR activation as was present in wild-type mice (Habegger et al., 2013). The mechanism by which FGF-21 increases EE is not clear but seems to be independent of BAT mediated thermogenesis.

FGF-21 plays a significant role in the regulation of energy and glucose homeostasis (Xu et al., 2009, Kharitonenkov et al., 2005). It is expressed in adipose tissue, liver, pancreas and muscle (Nishimura et

al., 2000), and appears to have differential effects centrally and peripherally. Continuous intracerebroventricular (ICV) infusion of FGF-21 increases EE, and increases insulin sensitivity via improved hepatic insulin sensitivity, but does not reduce body weight. In contrast, opposite findings are demonstrated following peripheral administration (Sarruf et al., 2010). Indeed, central administration increases food intake, but the increase in EE rendered this effect on weight, and body composition, neutral. The improvement in hepatic insulin sensitivity is mediated by an insulin-induced suppression of liver glucose production and hepatic gluconeogenesis, without any effect on glucose utilisation (Sarruf et al., 2010). Whilst the relationship between FGF-21 and UCP-1 has been documented in various studies (Véniant et al., 2015, Lee et al., 2014, Fisher et al., 2012), Samms et al showed that the effects of FGF-21 are not necessarily dependent on UCP-1 expression (Samms et al., 2015). This supports my findings that an increase in EE with GCGR agonism may be mediated by pathways other than BAT activation. One possibility for the elusive mechanism is that of WAT-mediated thermogenesis that is independent of UCP-1 or 'britening' (Granneman et al., 2003, Ukropec et al., 2006) and potentially facilitated via the β3 adrenoceptor. Further potential mechanisms are that of adaptive thermogenesis in skeletal muscle mediated by sarcolipin uncoupling calcium transport from the SERCA pump (Bal et al., 2012), and also futile cycling in BAT or WAT involving lipolysis and lipogenesis (Mottillo et al., 2014).

In summary, I have demonstrated an increase in EE which is mediated via the GCGR – either by glucagon itself (Chapter 3), or by a long acting analogue of OXM (Chapter 2). The mechanism of this increase in EE is unclear, but my experimental evidence points away from this being due to BAT mediated thermogenesis. It does, however, seem feasible that the increase in EE is mediated via FGF-21. Further work documenting the increase in EE in DIO mice treated with long acting analogues is required, and calorimetry studies using the comprehensive laboratory animal monitoring system (CLAMS) would be useful – in particular to assess any discrepancy in EE between the OXM-LA group and the triple combination treated group. If EE is mediated via the GCGR receptor then it should be comparable in both these groups, and an alternative explanation for the marked improvement in body

weight seen only in the triple therapy group needs to be sought, especially given there is no significant reduction in food intake in any of the treatment groups by the end of the study. Additional studies assessing the mRNA expression and phenotype of WAT in these rodents would be useful. It proved difficult to extract sufficient mRNA from many of the WAT samples for adequate analysis, and it may be that in fact there is increased UCP-1 expression and 'britening' in this tissue to account for some of the increased EE. Histology would be useful to look for 'brite' or 'beige' changes in the WAT deposits, particularly as this is known to be an effect of FGF-21 (Fisher et al., 2012) Furthermore, I examined mRNA expression in epididymal WAT, whereas inguinal WAT may have been a more appropriate depot to examine(Fisher et al., 2012, Li et al., 2014) Further experimental work examining the downstream effectors of FGF-21 in tissues other than BAT would also be interesting.

Glucose Homeostasis

The improvement of glycaemic control which occurs following gastric bypass surgery occurs well before this could possibly be attributable to increased insulin sensitivity due to weight loss – often within days of the surgery. This may therefore be, at least in part, due to elevations in the gut hormone profile post-operatively.

