The Effects of Amino Acids on Gut Hormone Release and Appetite

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Abstract

Obesity is a major health concern and a public health burden. Current pharmacological treatments have limited efficacy and are associated with significant side effects. Diets and life style changes remain the most effective strategy for treatment of obesity, but are often difficult to adhere to. High protein diets are among the most satiating diets, associated with the greatest satiety and weight loss, and with better weight management. The exact mechanisms by which high protein diets exert their effects are unclear. However, evidence suggests that amino acids produced as a result of protein digestion may play a role in appetite regulation and satiety.

Preliminary studies within our group demonstrated that specific amino acids can reduce food intake in rodents. The work carried out in this thesis examined the effect of the amino acids L-arginine and L-phenylalanine on appetite and explored the potential mechanisms by which these effects are mediated. In addition, the thesis investigated the effect of microencapsulation of these amino acids on their ability to suppress appetite.

Oral gavage of L-arginine significantly reduced food intake in mice and rats. This effect was not associated with any abnormal behavioural side effects. L-arginine significantly stimulated GLP-1 and PYY release from a murine primary intestinal culture, and oral L-arginine also significantly elevated plasma GLP-1 and PYY in rats. However, the anorectic effect of L-arginine appears unlikely to be mediated by changes in these gut hormones. L-arginine significantly reduced food intake in GPRC6A knockout and wild-type mice, suggesting its anorectic effect is not mediated by GPRC6A.

Oral gavage of L-phenylalanine significantly reduced food intake in mice and rats. Oral administration of L-phenylalanine also elevated circulating GLP-1 and PYY levels and suppressed plasma ghrelin levels in rats. Direct ileal administration of L-phenylalanine reduced food intake in rats, and this effect was blocked by a calcium sensing receptor

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antagonist. Chronic administration of L-phenylalanine also reduced food intake and body weight in diet-induced obese mice.

Encapsulated L-arginine and L-phenylalanine reduced food intake in rats. Specific encapsulation processes appeared to delay the anorectic effect of these amino acids. However, there was no significant difference in the magnitude of response compared to the un-encapsulated forms. Encapsulation may be a viable approach to facilitate targeted delivery of the amino acids in the gastrointestinal tract, but further matrix optimization is required to improve the controlled release of the amino acids in the gut.

These studies demonstrate the anorectic properties of L-arginine and L-phenylalanine, and contribute to the current understanding of amino acid sensing in the gut. Further studies are now required to determine the therapeutic potential of specific amino acids as novel treatments for obesity.

Copyright Declaration

Declaration of Contributors

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

All radioimmunoassays were carried out under the supervision of Professor Mohammad A. Ghatei (Division of Diabetes, Endocrinology and Metabolism, Imperial College London).

Chapter 2:

In vivo energy expenditure studies were performed in collaboration with Dr Anne McGavigan and Elly Spreckley.

GPRC6A knockout mouse colony was bred and maintained by Dr James Kinsey-Jones.

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Chapter 3:

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Chapter 4:

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*Dedicated to the memory of **Professor Mohammad Ali Ghatei** (1943 – 2015); a great mentor, teacher, and colleague.*

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT2c	Serotonin 2c receptor
7TM	Seven transmembrane
AA	Amino acid
AAV	Adeno-associated virus
ACTH	Adrenocorticotropic hormone
Adipo-R1	Adiponectin receptor 1
Adipo-R2	Adiponectin receptor 2
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AgRP	Agouti-related peptide
AP	Area postrema
APC	Anterior piriform cortex
ARC	Arcuate nucleus
ASL	Argininosuccinate lyase
ASS1	Argininosuccinate synthase 1
ATF4	Activating transcription factor 4
BH4	Tetrahydrobiopterin
BMI	Body mass index
BSA	Bovine serum albumin
CART	Cocaine and amphetamine-regulated transcript
CaSR	Calcium sensing receptor
CAT	Cationic amino acid transporter
CCK	Cholecystokinin
CCK-1R	Cholecystokinin receptor 1
CCK-2R	Cholecystokinin receptor 2
CLAMS	Comprehensive laboratory animal monitoring system
CLIP	Corticotrophin-like intermediate peptide
CNS	Central nervous system
CP	Carbamoyl phosphate
CPS I	Carbamoyl-phosphate synthase I
CPS1	Carbamoylphosphate synthetase 1
CRD	Cysteine rich domain
DIO	Diet-induced obese
DIT	Dietary induced thermogenesis
DMEM	Dulbecco's Modified Eagle Medium
DMN	Dorsomedial nucleus
DMOS	Dimethyl sulfoxide
DMX	Dorsal motor nucleus of the vagus
D-Phe	D-phenylalanine
DPP-4	Dipeptidyl peptidase 4
DVC	Dorsal vagal complex
EDTA	Ethylenediaminetetraacetic acid

elf2α	Eukaryotic initiation factor 2 alpha
ENS	Enteric nervous system
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FDA	Food and Drug Administration
FTO	Fat mass and obesity-associated protein
GABA	Gamma-aminobutyric acid
GCGR	Glucagon receptor
GCN2	General control nonrepressed 2 protein
GDP	Guanosine diphosphate
GHSR-1a	Growth hormone secretagogue receptor 1a
GI	gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GLP-2	Glucagon-like peptide 2
GOAT	Ghrelin O-acyltransferase
GPCR	G-protein coupled receptor
GPRC6A	G-protein coupled receptor group C member 6A
GPRC6a-KO	G-protein coupled receptor group C member 6A knockout
GTP	Guanosine triphosphate
HFD	High fat diet
HPLC	High pressure liquid chromatography
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IBMX	3-isobutyl-1-methylxanthine
ICV	Intracerebroventricular
IP	Intraperitoneal
IP-1	Intervening peptide 1
IP-2	Intervening peptide 2
IP3	Inositol trisphosphate
KOH	Potassium hydroxide
KOMP	Knockout Mouse Project
L-15	Leibovitz-15
L-Arg.HCl	L-arginine monohydrochloride
LDH	3-Lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
LHA	Lateral hypothalamic area
L-Phe	L-Phenylalanine
MBH	Mediobasal hypothalamus
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MCH	Melanin concentrating hormone
MCH-R1	Melanin concentrating hormone receptor 1
MCH-R2	Melanin concentrating hormone receptor 2
MEF	Mouse embryonic fibroblast

MSH	Melanocyte stimulating hormone
mTORC1	Mammalian target of rapamycin complex 1
NAGS	N-acetylglutamate synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
Npy6r	Npy6 receptor
NSB	Non-specific binding
NTS	Nucleus of the solitary tract
OAA	Oxaloacetate
OAT	Ornithine aminotransferase
Ob-R	Leptin receptor
OG	Oral gavage
OH-L-Arg	N(G)-hydroxy-L-arginine
OTC	Ornithine carbamoyltransferase
OXM	Oxyntomodulin
OXR1	Orexin receptor 1
OXR2	Orexin receptor 2
P5C	L-Δ1-pyrroline-5-carboxylate
P5CS	L-Δ1-pyrroline-5-carboxylate synthetase
PAH	Phenylalanine hydroxylase
PBN	Parabrachial nucleus
PEG	Poly ethylene glycol
PKU	Phenylketonuria
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PROHD	Proline dehydrogenase
PVN	Paraventricular nucleus
PYY	Peptide YY
qBH_2	Quinonoid dihydrobiopterin
QC	Quality control
RER	Respiratory exchange rate
RIA	Radioimmunoassay
SEM	Standard error of the mean
SLC	Solute-linked carrier
SNAT2	Sodium-coupled neutral amino acid transporter 2
T1R1-T1R3	Taste receptor 1 member 1- taste receptor 1 member 3
TRPM5	Transient receptor potential cation channel subfamily M member 6
TSC	Tuberous sclerosis
VCO_2	Carbon dioxide production
VFT	Venus fly trap
VMN	Ventromedial nucleus
VO_2	Oxygen consumption

WHO	World Health Organization
WT	Wild-type
Y4R	Y4 receptor

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Chapter I:
General Introduction

1.1 Obesity

Obesity is a major health problem. In 2014, more than 1.9 billion adults worldwide were overweight, of which approximately 600 million were estimated to be obese (WHO, 2015). Obesity was previously thought to only be a health problem in developed countries. However, the prevalence of obesity and overweight is dramatically rising in developing countries, and obesity is now becoming a global epidemic.

The World Health Organization (WHO) defines obesity as an abnormal or excessive accumulation of fat that can affect health (WHO, 2015). Obesity is associated with increased risks of developing a number of chronic diseases, including type II diabetes, cardiovascular disease and cancer. Currently, individuals with body mass index (BMI) of $30Kg/m^2$ or over are classified as obese. A recent systemic analysis study suggested that 67% of men and 57% of women in the UK are either overweight (BMI of over 25, but under 30) or obese (BMI of 30 or over) (Ng et al., 2014).

Excess weight gain within a population increases the financial burden associated with treating such conditions. A recent study projected that by 2030 there will be 65 million more obese adults in the USA and 11 million more obese adults in the UK, consequently increasing the cases of diabetes, heart disease, stroke and cancer to alarming rates and increasing the economic costs associated with treating such diseases by approximately 48-66 billion dollars per year in the USA and by 1.9-2 billion pounds per year in the UK (Wang et al., 2011b). Despite this, there is currently a lack of effective and safe treatments for obesity.

1.1.1 Current obesity treatment

Current treatments for obesity include life style modifications, pharmacological intervention and bariatric surgery. Life style modifications, including increased physical activity and dietary restriction, remain the first line treatment option. Although such changes have proven

effective in the short term, they are often difficult to adhere to and it is therefore challenging for individuals to sustain initial weight loss over longer time periods (Weiss et al., 2007).

The pharmacological agents available for treatment of obesity are very limited. Currently, Orlistat is the only licensed prescription medication for obesity in the UK. Orlistat is a gastric and pancreatic lipase inhibitor which acts by inhibiting the absorption of fat in the gastrointestinal (GI) tract. Orlistat is associated with modest weight loss in obese individuals. In a systemic review of Orlistat randomised clinical trials, only 30% of patients on Orlistat achieved weight loss of greater than 5% after one year of treatment, suggesting only modest efficacy. Furthermore, as a consequence of its mechanism of action, Orlistat can result in unpleasant GI side effects (Powell et al., 2011).

The Food and Drug Administration (FDA) has recently approved three new medicines, Belviq, Qsymia, and Saxenda (which was originally licensed for the treatment of diabetes), for the treatment of obesity in the USA.

Belviq (Lorcaserin) is a selective 5-HT_{2c} (5-hydroxytryptamine; serotonin) receptor agonist which is thought to act on the melanocortin system within the central nervous system (CNS) (Lam et al., 2008). The FDA currently recommends a weight loss of greater 5% for a novel agent in order to be approved for the treatment of obesity. However, reports from a phase III clinical trial demonstrate 3.6% weight loss with Lorcaserin compared to the placebo, suggesting relatively low efficacy (Smith et al., 2010). In addition Lorcaserin is associated with adverse side effects, including nausea, headache and dizziness (Chan et al., 2013).

Qsymia (Qnexa) is a combination of phentermine, an amphetamine derivative, and the anticonvulsant topiramate, a weak carbonic anhydrase inhibitor. Two clinical studies, EQUIP and CONQUER, investigated the efficacy and safety of Qsymia and were subsequently used in the approval process. The CONQUER study was later extended for a second year of observations in patients, which was called the SEQUEL study. The SEQUEL study reported 79% of patients treated with Qsymia demonstrated weight loss greater than 5% (Garvey et

al., 2012). The CONQUER study also reported significant improvements in blood pressure and glycaemic parameters in patients treated with Qsymia (Gadde et al., 2011). However, there are safety concerns associated with Qsymia, including possible teratogenic complications and eye problems. In addition, side effects often associated with centrally acting drugs, such as depression and anxiety, were reported by some patients taking Qsymia.

In December 2014 the FDA approved Saxenda (Liraglutide) as an obesity treatment for chronic weight loss management in addition to a reduced calorie diet and increased physical activity (FDA, 2014). Liraglutide, a glucagon like peptide 1 (GLP-1) receptor agonist, has been extensively used at lower doses for treatment of type II diabetes. The clinical trial results reported an average 4.5% weight loss compared to placebo at one year. Safety concerns have previously been raised regarding the clinical use of GLP-1 analogues. Liraglutide has been associated with acute pancreatitis in diabetic patients treated with the drug (Lee et al., 2011). However, this effect has not been observed in rodent studies (Koehler et al., 2009), and recent reports from human trials suggest that GLP-1 agonist-based drugs are not associated with pancreatitis or pancreatitis cancer any more than other available anti-diabetic medications (Funch et al., 2014). In addition, evidence from rodent studies suggests that Liraglutide can result in thyroid C-cell tumours (Bjerre Knudsen et al., 2010). However, this has not been reported in humans. The safety and the relatively limited efficacy of the pharmacological agents currently available or in late phase III clinical trials remain the biggest impediments in drug development for the treatment of obesity (McGavigan and Murphy, 2012).

Bariatric surgery is the most effective obesity treatment to date, leading to sustained weight loss in patients (Maggard et al., 2005). However, it is an impractical choice for the majority of patients due to its invasive nature, the risks associated with surgery, and the resources required. Currently, bariatric surgery is only available to morbidly obese individuals with a secondary life threatening disease such as diabetes or cardiovascular complications.

1.2 Energy Homeostasis

The ability of an organism to maintain a relatively stable internal environment is a vital process that requires precise regulatory mechanisms. Specific regions within the brain are involved in regulating homeostatic processes including energy expenditure and food intake. The hypothalamus is the most important region involved in regulation of food intake and energy homeostasis. In addition, the caudal brainstem and parts of cortex are also involved. These brain regions integrate neuronal and hormonal signals from other regions of the brain and from peripheral organs including the GI tract and adipose tissue.

1.2.1 The hypothalamus

Hypothalamus has a major role in the CNS, integrating information from endocrine and autonomic systems. The hypothalamus is located below the thalamus at the base of the brain and is comprised of several distinct nuclei including the arcuate nucleus (ARC), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) (Fig. 1.1). These hypothalamic nuclei have extensive connections and neuronal projections between each other and extra-hypothalamic regions that collectively co-ordinate and regulate energy homeostasis (Molina, 2013).
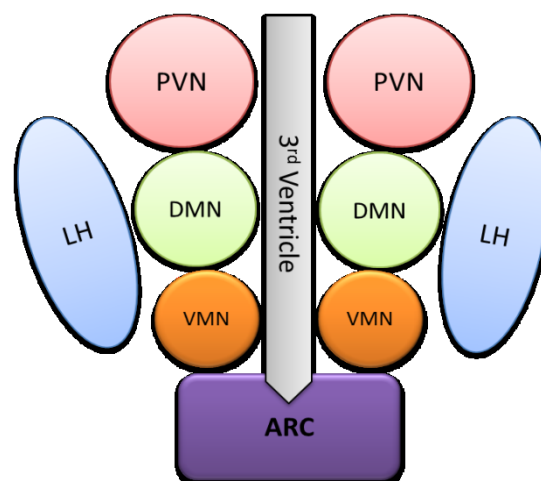
Figure 1.1 Schematic diagram of a coronal section of rat hypothalamus displaying the relative locations of the hypothalamic nuclei implicated in appetite regulation. PVN, Paraventricular nucleus; LH, Lateral hypothalamus; DMN, Dorsomedial nucleus; VMN, Ventromedial nucleus; ARC, Arcuate nucleus. Adapted from (Druce and Bloom, 2006)

The role of the hypothalamus in regulating the body weight and energy homeostasis was first conclusively demonstrated in early rodent studies in which VMN lesioning resulted in hyperphagia and weight gain (Hetherington and Ranson, 1940), and ablation of LHA conversely led to diminished appetite and weight loss in rats (Anand and Brobeck, 1951). Such early observations led to the 'dual centre' hypothesis and the belief that specific regions in the brain such as VMH and LHA control specific feeding behaviours. However, the notion of a specific appetite regulatory 'centre' of the brain has since been superseded by the concept of complex integrated neuronal networks and subpopulations within the hypothalamic nuclei that regulate energy balance (Murphy and Bloom, 2006). Within the hypothalamus, the ARC has emerged as a key region involved in the control of energy balance.

The ARC is situated at the base of the hypothalamus and sits in close proximity to the median eminence, an area with incomplete blood-brain barrier, exposing the ARC to circulating nutrients and metabolic signals (Schwartz et al., 2000). The ARC contains two functionally distinct and well characterised neuronal populations involved in energy homeostasis; the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurones, which stimulate food intake, and anorexigenic neurones co-expressing proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART), which inhibit food intake (Fig. 1.2). These neuronal circuits extensively communicate with neurones in other hypothalamic nuclei and extra-hypothalamic regions of CNS to regulate energy homeostasis.(Bagnol et al., 1999).
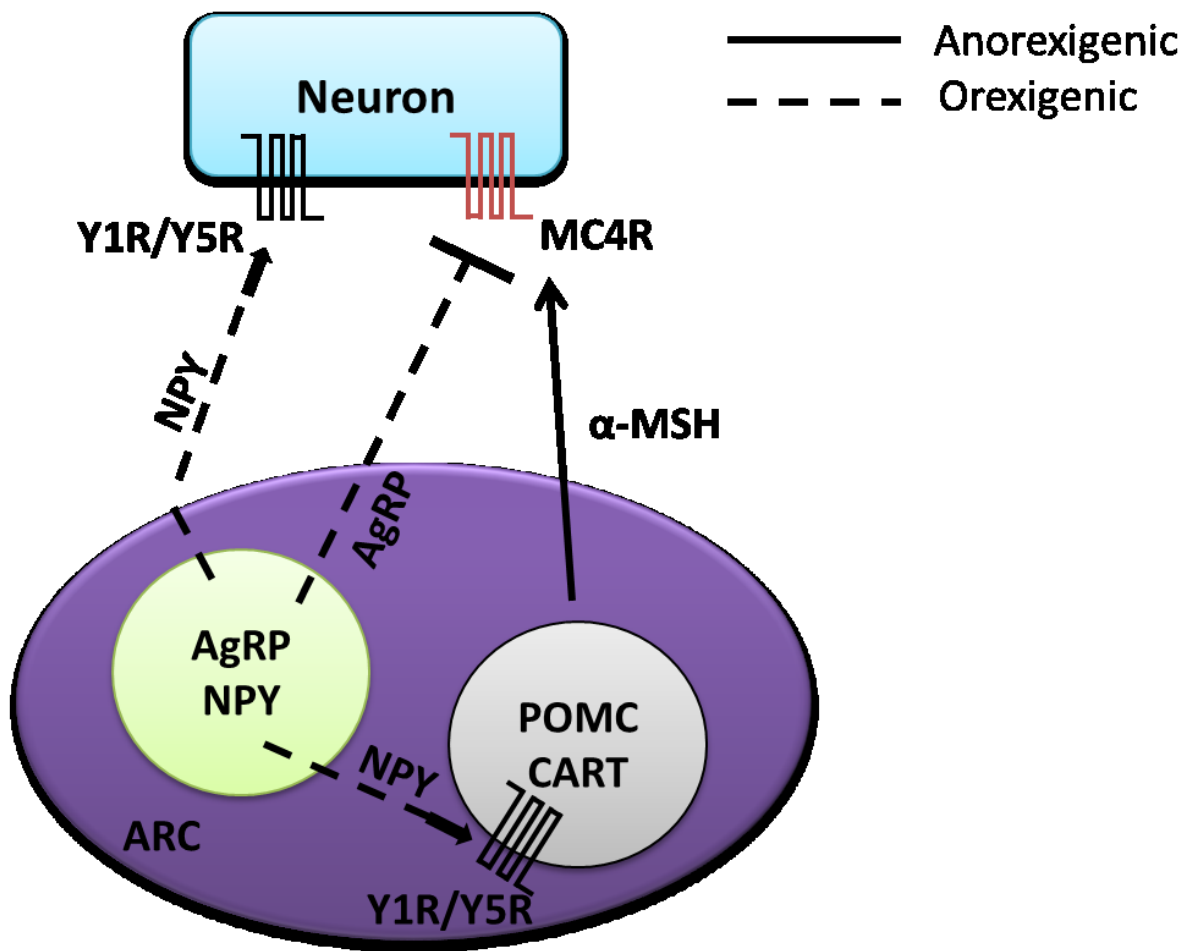
Figure 1.2 Schematic diagram of the hypothalamic arcuate nucleus neuronal populations involved in appetite regulation.
ARC: arcuate nucleus, NPY: neuropeptide Y, AgRP: agouti related peptide, POMC: proopiomelanocortin, CART: cocaine amphetamine regulated transcript, MC4R: melanocortin 4 receptor, Y1R: Y1 receptor, Y5R: Y5 receptor, α-MSH: alpha-melanocyte stimulating hormone. Adapted from (Schwartz and Morton, 2002).

1.2.1.1 Neuropeptide Y (NPY):

NPY is a neuropeptide widely expressed in the brain. It is highly expressed in the ARC (Morris, 1989), and is the most potent orexigenic neuropeptide known, acting via Y1 and Y5 receptors to increase food intake (Rudolf et al., 1994, Criscione et al., 1998). Both Y1 and Y5 receptors are widely expressed in the brain, and notably within hypothalamic nuclei including the ARC and PVN (Kopp et al., 2002, Durkin et al., 2000). Central administration of NPY, or specific Y1 or Y5 agonists increases food intake (Cabrele et al., 2000, Mullins et al., 2001). Arcuate NPY neurones modulate the activity of neighbouring POMC neurons by releasing NPY to act on Y1 receptors expressed on POMC neurones (Roseberry et al., 2004). In addition, ARC NPY is co-expressed with the Y2 receptor, which is thought to act as an inhibitory autoreceptor modulating the release of NPY and is a target receptor for the anorexigenic gut hormone peptide YY (PYY) (Broberger et al., 1997).

Pharmacological evidence suggests that NPY plays a role in appetite regulation. Chronic central administration of NPY causes hyperphagia and obesity (Stanley et al., 1986). Recombinant adeno-associated virus (AAV)- mediated overexpression of NPY in the LHA or the PVN increases food intake in rats (Tiesjema et al., 2007). In addition, NPY is upregulated in the ARC following fasting; suggesting a physiological role in appetite regulation (Sahu et al., 1988). NPY knockdown in the ARC resulted in reduction in food intake and body weight in rats (Gardiner et al., 2005), however germline NPY knockout has no appetite or body weight phenotype (Erickson et al., 1996), which suggests developmental compensation.

NPY signalling has been explored as a target for treatments for obesity. Specific Y1 and Y5 receptor antagonists have been developed and studied. However, most of these agents have failed to progress into clinical trials due to poor brain penetrability, low oral bioavailability, and lack of selectivity. The few that have progressed to clinical trials have shown poor long term efficacy (Erondu et al., 2006, Antal-Zimanyi et al., 2008).

1.2.1.2 Agouti related peptide (AgRP)

AgRP is highly co-expressed with NPY in the ARC (Broberger et al., 1998), and like NPY, it is an orexigenic peptide (Rossi et al., 1998). The mechanisms behind the orexigenic effects of AgRP are mediated through the melanocortin system. AgRP is an antagonist of the melanocortin 3 receptor (MC3R) (Ollmann et al., 1997) and an inverse agonist of the melanocortin 4 receptor (MC4R) (Nijenhuis et al., 2001). Central administration of AgRP increases food intake in rodents (Rossi et al., 1998), and its expression in the ARC is up-regulated in the fasted state (Bi et al., 2003). Overexpression of AgRP leads to obesity in rodents (Graham et al., 1997). As with NPY, global germline knockout of AgRP results in a very limited body weight or appetite phenotype (Qian et al., 2002). However, post-natal destruction of AgRP-expressing neurons leads to a significant reduction in food intake and body weight in rodents (Gropp et al., 2005), suggesting this neuronal population is critical to energy homeostasis. The NPY/AgRP neurones also release neurotransmitter gamma-aminobutyric acid (GABA), by which pathway they can inhibit the activity of ARC POMC neurons, and influence other neuronal populations. Administration of a $GABA_A$ receptor agonist into the parabrachial nucleus (PBN) reverses the effect of post natal AgRP neuron deletion on food intake and survival, suggesting an important role for the GABA released from AgRP neurones in the PBN in the regulation of food intake and body weight (Wu et al., 2009).