Acutely, I have shown in chapters 3 and 4 that a rise in glucose due to GCGR agonism can be ameliorated and indeed, in chapter 4 with a lower dose of glucagon, abolished by the co-infusion of GLP-1. This suggests that using OXM and GLP-1, or analogues thereof in combination is a potentially feasible therapy for obesity, giving weight favourable effects without any deterioration in glucose control. The amelioration of the glucagon-mediated rise in blood glucose in the combination GLP-1/glucagon group is likely to be due to the greater insulinotropic effect of the two hormones in combination. GLP-1 is an incretin hormone, with direct action on the beta cell to stimulate insulin secretion. Glucagon also has a direct stimulatory effect on the beta cell (Huypens et al., 2000, Kawai et al., 1995), despite its prominent role in increasing blood glucose through hepatic glycogenolysis and gluconeogenesis. GLP-1 alone has little effect on circulating insulin concentrations in the absence of a

glucose load, and thus the increase in glucose due to glucagon is important in mediating the synergistic rise in insulin due to GLP-1 and glucagon in combination seen in these studies. The effects observed in chapters 3 and 4 are acute, and it will be important to investigate chronic co-administration of these hormones if they are to be employed as a feasible therapy for obesity (and potentially diabetes). Specifically, the insulin-glucose profiles may be altered once hepatic glycogen stores are exhausted.

Further to the effects of GLP-1 and glucagon in combination on glucose and insulin levels, I went on to investigate the contribution of PYY(3-36) plus GLP-1 alone and in combination on glucose handling when infused acutely (Chapter 5). These hormones are both elevated following gastric bypass, and in the past it has been suggested that PYY(3-36) may have a favourable effect on insulin sensitivity (Van den Hoek et al., 2004), thus I have evaluated the contribution of GLP-1 and PYY(3-36) on first phase insulin response and glucose disposal. GLP-1, as expected from its role as an incretin, was able to augment the acute insulin response to glucose in a FSIVGTT, but PYY(3-36) had no effect on insulin secretion itself and did not synergistically nor additively improve the insulinotropic effect of GLP-1. This is in keeping with the lack of Y2 receptor expression on pancreatic beta cells (Chandarana et al., 2013). In this study, neither hormone had a measurable effect on glucose disposal or insulin sensitivity, and alone are not able to fully elucidate the mechanism for the acute improvement in glucose control after bypass surgery. When used chronically, GLP-1 plus PYY(3-36) could feasibly result in weight loss which would confer an improvement in insulin sensitivity.

The longer term effects of these gut hormones on glucose homeostasis were examined in chapter 2. Following 70 days of treatment with long acting analogues of GLP-1, PYY and OXM, DIO mice underwent an IPGTT, to assess the ability to clear a glucose load. I demonstrated that, after chronic treatment, the triple analogue group and the group receiving OXM had a significantly reduced basal insulin concentration, although there was no difference in basal glucose levels across all treatment groups. The response to the intraperitoneal (IPe) glucose load demonstrated no significant increase in insulin secretion in any of the groups (AUC (insulin), Fig 2.4A), but the insulin response in the OXM-LA

group was significantly lower than placebo. This may represent improved insulin sensitivity, a theory which could be explained by the increase in hepatic FGF-21 expression in this group. FGF-21 is known to improve both hepatic and peripheral insulin sensitivity (Camporez et al., 2013), both facilitating an increase in peripheral glucose uptake in BAT, WAT and skeletal muscle, (a phenomenon which is UCP-1 dependent (Kwon et al., 2015)) and reducing endogenous glucose production under hyperinsulinaemic conditions suggesting improved hepatic insulin sensitivity(Camporez et al., 2013, Berglund et al., 2009). My data showing a lack of up-regulation of the enzymes G-6-Pase and PEPCK1 which are intrinsic to gluconeogenesis supports this theory of reduced hepatic endogenous glucose production. Furthermore, the triple analogue, OXM-LA and GLP-1-LA groups all had a significantly lower glucose response during the IPGTT compared to the placebo group. In the triple analogue group this may feasibly be due to reduced insulin resistance due to greater weight loss, and in the triple analogue and OXM-LA groups, there may be an effect of FGF-21 as discussed above. However, this does not explain the favourable glucose profile seen in the GLP-1-LA group, especially as the GLP-1-LA group did not exhibit weight loss. Interestingly, although both GLP-1 and OXM are insulinotropic in response to a glucose load (Du et al., 2012, Kreymann et al., 1987), this effect was not marked compared to the PYY-LA and placebo groups in the IPGTT. The incretin effect may potentially have been greater following an OGTT rather than IPGTT.

The mechanism for the favourable glucose homeostasis results seen in the triple analogue group could be explored further by looking at glucose uptake in tissues such as BAT, WAT and skeletal muscle using PET CT, and euglycaemic-hyperinsulinaemic clamp studies would also be interesting to clarify whether the combination of triple analogues improves hepatic insulin resistance.

In summary, acute GLP-1R and GCGR agonism in combination can deliver a glucose-neutral increase in EE, although the chronic effect remains to be investigated. PYY(3-36) was not able to show any favourable effect on insulin secretion or glucose disposal acutely, but may improve long-term insulin sensitivity through weight loss. Chronic administration of OXM-LA and the triple combination of

analogues were able to significantly improve glucose homeostasis, likely to be at least partially via an FGF-21 mediated mechanism, as OXM-LA did not cause significant weight loss. However the weight loss in the triple analogue group may have conferred an improvement in insulin sensitivity.

Lipid Metabolism

Agonism at the GCGR is traditionally thought to promote lipolysis (Richter et al., 1989, Habegger et al., 2010). However in the study in chapter 3, I have demonstrated both a fall in fat oxidation and a reduction in non-esterified fatty acid levels following glucagon and glucagon + GLP-1 infusions. These findings would correlate with a reduction in lipolysis, which is possibly secondary to inhibition of the enzyme HSL by the supraphysiological insulin levels seen in this study. This finding in my acute study is in contrast to the findings of Day et al who demonstrated increased activation of HSL following combined glucagon and GLP-1 receptor agonism (Day et al., 2009), albeit in the context of reduced circulating insulin levels after chronic receptor agonism, and not therefore directly comparable. However, Day's results can be compared to the findings in chapter 2, wherein I measured HSL mRNA expression in WAT after chronic agonism at the GLP-1, glucagon and PYY receptors. Despite the chronicity of the study, and reduced basal insulin levels in the triple analogue group, I was unable to replicate an increase in HSL expression. Indeed, whilst not significant, there was a trend towards a decrease in HSL expression in the triple analogue group. This suggests that the chronic administration of the triple analogues did not induce significant lipolysis in WAT.

GLP-1-LA caused a reduction in liver weight, and also a reduction in circulating triglycerides. This is in keeping with documented findings that GLP-1 agonism is able to reduce features of NAFLD, including liver weight, hepatic lipids and plasma triglycerides (Trevaskis et al., 2012). Further studies on body composition with magnetic resonance imaging (MRI) would be interesting to investigate the effect of GLP-1-LA and the triple analogue combination on hepatic lipids, particularly as a reduction in hepatic lipids may confer improved hepatic insulin resistance, and thus contribute to the favourable glucose profile seen in the triple analogue group. One of the postulated mechanisms for the increase in EE

seen in this study is that of futile cycling, and one possibility for this is triglyceride hydrolysis and subsequent resynthesis in WAT (Granneman et al., 2003, Ukropec et al., 2006) – this carries a large ATP requirement and could account for some of the increase in EE as well as the increase in WAT weight seen in the GLP-1-LA group. However, there was no up-regulation of PPARγ in WAT to support increased lipogenesis. I also investigated the expression of ACC1 mRNA in liver, as this enzyme is important in fatty acid metabolism. However, as in WAT, there was no change in expression in any of the treatment groups suggesting no increase in hepatic lipogenesis.