1.2.1.3 Pro-opiomelanocortin (POMC)

POMC neurons are found in the ARC and in the nucleus of the solitary tract (NTS) in the brainstem. POMC is a precursor molecule that undergoes post-translational modifications to generate melanocyte stimulating hormones (α-, β-,and γ-MSH), β-endorphin, and adrenocorticotrophic hormone (ACTH) (Fig. 1.3) (Cone, 2005). POMC expression in the ARC is significantly increased following food deprivation in rodents (Mizuno et al., 1998).
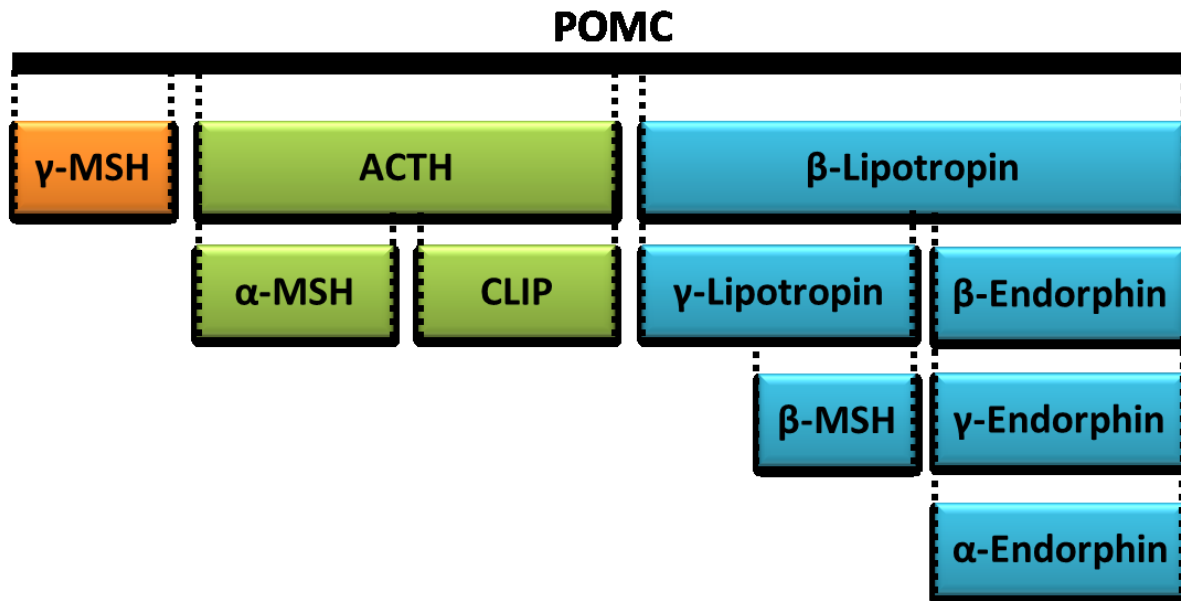
POMC

Figure 1.3 Schematic diagram illustrating pro-opiomelanocortin and its products
POMC undergoes a series of enzyme-dependent post-translational changes.
MSH, melanocyte stimulating hormone; ACTH, adrenocorticotropic hormone; CLIP, corticotrophin-like intermediate peptide.

α-MSH is an anorexigenic neuropeptide produced from central POMC neurons which acts as an agonist of the MC3R and MC4R receptors. Central administration of α-MSH in rodents inhibits food intake (Tsujii and Bray, 1989). MC3R has limited expression in the CNS, but it is expressed in the ARC (Roselli-Rehfuss et al., 1993). MC3R knockout mice exhibit a phenotype which includes mildly increased body weight and fat mass, but without hyperphagia (Chen et al., 2000). It is therefore speculated that the MC3R plays a greater role in energy expenditure than in the regulation of food intake.

In contrast, MC4R knockout mice are hyperphagic and obese (Huszar et al., 1997). MC4R is widely expressed in the brain in regions including the ARC, PVN and DMN in the hypothalamus, and in the hippocampus and brainstem (Gantz et al., 1993). Evidence suggests that MC4R signalling is a key regulator of appetite and energy homeostasis. Selective re-expression of MC4R in MCR4 knockout mice in the PVN and central amygdala attenuates approximately 60% of the obese phenotype (Balthasar et al., 2005). In addition, human mutations in MC4R gene are the most common known cause of monogenic obesity

(Farooqi et al., 2000). These studies suggest that MC4R is a key player in appetite regulation, providing an important inhibitory tone that results in obesity and hyperphagia in both human and rodents when disrupted.

MC4R agonists have been developed as potential treatments for obesity. However, they have failed in clinical trials due to poor efficacy and adverse side effects such as pressor activity (Krishna et al., 2009, Fehm et al., 2001). A recent MC4R agonist has been reported to suppress food intake without cardiovascular effects in non-human primates, and this agent is currently undergoing clinical trial (Greenhill, 2012).

1.2.1.4 Melanin-concentrating hormone (MCH)

Melanin concentrating hormone is an orexigenic neuropeptide highly expressed in neurons in the LHA and zona incerta (Bittencourt et al., 1992). In humans, MCH acts on the MCH receptor 1 (MCH-R1) (Chambers et al., 1999), and the MCH receptor 2 (MCH-R2) (Sailer et al., 2001). Central administration of MCH increases food intake and weight gain in rodents (Della-Zuana et al., 2002). Overexpression of MCH results in hyperphagia and obesity in rodents (Ludwig et al., 2001), whereas MCH knockout mice are hypoghagic, lean, and have an elevated metabolic rate (Shimada et al., 1998).

1.2.1.5 Orexins

The orexins are orexigenic neuropeptides also expressed in the LHA (Sakurai et al., 1998), though in a neuronal population distinct from that expressing MCH. Orexins exist in two forms; orexin A and orexin B, which have approximately 50% sequence identity and are produced by cleavage of a single precursor protein molecule (Nixon et al., 2015). There are two orexin receptors: orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R). Orexin A binds to both receptors with high affinity, while orexin B preferentially binds to OX2R (Trivedi et al., 1998). Central administration of orexin A or B stimulates food intake in rats (Sakurai et al., 1998). However, it is unclear to whether this represents pharmacological activation of a physiological feeding pathway, or a secondary effect as a result of increased arousal. Orexin knockout models have also been generated. These models have hyperphagia and obesity

phenotype, but their predominant phenotype is narcolepsy (Chemelli et al., 1999), suggesting that orexins may be more important in arousal in general than in energy homeostasis specifically.

1.2.2 The caudal brainstem

The brainstem is another key region involved in the regulation of energy balance. In the absence of hypothalamic and forebrain inputs, the caudal brainstem is still capable of controlling some aspects of feeding behaviour in rats (Grill, 2006). There are three major areas of the caudal brainstem involved in the control of energy balance. These are the NTS, the dorsal motor nucleus of the vagus (DMX) and the area postrema (AP), which together comprise the dorsal vagal complex (DVC) (Schwartz, 2006). A combination of mechanosensory, chemosensory and endocrine signals from the GI tract is received by the vagal afferent synapses in the NTS of the caudal brainstem (van der Kooy et al., 1984). The DMX provides parasympathetic motor output to peripheral organs, including the upper GI tract, and receives signals from the NTS (Ito and Seki, 1998). The NTS itself receives input from the AP, a circumventricular organ with an incomplete blood-brain barrier, which is thus accessible to circulating stimuli including nutrients and peptide hormones. The AP has projections to other areas of the brainstem besides the NTS, including the DMX and the parabrachial nucleus (Ito and Seki, 1998). The NTS contains neurons which express POMC and MC4R, which are thought to be involved in the regulation of appetite and energy balance within the brainstem (Mountjoy et al., 1994). In addition, evidence suggests that many of the gut peptides that influence appetite at least in part act via vagal afferents and the brainstem (Date et al., 2002, Abbott et al., 2005).

1.3 Adiposity Signals

The CNS plays a vital role in the regulation of energy homeostasis by integrating signals from the periphery which communicate information about the overall energy state of an individual. These signals can be divided into short term episodic signals which respond to

acute nutrient state, such as GI hormones, or long term tonic signals, such as leptin and insulin, that circulate in levels proportional to adipose tissue mass.

1.3.1 Insulin

Insulin is a 51 amino acid peptide hormone and product of *INS* gene that is primarily produced from the precursor pro-insulin peptide by the pancreatic β-cells. It is essential for both glucose homeostasis and energy balance. Insulin levels are rapidly elevated post prandially to stimulate the uptake of excess glucose from the circulation by acting on tissues such as liver, adipose tissue and skeletal muscle.

In addition to its classical role in maintaining the glucose homeostasis, insulin is also known to be essential in regulating energy balance. Plasma insulin levels are proportional to both the adiposity (Bagdade et al., 1967) and body fat mass (Polonsky et al., 1988), suggesting insulin has a role in relaying peripheral energy availability to the CNS. The insulin released into circulation crosses the blood-brain barrier via a saturable transport mechanism, to act as an anorexigenic signal to central brain circuits (Woods et al., 2003). Insulin acts by binding to the insulin receptor, which is widely expressed throughout the peripheral tissues and the CNS, including areas implicated in energy homeostasis such as the hypothalamic ARC (Marks et al., 1990). Central administration of insulin reduces food intake and body weight in rodents (Chavez et al., 1995). Evidence suggests that insulin modulates the expression of ARC NPY and POMC. Third ventricle administration of insulin in rats reduces preproNPY expression in the ARC, decreases the NPY release induced by fasting in the PVN, and increases the expression of POMC (Schwartz et al., 1992). Peripheral administration of insulin at levels that do not cause hypoglycaemia reduces food intake in rats (McGowan et al., 1990). Neuronal-specific disruption of the insulin receptor results in increased food intake in female mice and leads to diet-induced obesity (Bruning et al., 2000).

1.3.2 Leptin

Leptin was discovered in 1994 as an adipocyte-derived circulatory protein hormone with a pivotal role in regulating food intake (Zhang et al., 1994). Leptin is the product of *ob* gene and has multiple physiological roles, primarily acting as a long term adiposity signal. Mice lacking the *ob* gene are severely hyperphagic, obese and prone to diabetes. Exogenous recombinant leptin administration to ob/ob mice reduces food intake and reverses the metabolic phenotype associated with the lack of leptin (Campfield et al., 1995, Halaas et al., 1995, Pelleymounter et al., 1995).

Similar to insulin, plasma leptin levels directly correlate with body adiposity (Maffei et al., 1995). Leptin penetrates through the blood-brain barrier, relaying information on peripheral energy state to the appetite regulatory regions in the CNS (Schwartz et al., 1996a). Increased leptin signalling reduces food intake and increases energy expenditure (Weigle et al., 1995, Halaas et al., 1995). In contrast, a reduction in leptin signalling due to low adiposity levels or defects in the leptin gene or its receptor has a significant effect on many regulatory systems, including the reproductive system and energy homeostasis (Boden et al., 1996, Chehab et al., 1996). Leptin exerts its anorexigenic effects by acting on the leptin receptor (Ob-R), which is expressed in the ARC NPY/AgRP and POMC neurons, and in the PVN, DMN and VMN (Baskin et al., 1999, Cheung et al., 1997, Schwartz et al., 1996b). Leptin signals to reduce food intake in part by upregulating the activity of ARC POMC neurons (Seeley et al., 1997), and inhibiting NPY/AgRP neurones (Wang et al., 1997).

The discovery of leptin transformed the field of obesity research. The initial observations in leptin deficient mice led to the suggestion that leptin is a likely aetiological factor in the development of human obesity. However, homozygous mutations of the human leptin gene have so far only been reported in a few families in the world (Montague et al., 1997, Farooqi et al., 1999). The phenotypes of families with the leptin gene mutations resemble that of the ob/ob mouse, and as in these mice, the administration of the exogenous recombinant leptin

has been successful in reversing the obesity phenotype (Farooqi et al., 2007a). Mutations in the leptin receptor gene, though rare, also occur in humans (Farooqi et al., 2007b).

Exogenous leptin therapy was proposed and trialled as a potential treatment in obese individuals. However, it has been shown that most obese individuals have chronically elevated plasma leptin levels and that obesity is associated with leptin resistance rather than leptin deficiency (Halaas et al., 1997, Heymsfield et al., 1999).

1.3.3 Adiponectin

Adiponectin is another protein hormone secreted from adipose tissue (Scherer et al., 1995). Two adiponectin receptors, adiponectin receptor 1 (Adipo-R1) and adiponectin receptor 2 (Adipo-R2), have been identified. Adipo-R1 is particularly highly expressed in skeletal muscle, whereas Adipo-R2 is primarily expressed in the liver (Yamauchi et al., 2003a). Adiponectin circulates at higher levels than most other adipokine hormones, and its concentrations are negatively correlated with adiposity (Arita et al., 1999). The role of adiponectin in glucose homeostasis has been extensively studied (Hotta et al., 2001, Ryo et al., 2004). Peripheral administration of adiponectin results in insulin sensitisation via an Adipo-R1 dependent mechanism (Yamauchi et al., 2003b). Adiponectin knockout mice have severe insulin resistance and elevated tissue triglyceride levels when placed on a high sucrose and fat diet, suggesting a primary role for adiponectin in the regulation of glucose homeostasis (Yamauchi et al., 2007).