In summary, GLP-1-LA appears to have favourable effects on hepatic lipids and plasma triglycerides, which are in keeping with those described in other studies. This may play a role in improving hepatic insulin resistance and thus improve glucose homeostasis. I was unable to demonstrate any experimental evidence of an increase in lipolysis or lipogenesis in any of my studies.

Food Intake

The combination of glucagon and GLP-1 infused acutely at low doses (chapter 4) was able to reduce food intake by 13% compared to placebo. Neither hormone alone at these low doses was able to replicate the anorectic effect, which may be centrally mediated - either via the circulation or via vagal afferents. A sustained 13% reduction in food intake in the long term could lead to significant weight loss, making this combination very interesting as a therapeutic option for obesity. There was some nausea reported in the study, and indeed in the group receiving glucagon alone, two participants vomited. All nausea and vomiting episodes were post-prandial, suggesting that it is mediated by a delay in gastric emptying, a known effect of both peptides (Chernish et al., 1978, Flint et al., 1998). The ability of the combination of hormones to sustain a reduction in food intake in the long term is an important point to investigate, as there is a potential for tachyphylaxis. This has been documented with PYY(3-36) administration when assessing inhibition of food intake (Van den Hoek et al., 2007), and there have been reports of tachyphylaxis to GLP-1 mediated delay in gastric emptying itself (Jelsing et al., 2012, Nauck et al., 2011). In addition, chronically administered peptides may be subject

to desensitisation, receptor down-regulation or neutralising antibody formation, all of which could play a role in negating the effect on food intake.

Interestingly, in chapter 2, there was no reduction of food intake using the combination of GLP-1-LA, OXM-LA and PYY-LA. Despite this there was a significant reduction in body weight in the group receiving the triple analogue treatment, which has been discussed previously. It is possible that the peptides in the chronic DIO study underwent desensitisation by one of the above mechanisms, and further work to elucidate this would be useful.

In summary, the acute co-infusion of GLP-1 and glucagon is able to reduce food intake additively to give a 13% overall reduction in energy intake. However, in the chronic DIO study a reduction of food intake was not seen after administration of any of the analogues alone or in combination despite a marked weight loss in the triple analogue group. This suggests that the predominant determinant of weight loss was an increase in EE.

In conclusion, administration of the gut hormones GLP-1, OXM and PYY in various combinations is able to acutely reduce food intake, increase EE and augment the acute insulin response to glucose. Chronically, the combination of all three hormones is able to instigate and maintain weight loss, with associated favourable effects on glucose and lipid homeostasis. In the face of the obesity pandemic, the development of new treatments for obesity is critical, particularly for those patients who cannot undergo gastric bypass surgery. Increasingly, it has become apparent that combinations of two or more drugs or peptides have beneficial weight loss effects when compared to single agents, thus the development of this triple combination of gut hormones offers a realistic future treatment option for obesity.

Future Work

Some additional work has been outlined above which would provide more insight into the mechanisms behind the results I have presented here. In the DIO mice, further useful information

would be gained by examining glucose uptake using PET-CT, body composition studies using MRI, and calorimetry studies using the CLAMS system. Additional studies into the downstream effectors of FGF-21 on EE independent of UCP-1 would also be very interesting.

Ultimately, the next step following these studies is to translate this research into man, and investigate the effects of a combination of the three hormones GLP-1, PYY(3-36) and OXM on food intake, EE, body weight, carbohydrate tolerance and brain areas activated by food cues using functional MRI. This is currently underway, funded by an Experimental Medicine Grant from the MRC.

A 28 day chronic infusion of the above hormones by subcutaneous pump (to avoid the consequences of the short half-lives of these peptides) will be carried out and the above parameters assessed. This data will then be compared to the same data recorded in subjects who have undergone gastric bypass surgery.