In addition to its peripheral effects, central adiponectin has been proposed to centrally regulate food intake and energy balance. However, its precise role is unclear. Some findings suggest an increase in food intake following intracerebroventricular (ICV) administration of adiponectin in rodents (Kubota et al., 2007), whilst other studies have found central administration of adiponectin reduces food intake and increases energy expenditure (Qi et al., 2004, Coope et al., 2008). Both Adipo-R1 and Adipo-R2 are highly expressed in the

ARC, where they are co-localised with NPY and POMC, and in the brainstem (Hoyda et al., 2007, Fry et al., 2006, Guillod-Maximin et al., 2009).

1.4 The Enteric Nervous System: Role of Vagus Nerve

The enteric nervous system (ENS) is one of the major divisions of the nervous system, and is responsible for regulating the function and activity of the GI tract, with important roles in gastric and exocrine secretion, blood supply and gut hormone secretion. The ENS contains two main nerve plexuses, the submucosal plexus and myenteric plexus (Furness, 2012).

The vagus nerve is one of the major extrinsic nerves with a key role in the gut-brain axis. The mucosal and submucosal layers within the GI tract relay mechanosensory and chemosensory signals via vagus afferents to the NTS within the brainstem. In addition, gut hormones including ghrelin, PYY, GLP-1 and cholecystokinin (CCK) have been reported to exert their effects on appetite and food intake via vagal afferents, though other data suggests that ghrelin, PYY and GLP-1 can act directly on the brain (Fig. 1.4) (Date et al., 2002, Koda et al., 2005, Imeryuz et al., 1997, MacLean, 1985).

1.5 Gut Hormones

The GI tract is the largest endocrine organ in the body, secreting more than one hundred bioactive peptides (Ahlman and Nilsson, 2001). These peptide hormones are secreted from a range of enteroendocrine cells that are embedded in the GI epithelium along the length of the GI tract. These cells are capable of sensing changes in luminal nutrient content, pH and GI tract distension, either directly or via other cell types, and modulate their release of appetite-regulating peptide hormones in response to these changes (Fig 1.4) (Dhillo and Bloom, 2004).
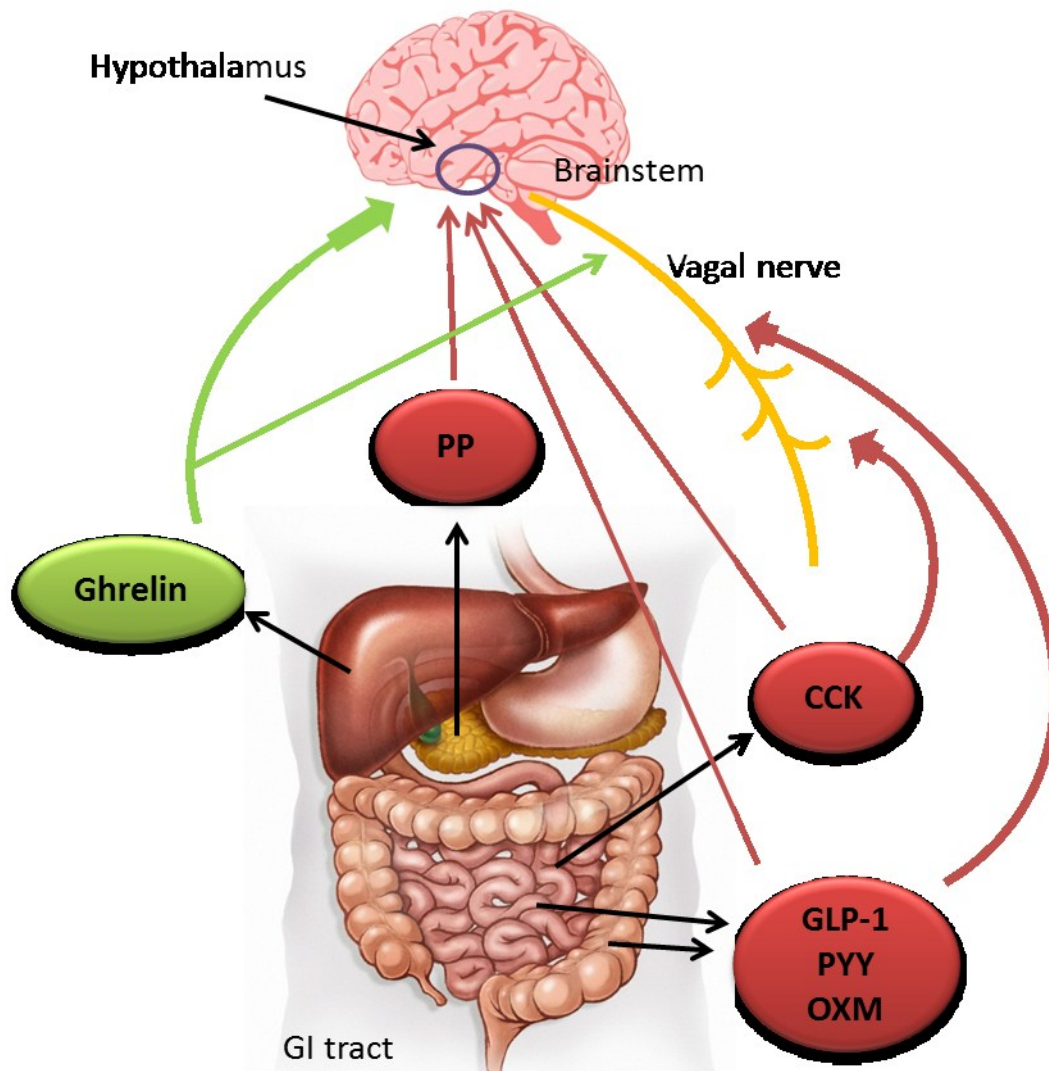
Figure 1.4 Schematic diagram showing the putative pathways by which gut hormones regulate appetite.
Gut hormones are released from specific cells from various levels of the GI tract and stimulate the anorectic or orexigenic pathways in the CNS directly or via signalling through vagus. GLP-1: glucagon like peptide-1, PYY: peptide YY, OXM: oxyntomodulin, PP: pancreatic polypeptide, CCK: Cholecystokinin. Adapted from (Murphy and Bloom, 2006)

1.5.1 Ghrelin

Ghrelin is an orexigenic gut peptide hormone secreted from X/A-like enteroendocrine cells in

the stomach and upper small intestine (Date et al., 2000). It was first isolated from rat

stomach and was identified as the endogenous ligand for the growth hormone secretagogue

receptor (GHSR-1a) (Date et al., 2000). The ghrelin gene encodes the pre-proghrelin

peptide which is cleaved and processed to form the 28 amino acid ghrelin peptide. Ghrelin exists in two distinct forms: acyl and des-acyl ghrelin peptides. Acylated form of ghrelin has an n-octanoyl group attached to the serine at position 3 (Kojima et al., 1999). Ghrelin acylation is facilitated by the enzyme ghrelin O-acyltransferase (GOAT) (Yang et al., 2008), and is crucial for the biological activity of ghrelin. Acyl-ghrelin acts as an orexigenic agent. Central or peripheral administration of acyl-ghrelin increases food intake and adiposity in rats (Wren et al., 2000). In addition to its orexigenic role, ghrelin is thought to be involved in maintaining fasting glucose levels (Scott et al., 2012), gastric motility (Levin et al., 2006) and adipogenesis (Thompson et al., 2004). The physiological role of des-acyl ghrelin is not clear; it has been reported to have no effect on food intake (Asakawa et al., 2005, Neary et al., 2006), but other groups have found it to have some biological activity (Broglio et al., 2004, Heppner et al., 2014). The orexigenic effects of acyl-ghrelin are thought to be mediated by NPY/AgRP neurones in the ARC. Ghrelin administration has shown to increase the expression of NPY and AgRP in the ARC (Kamegai et al., 2001), and the orexigenic effects of ghrelin are abolished in NPY/AgRP null mice (Chen et al., 2004).

Blocking ghrelin signalling has been investigated as a potential approach to treat obesity. Ghrelin and ghrelin receptor knockout mice models are resistant to diet-induced obesity (Sun et al., 2008). GHSR antagonists and ghrelin antibodies have been developed as potential treatments for obesity (Asakawa et al., 2003a, Vizcarra et al., 2007). However, so far these approaches have been unsuccessful, which may reflect the adaptive reduction in circulating ghrelin which occurs in obesity, and which presumably reduces the effectiveness of additional attempts to reduce ghrelin signalling (Alvarez-Castro et al., 2013). GOAT antagonists have been explored as alternative targets and initial results have been promising, demonstrating reduced food intake, body weight and fat mass in high fat fed rodents (Barnett et al., 2010).

1.5.2 Cholecystokinin (CCK)

CCK was the first gut hormone discovered to be involved in the regulation of energy homeostasis (Jorpes and Mutt, 1959). It is an anorexigenic hormone secreted from enteroendocrine I-cells predominantly located in the proximal intestine (Buchan et al., 1978). The pre-procholecystokinin protein is processed to generate several CCK isoforms. Two major forms, CCK-8 and CCK-33, are secreted by the I-cells in response to fats and proteins (Polak et al., 1975, Liddle et al., 1985). Besides inhibiting appetite, active forms of CCK induce gallbladder contraction, stimulate pancreatic enzyme secretion and slow gastric motility (Liddle et al., 1985, Brugge et al., 1986). CCK acts via two receptors, CCK receptor 1 (CCK-1R) and CCK receptor 2 (CCK-2R) (Wank et al., 1992). The anorexigenic effects of CCK are mainly mediated through the binding of CCK-8 isoform to CCK-1R on the surface of vagal afferent fibres (Moran et al., 1990); vagal lesioning in rats abolishes the inhibitory effects of CCK on food intake (Lorenz and Goldman, 1982).

CCK infusion reduces food intake in human subjects (Muurahainen et al., 1988). Studies in rodents have demonstrated that CCK produces a short term signal leading to an acute reduction in calorie intake during meals (West et al., 1984). CCK-1R antagonism has shown to increase food intake in both rodents (Hewson et al., 1988) and humans (Beglinger et al., 2001), demonstrating that CCK plays a physiological role in the control of food intake. However, chronic administration of CCK does not chronically reduce food intake or body weight (West et al., 1987), as feeding patterns adapt to ensure adequate calorie intake. These findings suggest the therapeutic potential of CCK as a stand-alone anorectic agent is limited. However, CCK seems to have the ability to enhance the response to other anorectic hormones such as leptin and amylin (Matson et al., 2000, Bhavsar et al., 1998), suggesting that CCK may be a viable candidate as a component of combination therapy for the treatment of obesity.

1.5.3 Peptide YY (PYY)

PYY belongs to the PP-fold family of proteins, which also includes NPY. It is 36 amino acids in length and is secreted from the enteroendocrine L-cells in response to an oral nutrient load. Two native forms of PYY are found in circulation; PYY_{1-36} and PYY_{3-36} (Grandt et al., 1994). PYY_{3-36} is the major circulating peptide, and is produced upon the enzymatic N-terminal cleavage of PYY_{1-36} by dipeptidyl peptidase-4 (DPP-4) (Mentlein et al., 1993). PYY_{1-36} binds to Y1, Y2 and Y5 receptors whereas PYY_{3-36} is a selective Y2 receptor agonist (Walther et al., 2011). Whilst PYY_{1-36} does not seem to have a major effect on food intake (Sloth et al., 2007), PYY_{3-36} is an anorexigenic gut hormone that reduces food intake in both lean and obese rodents and humans following peripheral administration (Challis et al., 2003, Batterham et al., 2002, Batterham et al., 2003). PYY_{3-36} can also inhibit gastric emptying and intestinal transit time (Savage et al., 1987). PYY_{3-36} mediates its anorectic effects via the Y2 receptor, likely including those expressed on NPY/AgRP neurones within the ARC (Dumont et al., 1995, Batterham et al., 2002). It has also been suggested that PYY_{3-36} may exert its anorexigenic effects via Y2 receptor expressed on the vagal afferent fibres and in the brainstem (Koda et al., 2005), as vagotomy can abolish the anorexigenic effects of peripherally administrated PYY_{3-36} in rodents (Abbott et al., 2005).

Administration of exogenous PYY_{3-36} has been studied as a potential therapeutic agent for the treatment of obesity. However, like many gut peptides, supraphysiological concentrations of PYY_{3-36} can cause nausea (le Roux et al., 2008). In addition, PYY_{3-36} is rapidly metabolised (Lluis et al., 1989), thus making endogenous PYY_{3-36} peptide an unsuitable therapeutic agent. Long-acting PYY_{3-36} analogues are being explored as alternative strategies.

1.5.4 Pancreatic polypeptide (PP)

PP also belongs to the PP fold peptide family. It is a 36 amino acid peptide synthesised and secreted post prandially by the endocrine pancreatic PP (F cells) cells (Adrian et al., 1976,

Adrian et al., 1977). PP primarily binds to the Y4 receptor (Y4R) and to a lesser extent to Y1 and Y5 receptors (Berglund et al., 2003). Peripheral administration of PP reduces food intake in both humans and mice (Asakawa et al., 1999, Asakawa et al., 2003b). Moreover, overexpression of PP in mice results in hypophagic and lean phenotype (Ueno et al., 1999). It is thought that PP exerts its anorexigenic effects via binding to Y4R, which is highly expressed in central regions involved in appetite regulation, including the AP, the NTS and the ARC (Lin et al., 2009). Peripheral administration of PP increases c-fos expression in these areas. In addition, the anorectic properties of PP are abolished in Y4R knockout mice (Lin et al., 2009). It has been shown that PP release is tightly regulated by the vagus (Schwartz et al., 1978). Vagotomy significantly inhibits the release of PP (Asakawa et al., 2003b) and reduces its anorexigenic effects, suggesting an important role for the vagus and brainstem signalling in PP-mediated appetite regulation. Moreover, a recent study using Npy6 receptor (Npy6r) knockout mice model demonstrated a central regulatory mechanism for PP in body composition and growth hormone axis. Peripheral administration of PP increased c-fos expression in the hypothalamic suprachiasmatic nucleus and resulted in reduced food intake and increased energy expenditure in wild-type (WT) mice, but not in *Npy6r* knockout mice, suggesting that PP's anorectic effects require Npyr6 signalling. (Yulyaningsih et al., 2014).

1.5.5 Preproglucagon products

Preproglucagon is synthesised by pancreatic α-cells, the enteroendocrine L-cells of the GI tract and specific neurons in the NTS (Kieffer and Habener, 1999). Preproglucagon undergoes post translational modification in the gut resulting in production of the active peptides glucagon, GLP-1, oxyntomodulin, GLP-2 and glicentin (Fig. 1.5) (Holst, 2007).
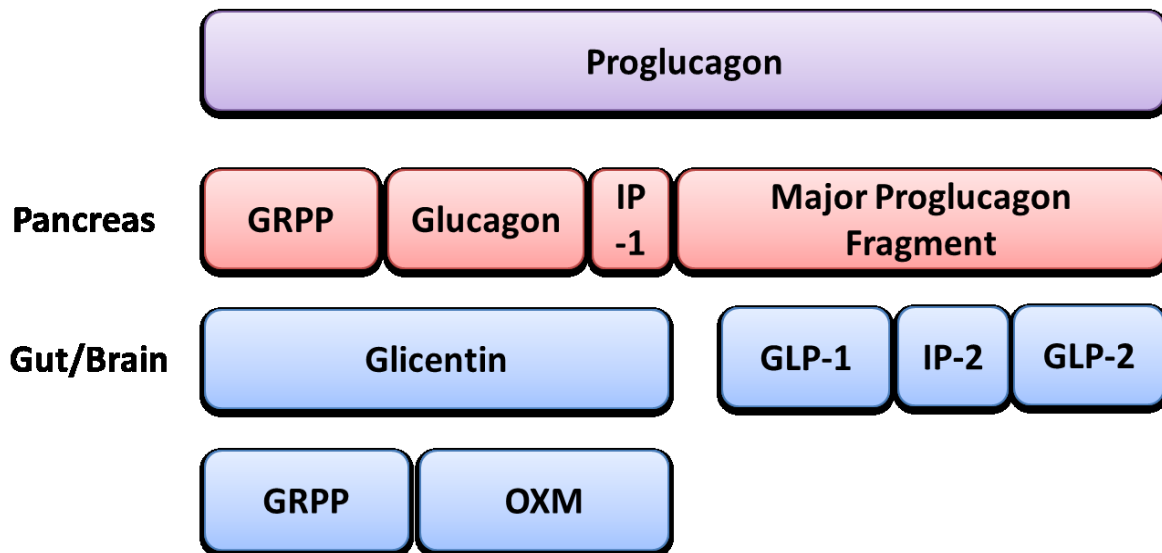
Figure 1.5 Schematic diagram illustrating pro-glucagon processing in neuroendocrine cells.
GRPP: glicentin-related polypeptide, IP-1: intervening peptide 1, IP-2: intervening peptide 2, GLP-1: glucagon-like peptide-1, GLP-2: glucagon-like peptide-2. Adapted from (Holst, 2007).

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

GLP-1 is an anorexigenic peptide synthesised in enteroendocrine L-cells and secreted post prandially. GLP-1 has a well characterised incretin role, stimulating the release of insulin following nutrient ingestion. It also slows gastric emptying and inhibits food intake (Holst, 1999, Holst, 2007). GLP-1 is co-localised with other pre-proglucagon products, including oxyntomodulin (OXM), and is co-expressed with PYY in L-cells, particularly in the distal small intestine and the large intestine (Eissele et al., 1992). GLP-1 exists in two major bioactive forms, GLP-1$_{7-37}$ and GLP-1$_{7-36\ amide}$. Both forms are potent agonists of the GLP-1 receptor (GLP-1R), though GLP-1$_{7-36\ amide}$ is thought to be the major bioactive form in circulation (Orskov et al., 1994). The GLP-1R is expressed in several appetite regulatory regions within the hypothalamus and the brainstem, including the ARC, the PVN and the NTS (Bullock et al., 1996, Campos et al., 1994). Peripheral administration of GLP-1 increases c-fos expression in the PVN and brainstem (Baggio et al., 2004). In addition, GLP-1R is also found in the peripheral nervous system, heart, kidney, pancreatic islets and the GI tract (Bullock et al., 1996). Central administration of exendin$_{9-39}$, a competitive inhibitor of the

GLP-1R, increases food intake and promotes body weight gain in rats (Meeran et al., 1999). Exendin-4, a well characterised GLP-1R agonist, reduces food intake and body weight gain (Baggio and Drucker, 2007). Despite these observations, the physiological significance of GLP-1R activity in the regulation of energy homeostasis is still disputed, as GLP-1R knockout mice do not exhibit hyperphagia or an obese phenotype (Scrocchi et al., 2000).

The anorectic effects of GLP-1 have been explored as a potential anti-obesity treatment. However, like most gut hormones, GLP-1 has a short half-life in circulation, being rapidly degraded by DPP-4 enzyme activity (Holst, 2007). However the incretin properties of GLP-1 have been exploited by anti-diabetic drugs. Exenatide and Liraglutide are long-acting GLP-1 analogues that are extensively used for the treatment of type II diabetes (Buse et al., 2009). They have both been shown to cause weight loss in clinical trials (Neff and Kushner, 2010). Recently, the USA FDA has approved Liraglutide as a novel treatment for chronic weight management in obese individuals with associated co-morbidities including type II diabetes (FDA, 2014)

GLP-1 analogues have proven to be effective drugs in treatment of type II diabetes which can also help to reduce body weight chronically in obese patients. However, there have been reported cases of acute pancreatitis in patients treated with Exanatide or Liraglutide, raising safety concerns (Anderson and Trujillo, 2010, Lee et al., 2011).

1.5.5.2 Oxyntomodulin (OXM)

Oxyntomodulin is another product of post translational processing of preproglucagon. It is an anorexigenic peptide hormone secreted from gut entroendocrine L-cells in response to a meal and in proportion to the energy content of the meal (Wynne et al., 2005, Baggio et al., 2004). Similar to GLP-1, OXM inhibits gastric emptying and reduces food intake (Cohen et al., 2003). The exact mechanisms underlying the anorexigenic effects of OXM are unclear. There is no specific receptor identified for OXM. OXM has relatively low affinity for glucagon receptor (GCGR) and it also binds GLP-1R, though with lower affinity than GLP-1 itself

(Fehmann et al., 1994). The anorectic effects of OXM are thought to be mediated by the GLP-1R. Pharmacological inhibition of GLP-1R by Exendin$_{9-39}$ blocks the anorectic effects of OXM (Dakin et al., 2001). Similarly, the effects of OXM on food intake are abolished in GLP-1R knockout mice (Baggio et al., 2004). However, OXM is a more potent anorectic agent when compared to GLP-1 at similar doses, which may suggest additional mechanisms are involved in mediating its effects on food intake (Dakin et al., 2001).

OXM also increases energy expenditure (Wynne et al., 2006). The dual actions of OXM in reducing food intake and increasing energy expenditure have made this system a popular target for obesity drug discovery programs (Pocai, 2014, Liu et al., 2010). OXM analogues may thus have utility in the treatment of obesity.

1.6 Nutrient Sensing

Nutrient sensing is defined as the ability of cells and tissues to recognise and respond to the presence of fuel molecules, including glucose, fatty acids and amino acids (Lindsley and Rutter, 2004). Such cells are often specialised, and express specialised molecular machinery, including receptors and downstream signalling molecules to allow them to carry out this function. Nutrient sensing can contribute towards the regulation of energy balance. Specific regions within the CNS such as the hypothalamus are actively involved in nutrient sensing, as are peripheral tissues including the GI tract. Nutrient sensing receptors are emerging as new targets for the treatment of metabolic conditions such as obesity and type II diabetes. Stimulation of specific nutrient-sensing receptors within the GI tract has been shown to stimulate anorectic gut hormone release (Fig. 1.7) (Edfalk et al., 2008, Hirasawa et al., 2005). While this is a relatively new field of research which requires further exploration, the ability to directly target these nutrient sensing mechanisms to stimulate the release of endogenous anorectic gut hormones may provide a novel therapeutic option to facilitate appetite regulation.

1.7 Macronutrient Diets

The first step, and perhaps the most important factor involved in regulating energy balance, is the control of food intake. Maintaining a balanced energy intake by means of nutritional interventions and dietary modifications has been the focus of many weight loss studies. Despite their initial success, these interventions are usually only effective for short periods of time, as they are difficult to adhere to. The majority of such dietary interventions are associated with increased hunger levels in individuals, making the reduced energy intake more difficult to sustain over time. This has led to the introduction of novel dietary intervention programs which focus on long term weight management and improving obesity-related metabolic disturbances, as well as on initial weight loss (Abete et al., 2010). Amongst these strategies, low glycemic index diets have proven effective. Low glycemic index diets cause weight loss and help maintain blood glucose homeostasis, reduce blood pressure and triglyceride levels (Goff et al., 2013). Similarly, diets rich in omega-3 fatty acids have been reported to help improve cardiovascular health, blood pressure and glucose homeostasis (Due et al., 2008). However, high protein diets have proven to be the most effective diets amongst other macronutrient diets in driving weight loss and improving body weight management (Halton and Hu, 2004).

1.7.1 High protein diets

A normal diet typically provides 55% of its energy from carbohydrates, 30% from fats and 15% from proteins. Increasing protein content to approximately 25-30% of dietary energy has been shown to increase satiety and promote weight loss in both rodents and humans (Soenen et al., 2013, Layman et al., 2009). Evidence suggests that protein induces greater satiety than fats and carbohydrates, and high protein diets are typically associated with reduced calorie intake compared to other macronutrient rich diets (Weigle et al., 2005). In a study where subjects were given *ad libitum* access to high protein diets, they consistently consumed fewer calories compared to control individuals with *ad libitum* access to isocaloric normal protein diets, but still reported similar levels of fullness (Weigle et al., 2005). Several

other studies have demonstrated the satiating power of protein by using a macronutrient preload (Poppitt et al., 1998), comparing high or low protein preloads (Veldhorst et al., 2009c, Veldhorst et al., 2009e), or comparing the effects of different types of protein on appetite (Veldhorst et al., 2009a, Veldhorst et al., 2009b). The exact mechanisms by which high protein diets exert their anorectic effects are not fully understood. Several mechanisms have been proposed, including the thermic effects of protein, the stimulation of gluconeogenesis, and changes in plasma gut hormone levels (Blom et al., 2006).