APPENDICES

APPENDIX A Visual Analogue Scale

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?

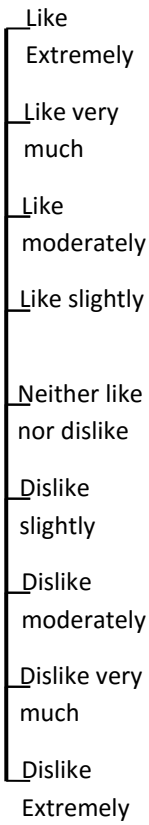
NOT AT ALL EXTREMELY

APPENDIX B Food Preference score

Food Preference

Name:	**Date:**

You will be given a serving of food to eat and you are asked to say how much you *like* or *dislike* it. Use the scale to indicate your attitude by checking at the point which best describes your feeling about the food. Keep in mind that you are the judge. You are the only one who can tell what you like. An honest expression of your personal feeling is important to the study.



Comments:

Food tasted:

APPENDIX C Note on imputed values

- For baseline data (pre-infusion), a mean of the other study visits for that participant at that time point has been calculated
- Missing data between two other values are calculated as the arithmetic mean of the two adjacent values
- Missing data at the beginning of a series is imputed as the same value as the following value. (First Observation Carried Backward)
- Missing data at the end of a series is imputed as the same value as the preceding value. (Last Observation Carried Forward)
- Values >2 standard deviations from the mean are excluded in analysis as erroneous outliers

APPENDIX D1 IC_{50} and EC_{50} of long-acting gut hormone analogues

Receptor binding assays for determination of peptide receptor affinity (IC_{50} see below) were carried out in Chinese hamster ovary (CHOv) cells transfected with human GLP-1 or human glucagon receptors, and in human embryonic kidney (HEK) cells transfected with human Y2 receptor. The competing radiolabels were I^{125}GLP-1(7-37), I^{125}Glucagon and I^{125}PYY(1-36).

cAMP concentration was used as a measure of receptor efficacy (EC_{50} see below). This was determined in vitro in cell membranes from the same cells outlined above, using a direct cAMP ELISA (Enzo life sciences UK).

Peptide	Receptor Affinity (IC_{50})				Receptor Efficacy (EC_{50})		
	hGLP-1r (nM)	hGCGr (nM)	Ratio GLP-1:GCG	hY2r (nM)	hGLP-1r (nM)	hGCGr (nM)	Ratio GLP-1:GCG
GLP-1	0.33	>10,000	1:>30,000		0.61	369	1:605
OXM	115	6.52	18:1		14.6	2.0	7:1
GCG	>10,000	0.53	19,000:1		303	0.01	30,000:1
GLP-1LA	2.15	-	-		0.53	>10,000	1:>19,000
OXM-LA	161	1.62	99:1		16.1	0.06	268:1
PYY_{3-36}				0.21			
PYY-LA				0.28			

Table D1 Receptor Affinity (IC_{50}) and receptor Efficacy (EC_{50}) of the long-acting analogues PYY-LA, GLP-1-LA and OXM-LA compared to their parent peptides. Abbreviations **hGLP-1r: human GLP-1 receptor, hGCGr: human glucagon receptor, hY2r: human Y2 receptor, GCG: glucagon**

IC_{50} is the concentration of the analogue needed to inhibit 50% of binding of the competing radiolabelled peptide

Thus: At the human GLP-1 receptor (hGLP-1R), only 0.33nM of GLP-1 is required to inhibit binding of the label – it therefore has effective receptor binding. However, at the same human GLP-1 receptor, >10,000 nM of glucagon is needed to inhibit label binding to the receptor – glucagon is not an effective competing peptide at this receptor and has little binding affinity.