1.7.1.1 Thermic effect of protein

The body requires energy to process ingested nutrients. The energy required for digestion, absorption and disposal of ingested nutrients is termed dietary induced thermogenesis (DIT), and is associated with energy expenditure increased above basal fasting levels. Protein requires more energy to process than other macronutrients. The DIT for protein is approximately 20-30% of energy consumed, which is considerably higher than that for carbohydrates (5-10%) and fats (0-3%) (Westerterp et al., 1999, Tappy, 1996). Ingested protein requires immediate metabolic processing, as it cannot be stored. Proteins are therefore utilised in a variety of metabolic processes, including protein synthesis, gluconeogenesis and urea production, all of which require energy. High protein diets are thus associated with increased dietary-induced energy expenditure (Luscombe et al., 2002, Luscombe et al., 2003). Levels of increased DIT associated with protein are greatly influenced by the protein source in the diet. Animal proteins, which often contain all of the essential amino acids, induce a greater thermogenic response than vegetable proteins such as soy (Mikkelsen et al., 2000). Several studies have investigated the effects of protein diets on DIT and the associated weight loss. Although high protein diets cause a significant increase in DIT and may contribute to the associated weight loss, these changes are relatively small, and are unlikely to account for the majority of the observed changes in body weight (Crovetti et al., 1998, Eisenstein et al., 2002).

1.7.1.2 Gluconeogenesis

Gluconeogenesis has been proposed to contribute to the effects of high protein diets. High protein diets increase gluconeogenesis. The satiating effects of high protein diets may be in part be due to improved glucose homeostasis through regulation of hepatic gluconeogenesis and the consequent glucose metabolism (Veldhorst et al., 2009f). There is also evidence to suggest that intestinal gluconeogenesis contributes to the anorectic effects of high protein diets (Mithieux et al., 2005). In addition evidence suggests that the increase in energy expenditure following ingestion of a high protein is in part due to increase in gluconeogenesis (Halton and Hu, 2004, Veldhorst et al., 2009f).

1.7.1.3 Protein-induced satiety

A number of studies investigating the satiating power of protein diets have demonstrated that protein induced satiety appears to be related to the timing of amino acids appearing in circulation (Luhovyy et al., 2007, Veldhorst et al., 2009d), suggesting a role for specific individual amino acids in protein induced satiety. Studies investigating the effects of ingestion of protein-based meals or the infusion of amino acids have established a correlation between plasma amino acid levels and satiety (Mellinkoff et al., 1956). A comprehensive study comparing the satiating effects of low or high soy, whey and casein proteins found that the satiety associated with such proteins is directly correlated with the appearance of specific amino acids in circulation. Their findings demonstrate that the soy protein-mediated satiety is associated with the appearance of taurine (Veldhorst et al., 2009e), whey protein's effects are associated with serine, threonine, isoleucine and alanine (Veldhorst et al., 2009a), and casein's effects with branched chain amino acids (Veldhorst et al., 2009c). These findings suggest an important role for amino acids in protein-mediated satiety. However, the appearance of these amino acids in circulation also likely correlates with other events within the gut lumen.

Changes in gut hormone profiles have been proposed as potential mechanisms involved in the satiating effects of protein. Plasma PYY levels are greatly elevated following a protein

meal compared to isocaloric high fat or high carbohydrate meal in normal and obese individuals (Batterham et al., 2006). Furthermore, PYY null mice are resistant to the specific satiating effects of high protein diets (Batterham et al., 2006). Ghrelin levels are more rapidly reduced following a high carbohydrate meal than by a meal high in fat or protein (Koliaki et al., 2010). However, a high protein meal causes a more sustained reduction in circulating ghrelin levels (Blom et al., 2006). The response of GLP-1 to protein seems to depend on the length of exposure; acute studies suggest that carbohydrate is a more potent stimulus for GLP-1 release than protein (Smeets et al., 2008). However, GLP-1 levels were higher in individuals chronically fed a high protein diet compared to those fed a normal protein diet (Lejeune et al., 2006).

In addition to protein-induced peripheral modulation of appetite, high protein diets have been associated with changes in neuronal activity within CNS. High protein diets reduce the activity of orexin neurones in the lateral hypothalamus when compared to a normal protein diet (Journel et al., 2012). High protein diets also increase the activation of noradrenergic neurons in the NTS and POMC neurones in the ARC compared to a normal protein diet (Faipoux et al., 2008). However, the exact mechanisms by which protein influences neuronal signalling are still unclear.

1.7.2 Protein digestion

Following ingestion, the protein content of a meal undergoes three distinct phases leading to its complete digestion: the gastric phase, the pancreatic phase and the intestinal phase (Fig. 1.6). Protein digestion begins with the gastric phase in the stomach, where pepsin enzymatic activity cleaves peptide bonds between hydrophobic and aromatic amino acids. This process generates oligopeptides, polypeptides and free amino acids in the stomach (Freeman and Kim, 1978). The pancreatic phase commences when the peptide molecules generated in gastric phase stimulate the release of hormones including CCK and secretin from the upper small intestine. These in turn stimulate the release of pancreatic juice, which contains protease precursors synthesised in pancreatic acinar cells (Meyer and Kelly, 1976). When

these precursors reach the duodenum, enterokinase produced by the GI epithelium cleaves inactive trypsinogen to generate active trypsin, which then further autocatalyses this reaction and converts other protease precursors into their active forms. The activity of these activated enteropeptidase enzymes, which include trypsin, chymotrypsin and elastase, results in the breakdown of poly- and oligo-peptides (Freeman and Kim, 1978). The final stages of protein digestion occur during the intestinal phase and at the brush border and inside the enterocytes of the intestinal mucosal wall. The jejunum is the main site for amino acid absorption in the small intestine. Membrane bound proteases break down many di- and tripeptides to their individual amino acids, which are then transported through a variety of mechanisms across the membrane. Amino- and carboxy-specific peptidases present within the small intestine ensure the complete cleavage of amide- and carboxyl terminal amino acids from the remaining oligo- or polypeptides. Some di- and tripeptides are also absorbed, though most are then subsequently broken down to individual amino acids within the enterocytes. However, a small portion of oligopeptides escape and enter the portal circulation (Freeman and Kim, 1978).

Small intestine: A variety of enzymes cleave and breakdown poly- and oligopeptides into smaller peptides and individual amino acids

Jejunum: Main site for individual amino acid absorption and release into circulation.

Stomach: pepsin breaks down the protein bonds generating oligo- and polypeptides

Pancreas: protein-digesting enzymes are secreted into the small intestine

Small amount of dietary protein is excreted

Figure 1.6 Protein digestion in the GI tract.
The diagram summarises the protein digestion process that leads to the release of individual amino acids in the gut lumen and into circulation.

1.8 Amino Acids

Amino acids are the ultimate product of protein digestion in the GI tract. Free amino acids enter the circulation from where they are transferred to different tissues throughout the body to serve as precursors for various molecular pathways, materials for protein synthesis, or as a source of energy.

A typical diet contains a variety of amino acids. Amino acids are divided into two major types; the proteinogenic and non-proteinogenic amino acids. There are only 20 proteinogenic amino acids that are naturally utilised in protein synthesis. Proteinogenic amino acids are sub-categorised into essential and non-essential amino acids (Table 1.1). Non-essential amino acids can be synthesised within the body, but essential amino acids cannot, and thus must be provided by dietary sources. Within the non-essential amino acids, a subset is classified as conditionally-essential. These are amino acids, including L-cysteine,

L-arginine and L-glutamine, which are highly required at certain stages in life such that *de novo* synthesis is insufficient to meet the biological requirements of the body (Wu, 2009). In addition, amino acids are also sub-categorised into different groups based on the chemical properties of their side chain (Table 1.2).

type	amino acids	3-letter symbol	single letter symbol	side chain formula
Essential amino acids	Histidine	His	H	$C_6H_7N_3O$
	Isoleucine	Iso	I	$C_6H_{11}NO$
	Leucine	Leu	L	$C_6H_{11}NO$
	Lysine	Lys	K	$C_6H_{12}N_2O$
	Methionine	Met	M	C_5H_9NOS
	Phenylalanine	Phe	P	C_9H_9NO
	Threonine	Thr	T	$C_4H_7NO_2$
	Tryptophan	Trp	W	$C_{11}H_{10}N_2O$
	Valine	Val	V	C_5H_9NO
Non-essential amino acids	Alanine	Ala	A	C_3H_5NO
	Arganine*	Arg	R	$C_6H_{12}N_4O$
	Asparagine	Asn	N	$C_4H_6N_2O_2$
	Aspartic acid	Asp	D	$C_4H_5NO_3$
	Cysteine*	Cys	C	C_3H_5NOS
	Glutamic acid	Glu	E	$C_5H_7NO_3$
	Glutamine*	Gln	Q	$C_5H_8N_2O_2$
	Glycine	Gly	G	$C_2H_3N_2O$
	Proline	Pro	P	C_5H_7NO
	Serine	Ser	S	$C_3H_5NO_2$
	Tyrosine	Tyr	Y	$C_9H_9NO_2$

Table 1.1 Essential and non-essential amino acids. Essential and non-essential amino acids with their three-letter and single-letter codes and the formula of their side chains. *Conditionally essential amino acids.

Aliphatic	Basic	Acidic	Amidic	-S or -OH containing	Aromatic
Alanine	Arganine	Aspartic acid	Asparagine	Cysteine	Histidine
Glycine	Lysine	Glutamic acid	Glutamine	Methionine	Phenylalanine
Isoleucine				Serine	Tryptophan
Leucine				Threonine	Tyrosine
Proline					
Valine					

Table 1.2 Amino acid classification according to side chain.

1.8.1 Amino acids and central mechanisms of appetite regulation

Amino acids can access the CNS through regions with an incomplete blood-brain barrier such as the brainstem. Specific amino acids are precursors for neurotransmitters. Tryptophan and tyrosine are the precursor molecules for the synthesis of serotonin and dopamine respectively. Histidine is also a precursor molecule for histamine synthesis (Fromentin et al., 2012). These neurotransmitter products have all been implicated in appetite regulation (Volkow et al., 2011, Bassil et al., 2007, Goto et al., 2007). Glutamate and aspartate are excitatory amino acids which agonise the N-methyl-D-aspartate receptor. Glycine also has partial activity at this receptor (Fromentin et al., 2012). In addition, branched chain amino acids such as leucine and isoleucine have been found to reduce appetite and food intake via central mechanisms discussed in detail in section 1.8.5 (Blouet et al., 2009).

1.8.2 Amino acids and peripheral appetite regulation: gut hormone release

There is evidence to suggest that individual amino acids are involved in the peripheral regulation of appetite via gut hormone-dependent mechanisms. *In vitro* studies using the human enteroendocrine cell line NCI-H716 demonstrated that a mixture of essential amino acids was more effective at stimulating GLP-1 release than a mixture of non-essential amino acids (Reimer, 2006). Furthermore, branched chain amino acids have been shown to

stimulate GLP-1 release in a dose dependent manner from NCI-H716 cells (Chen and Reimer, 2009). In a study in the GLP-1 secreting murine enteroendocrine GLUTag cell line, a range of individual amino acids, including L-glutamine, stimulated the release of GLP-1 (Reimann et al., 2004). Similarly, increased GLP-1 secretion has been reported from isolated murine primary colonic cultures stimulated with L-glutamine, L-phenylalanine or L-asparagine (Tolhurst et al., 2011). Aromatic amino acids, including L-phenylalanine and L-tryptophan, stimulate the release of CCK from isolated CCK-cells (Wang et al., 2011a). In addition, recent work in isolated rat small intestine preparations demonstrated that specific L-amino acids, including L-phenylalanine, L-tryptophan, L-arginine, L-glutamine and L-asparagine, stimulate the release of the gut hormones PYY, GLP-1 and glucose-dependent insulinotropic peptide (GIP) (Mace et al., 2012)

1.8.3 Amino acid sensing receptors

Three distinct promiscuous L-specific amino acids receptors are found in the gut: the calcium sensing receptor (CaSR), the heterodimer of taste receptor 1 member 1 and taste receptor 1 member 3 (T1R1-T1R3) and the G-protein coupled receptor group C member 6A (GPRC6A) (Fig. 1.7 and 1.8). All three belong to class C of the G protein coupled receptors (GPCR). The Class C receptors family typically have large extracellular domains that consist of an N-terminal Venus Flytrap (VFT) domain which contains a dimerization interface and an orthosteric binding site for the endogenous agonist. VFTs contain a cysteine rich domain (CRD) which connects the VFT to the seven transmembrane (7TM) domain, and can bind neurotransmitters or nutrient molecules. The 7TM domain is coupled to a G-protein via a C-terminal intracellular signalling domain (Smajilovic et al., 2014).
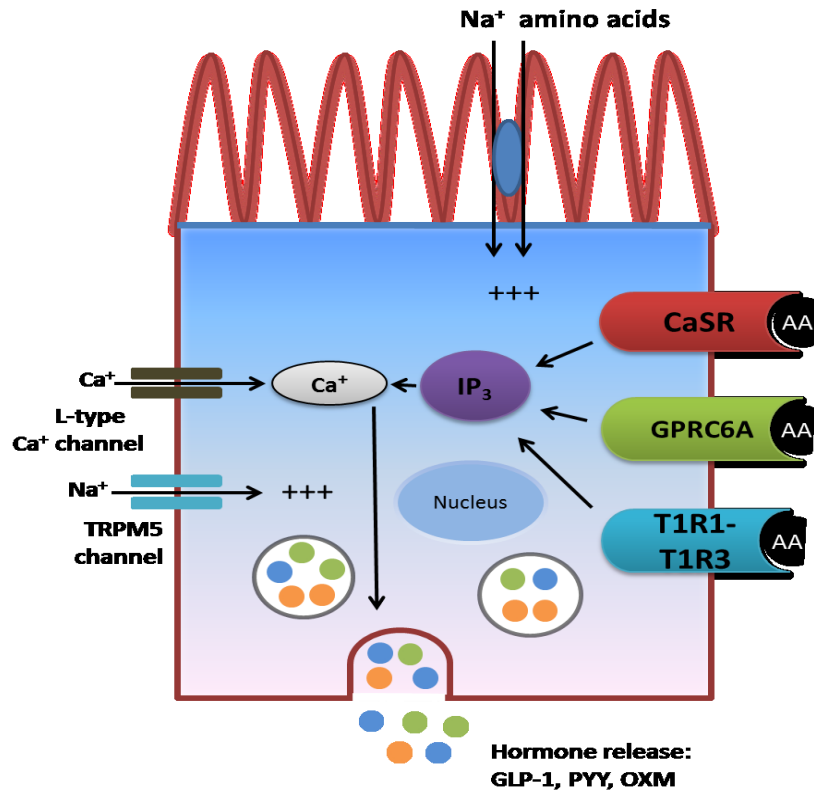
Figure 1.7 Schematic diagram illustrating putative amino acid sensing by enteroendocrine L-cells.

IP_3: Inositol trisphosphate, CaSR: calcium sensing receptor, T1R1-T1R3: taste receptor 1 member 1 and taste receptor 1 member 3, GPRC6A: G-protein coupled receptor group C member 6A, AA: amino acid, TRPM5: transient receptor potential cation channel subfamily M member 5. Adapted from (Psichas et al., 2015)
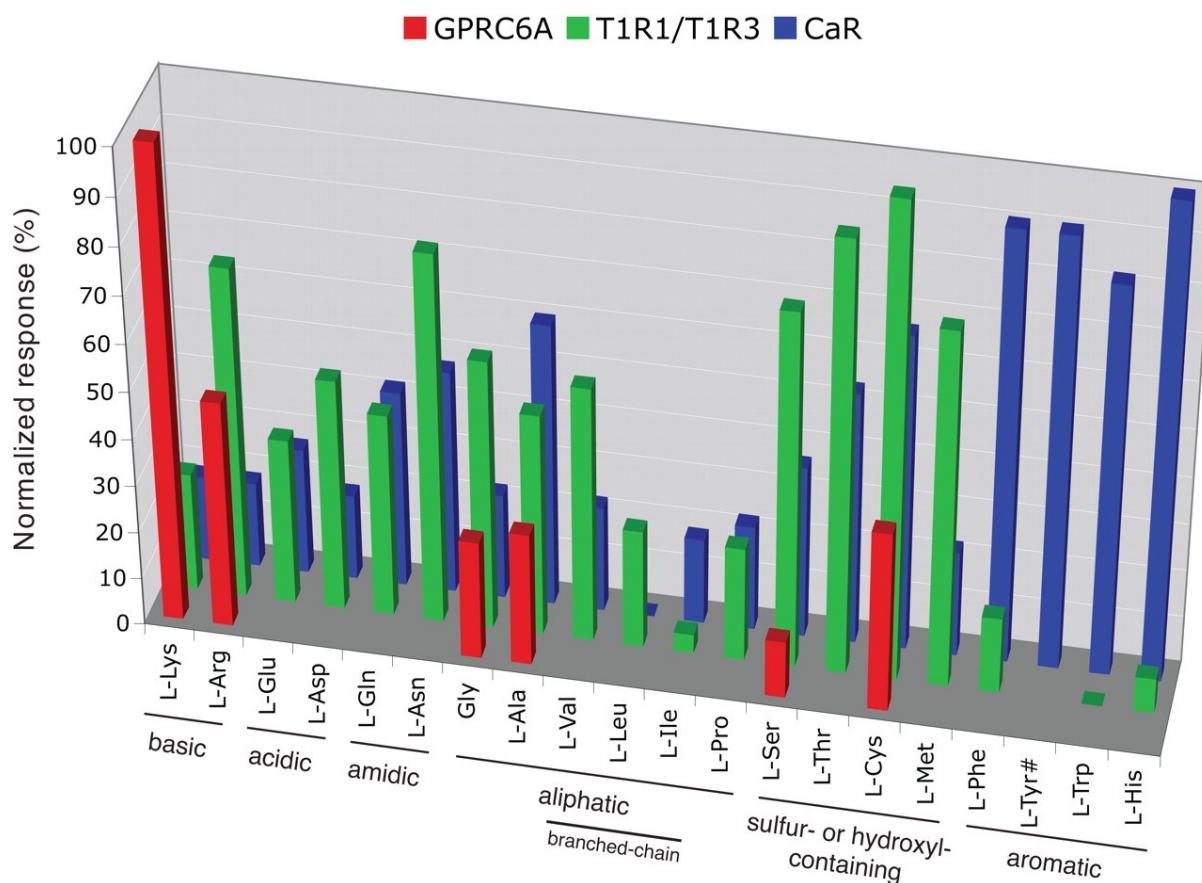
Figure 1.8 L-amino acid selectivity for L-specific amino acid receptors CasR, GPRC6A and T1R1-T1R3.
Effect of the 20 proteinogenic amino acids at 10mM on the mT1R1/T1R3 response in the presence of 2.5mM IMP expressed as a percentage normalized to L-cysteine response. CsSR percentage response is normalised to L-histidine. mGPRC6A values are based on EC50 values of the amino acids in the presence of 1mM Ca^{2+} and 1mM Mg^{2+} relative to L-lysine (Wellendorph et al., 2009a).

1.8.3.1 GPRC6A

GPRC6A human orthologue was first discovered in 2004 (Wellendorph and Brauner-Osborne, 2004) and was later characterised as an L-amino acid receptor (Wellendorph et al., 2005). The mouse and rat orthologues were subsequently cloned and characterised as receptors for L-amino acids (Wellendorph et al., 2005).There are three GPRC6A isoforms; isoform 1 is the most abundant form, has the longest structural sequence, and is expressed in a wide range of tissues. Isoform 2 is primarily expressed in kidney and isoform 3 is found at much lower levels than the other two isoforms in the brain, kidney and skeletal muscle (Wellendorph and Brauner-Osborne, 2004). Mouse GPRC6A has 80% homology to the human orthologue, but only one rat GPRC6A isoform has so far been identified (Wellendorph et al., 2007). Agonists and allosteric modulators of the receptor include calcium and other divalent cations, calcimimetics, osteocalcin and amino acids. Of the amino acids, it predominantly binds basic amino acids and, to a lesser extent, aliphatic amino acids. The L-amino acid specificity for GPRC6A varies between species. L-arginine is the most potent amino acid agonist for the human GPRC6A, whereas L-ornithine is the most potent amino acid agonist for the mouse and rat orthologues (Table 1.3) (Pi et al., 2005). GPRC6A signals by coupling to Gαq and consequently activating the phospholipase C pathway (Kuang et al., 2005).

L-amino acid	Mouse	Rat	Human
	EC50 (µM)		
L-Orn	63.6	264	112
L-Lys	135	>1000	169
L-Arg	284	>1000	44.1
L-Cys	356	>1000	>1000
K-Ala	486	>1000	173
Gly	538	455	263
L-Ser	1160	859	623
L-Met	>1000	>1000	854
L-Gln	>1000	>1000	590

Table 1.3 Amino acid affinity for GPRC6A orthologues.
EC50 values for mouse and rat GPRC6A were calculated from agonist-induced IP_3 accumulation in tsA cells expressing Gαq. EC50 values for human GPRC6A were obtained by transfection of a chimeric receptor (VFT/CRD-5.24 7TM goldfish domain) and measuring intracellular calcium levels using a Fluo-4 based intracellular calcium assay. Adapted from (Clemmensen et al., 2014)

GPRC6A is highly expressed in a wide range of tissues including GI tract, brain, kidney and pancreas, suggesting a diverse range of physiological roles (Wellendorph and Brauner-Osborne, 2004). Two knockout models have been independently generated to investigate the putative physiological roles of GPRC6A. However, there are discrepancies in the outcome of these studies.

The first knockout model was generated by Pi and colleagues by selective deletion of exon 2 (GPRC6A [exon2(-/-)]) that encodes the VFT domain of the receptor (Pi et al., 2008). The knockout model exhibited a number of metabolic and reproductive abnormalities compared to the WT mice. Although the knockout littermates appeared to have similar body weight and body length to WT mice, knockouts developed abnormal renal handling of calcium and phosphorus, feminization of the males, defective osteoblast-mediated bone mineralization, adiposity and glucose intolerance (Pi et al., 2008). These abnormalities were further studied and an association between hGPRC6A polymorphisms and bone mineral density reported (Pi et al., 2010). In addition, Pi and Colleagues used this model to demonstrate that

GPRC6A is implicated in L-arginine mediated insulin release from primary cultured beta-cells (Pi et al., 2012).

The second knockout model was generated by a selective deletion of exon 6 (GPRC6A$^{exon6(-/-)}$) which encodes the entire 7TM and C-terminal domains (Wellendorph et al., 2009b). In contrast to the first knockout model, this model was reported to have normal bone mineralisation and normal L-arginine-induced insulin secretion (Smajilovic et al., 2013). However, a recent study suggests that these GPRC6A$^{exon6(-/-)}$ mice are more susceptible to diet induced obesity, have higher fat mass, lower lean mass and decreased insulin sensitivity when placed on a high fat diet (Clemmensen et al., 2013a). This metabolic phenotype is similar to that reported in the GPRC6A$^{exon2(-/-)}$ model. It is possible that the 7TM region is not essential for all of the activities of the receptor and that GPRC6A functions as a heterodimer (Wellendorph et al., 2005). However, a recent study investigating the role of GPRC6A in protein-induced satiety used a third knockout model strain that had the entire GPRC6A locus deleted. Acute and chronic high protein feeding suppressed food intake and body weight in these GPRC6A knockout animals similar to WT mice, suggesting that these effects are not mediated by the GPRC6A, and that the GPRC6A is not necessary for protein-induced satiety (Kinsey-Jones et al., 2015). However, another study has suggested a role for GPRC6A in ornithine-induced GLP-1 release from the GLUTag cell line, though this effect could not be replicated in primary mice small intestine cultures, casting doubt on its physiological importance (Oya et al., 2013). Collectively these findings suggest a role for GPRC6A in energy homeostasis and metabolism, though GPRC6A may not be involved in protein-induced satiety.

1.8.3.2 Calcium sensing receptor (CaSR)

The CaSR is primarily involved in the regulation of calcium homeostasis, and regulates cellular processes including gene expression, proliferation, differentiation and hormone secretion. Calcium is the major activator of the receptor. However, physiologically, CaSR activity is modulated by pH, and by allosteric binding of other divalent cations, di- and tri-

peptides, aromatic L-amino acids, and, less potently, aliphatic amino acids. CaSR is widely expressed in various tissues throughout the body, Where it is expressed as a homodimer on the cell surface (Bai et al., 1998), and signals through $G\alpha_q$ and $G\alpha_i$ or $G\alpha_s$ in a cell specific manner (Handlogten et al., 2001).