EC_{50} is the concentration of the analogue needed to give 50% of its maximum effect - in this case stimulation of cAMP release

APPENDIX D2 Pharmacokinetic profiles of PYY-LA, GLP-1-LA AND OXM-LA

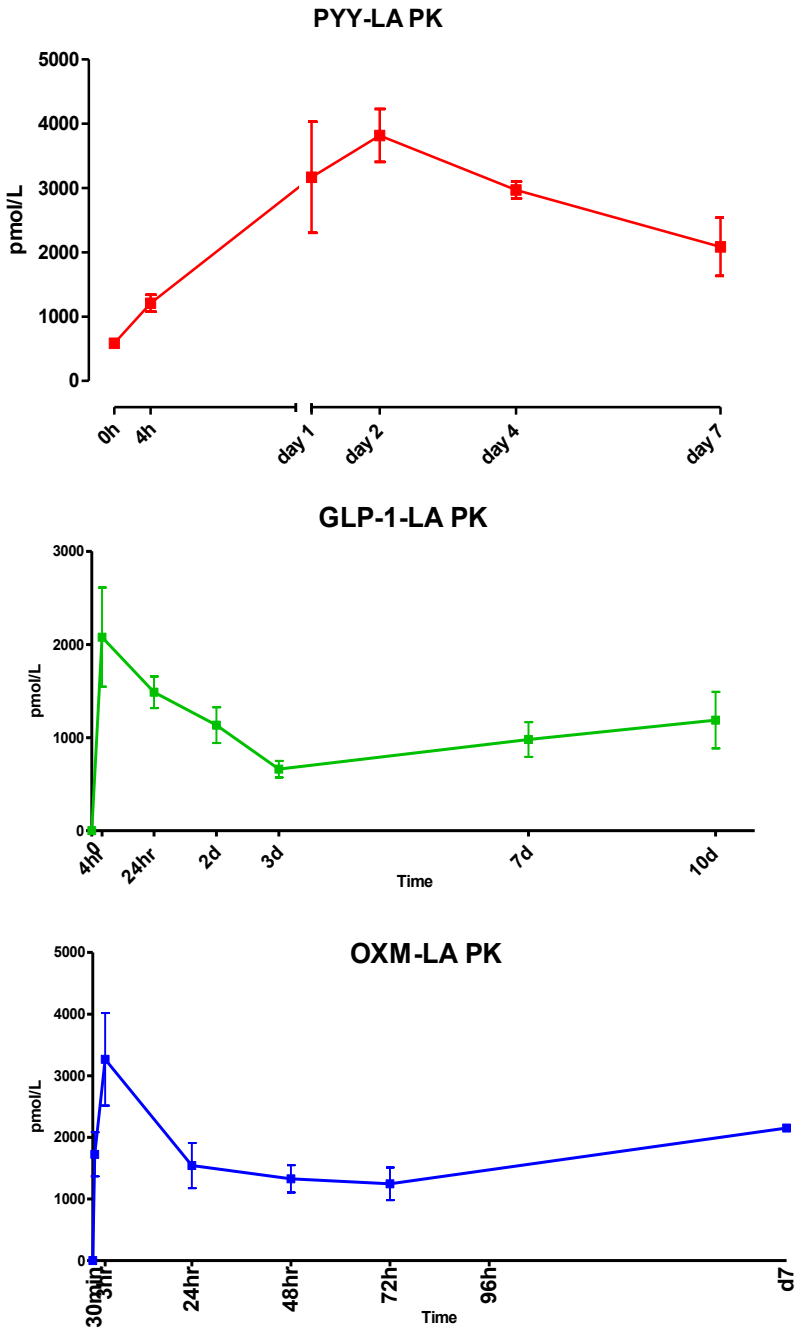


Figure D1 Pharmacokinetic profiles of the long acting peptide analogues PYY-LA, GLP-1-LA and OXM-LA. Peptides were administered to ad-libitum fed rats at 1mg/20µl/rat by subcutaneous injection at the start of the light phase. Tail vein blood was sampled at the time points shown for analysis of plasma peptide concentration n=3-5

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