CaSR is highly expressed in the gut and pancreas and has been implicated in regulation of energy homeostasis. It is expressed in pancreatic acinar and ductal cells, as well as in pancreatic islet cells, where it has shown to regulate the secretion of insulin and glucagon (Bruce et al., 1999, Squires et al., 2000). In the GI tract, CaSR is found in enteroendocrine G-cells (Ray et al., 1997), I-cells (Liou et al., 2011), D-cells (Haid et al., 2012) and L-cells (Diakogiannaki et al., 2013). The expression of CaSR on hormone secreting enteroendocrine cells suggests a role for the receptor in regulating the release of gut hormones from these cells. The aromatic amino acids L-phenylalanine and L-tryptophan stimulate CCK secretion from isolated I-cells (Wang et al., 2011a). In addition, specific L-amino acids including L-phenylalanine, L-tryptophan, L-arginine, L-glutamine and L-asparagine have been shown to stimulate the release of gut hormones PYY, GLP-1 and GIP from isolated rat small intestine through a CaSR-dependent mechanism. These effects were attenuated by a CaSR antagonist (Mace et al., 2012). Aromatic amino acids have also shown to stimulate gastric acid secretion from parietal cells in *ex vivo* stomach explants by allosteric modulation of CaSR activity (Busque et al., 2005). These findings suggest a role for CaSR in L-amino acid-induced hormone secretion in the GI tract, which may have implications for appetite regulation and energy balance.

1.8.3.3 T1R1-T1R3

Three taste receptor subunits have so far been identified: T1R1, T1R2 and T1R3. T1R receptors function as heterodimers, forming receptor complexes that sense sweet, bitter or umami taste. The T1R1-T1R2 receptor complex functions as a sweet taste receptor activated by a broad range of sweet molecules. T1R1-T1R3 receptor complex comprises the 'umami' taste receptor system, which is activated by mono-sodium glutamate and a range of

L-amino acids (Wellendorph et al., 2010). T1R3 has also been implicated in calcium sensing and calcium taste (Tordoff et al., 2008).

T1R1-T1R3 signals via a transient receptor potential cation channel subfamily M member 5 (TRPM5) and through the heterodimeric G-protein gustducin (Zhang et al., 2003). There are differences in T1R1-T1R3 receptor agonist selectivity between species, which likely reflect the relatively low homology (approximately 70%) between human and rodent T1R receptors. L-glutamate is the most potent agonist at the human T1R1-T1R3 receptor (Li et al., 2002), whereas L-cysteine has found to have the greatest agonist activity at the mouse T1R1-T1R3 receptor (Nelson et al., 2002). In addition, the mouse T1R1-T1R3 orthologue responds to a wider range of L-amino acid agonists compared to the human orthologue (Nelson et al., 2002).

The T1R1-T1R3 heterodimer is expressed in a number of tissues, including the tongue, the GI tract, pancreas, brain and skeletal muscle (Wauson et al., 2012). The exact role of the receptor in the non-gustatory tissues is not fully understood. T1R1-T1R3 receptor has been shown to regulate CCK release in the immortalized intestinal enteroendocrine STC-1 cell line, with T1R1 knockdown abolishing the stimulatory effects of L-phenylalanine, L-glutamate and L-leucine on CCK release. In addition, these stimulatory effects were modulated by allosteric activation or inhibition of the receptor in mouse small intestine tissue explants (Daly et al., 2013). The T1R1-T1R3 heterodimer has also been implicated in L-glutamate and L-arginine-induced insulin release from the MIN-6 immortalized mouse pancreatic beta-cell line (Oya et al., 2011). Furthermore, isolated beta-cells from T1R3 knockout mice showed delayed insulin secretion in response to glucose (Geraedts et al., 2012). However, a study by another group has suggested that T1R1-T1R3 regulates pancreatic beta-cell insulin content, rather than insulin secretion. In their proposed model, amino acids signal via T1R1-T1R3 to activate extracellular signal-regulated kinases (ERK1/2) and Mammalian target of rapamycin complex 1 (mTORC1) to control mRNA translation initiation via mechanisms

dependent on the availability of extracellular amino acids. This signalling cascade ultimately results either in cell growth, or in autophagy and apoptosis (Wauson et al., 2012).

1.8.4 Amino acid transporter systems

Amino acid transporters are present on both the apical and basolateral membranes of GI epithelial cells, and are involved in absorption of amino acids from the gut lumen and their transfer into the bloodstream. Amino acid transporters belong to the solute-linked carrier (SLC) family of the membrane proteins. There are several amino acid transporter systems and each system transports a range of amino acids, resulting in a certain amount of overlap in the activity of the different systems (Table 1.4). However, despite their promiscuity, specific transporters are known to be physiologically important, as defects in these systems are associated with particular diseases and disorders (Broer, 2008). Interestingly, recent studies suggest that these transporter systems also exhibit receptor like activity and may be involved in amino acid sensing. Amino acid transporter systems are capable of sensing the changes in the availability of extracellular amino acids, and may mediate nutrient-induced signalling. The sodium-coupled neutral amino acid transporter 2 (SNAT2) has recently been implicated in nutrient sensing and gut hormone release. SNAT2 acts as a secondary active transporter by coupling the transfer of amino acids against their concentration gradient to the simultaneous inward movement of Na^+ down its electrochemical gradient. This sodium-dependent transport mechanism has been shown to increase intracellular calcium levels and to consequently stimulate the release of gut hormones (Young et al., 2010). Of note, L-glutamine has been shown to stimulate GLP-1 release from intestinal L-cells via a SNAT2-mediated mechanism (Tolhurst et al., 2011).

System	Transporter	Substrates	Mechanism
A	SNAT2	G,P,A,S,C,Q,N,H,M,	Symport
	SNAT4	G,A,S,C,Q,N,M,AA$^+$	Symport
N	SNAT3	Q,N,H	Symport
	SNAT5	Q,N,H,S,G	Symport
y+	CAT-1	R,K,O,H	Uniport
L	4F2hc/LAT1	H,M,L,I,V,F,Y,W	Antiport
	4F2hc/LAT2	AA^0 (expect P)	Antiport
y+L	4F2hc/y+LAT1	K,R,Q,H,M,L	Antiport
	4F2hc/y+LAT2	K,R,Q,H,M,L,A,C	Antiport
X^-_c	4F2hc/xCT	E, Cystine	Antiport
asc	4F2hc/asc1	G,A,S,C,T	Antiport
b$^{0,+}$	rBAT/b$^{0,+}$AT	R,K,O, Cystine	Antiport
X$^-_{AG}$	EAAT1-5	E,D	Symport
ASC	ASCT1	A,S,C	Antiport
	ASCT2	A,S,C,T,Q	Antiport
T	TAT1	F,Y,W	Uniport
Gly	GLYT1,2	G	Symport
B$^{0,+}$	ATB$^{0,+}$	AA^0, AA$^+$	Symport
B^0	B^{0}AT1	AA^0	Symport
	B^{0}AT2	P,L,V,I,M	Symport

Table 1.4 Amino acid transporter systems.
Table summarises all the different amino acid transport systems, their amino acid substrates and mechanism of transport. AA^0: neutral amino acids, AA$^+$: cationic amino acids. Adapted from (Broer, 2008)

1.8.5 Intracellular amino acid sensing mechanisms

1.8.5.1 Mammalian target of rapamycin complex 1 (mTORC1)

mTORC1 is a serine/threonine protein kinase that functions as a key regulatory molecule in metabolism, protein synthesis and growth. The activity of the mTORC1 complex is regulated by insulin, growth factors, oxidative stress and amino acids. Branched chain amino acids and leucine are particularly implicated in mTORC1 signalling (Blouet et al., 2009). Administration of L-leucine into the mediobasal hypothalamus (MBH) reduces food intake and meal size. The anorectic effect of L-leucine is mediated by activating mTORC1 within the arcuate POMC neurones through an MBH-PVN-NTS circuit (Blouet et al., 2009). In a

similar fashion, L-leucine administration into the caudomedial NTS also reduces food intake and meal size via an mTORC1-dependent mechanism (Blouet and Schwartz, 2012).

The exact mechanisms by which amino acids activate mTORC1 are not fully understood. Rheb, a GTPase molecule is a key regulator involved in the activation of mTORC1 pathway (Stocker et al., 2003). The activity of Rheb is in turn regulated by the tuberous sclerosis (TSC) complex. The TSC complex includes TSC2, which activates the GTPase enzyme, and regulates mTORC1 activity by hydrolysing the guanosine triphosphate (GTP) of the active Rheb-GTP to the inactive guanosine diphosphate (GDP) bound form. The activity of the TSC complex itself is controlled through phosphorylation, which leads to its increased or decreased GTPase activating protein activity. In addition, mTORC1 activation is dependent on Rag GTPases. This mechanism is sensitive to the levels of available amino acids in the cell. Amino acid availability promotes the activity of a guanine nucleotide exchange factor named Ragulator. The Ragulator complex interacts with Rag GTPases which results in the accumulation of a $RagA/B^{GTP}-RagC/D^{GDP}$ complex. The presence of amino acids activates the Ragulator-Rags complex, which interacts with the lysosomal membrane protein v-ATPase, a protein pump. This complex recruits mTORC1 to the lysosomal surface where it can ultimately be activated by the Rheb (Efeyan et al., 2015).

1.8.5.2 Fat mass and obesity associated protein (FTO)

FTO is an mRNA demethylase enzyme that is encoded by the fto gene located on chromosome 16 in humans (Gerken et al., 2007). Mutations in *fto* genes are strongly linked with obesity, as several variants have been identified as obesity risk alleles (Frayling et al., 2007). Fto is expressed within the hypothalamic ARC. The expression within the ARC is regulated by nutritional state; fasting significantly reduces the expression of Fto whereas prolonged high fat feeding increases Fto expression (Tung et al., 2010). Amino acid availability has been shown to influence the expression of Fto. Studies suggest that essential amino acid deprivation down-regulates Fto expression both at mRNA and protein level within the ARC (Cheung et al., 2013). Furthermore, Fto has been implicated in the regulation of

mTOR1 activity. This is interesting, as mTOR1 activity is regulated by amino acid availability. Mouse embryonic fibroblasts (MEFs) derived from Fto knockout mice exhibit decreased mTOR1 activity and increased autophagy compared to cells expressing Fto (Gulati et al., 2013). Together these findings suggest a role for FTO in amino acid sensing via the regulation of mTORC1 activity and that FTO may influence body composition by playing a role in cellular amino acid sensing.

1.8.5.3 General control nonrepressed 2 protein (GCN2)

The amino acid content of a diet can greatly affect feeding behaviour in animals. Animals have been shown to prefer a diet containing essential amino acids over a diet lacking essential amino acids (Koehnle et al., 2003). The anterior piriform cortex (APC) region plays a key role in regulating such adaptive feeding behaviour (Leung and Rogers, 1971). GCN2 protein is the key molecule involved in sensing changes in amino acid availability. It is a serine/therionine protein kinase that binds to uncharged tRNAs that are accumulated as a result of amino acid deficiency within the APC. GCN2 phosphorylates eukaryotic initiation factor 2 alpha (elf2α), resulting in down-regulation of protein synthesis. In contrast, activating transcription factor 4 (ATF4) increases the concentrations of available intracellular amino acids (Hao et al., 2005). ATF4 is a transcriptional factor implicated in the activation of amino acid biosynthesis and transport by activating transporters systems such as SNAT2 (Blais et al., 2003).

1.9 Thesis Hypothesis and General Aims

Initial work within our group identified specific amino acids that could reduce food intake in rodents (McGavigan et al., 2015). Amongst tested amino acids, oral and intraperitoneal (IP) administration of L-cysteine and L-arginine reduced food intake in rats. In particular, the anorectic properties of L-arginine required further study to determine whether they reflected specific physiological effects rather than non-specific effects secondary to other roles. In addition, data suggested that L-phenylalanine stimulates the release of anorectic gut

hormones (Tolhurst et al., 2011); and that the release may require the activity of CaSR (Mace et al., 2012).

The sensing of such amino acids may occur in the distal small intestine, where there is relatively high expression of L-specific amino acid receptors and GLP-1 and PYY releasing cells. The targeted delivery of such amino acids to the distal small intestine may enhance the amino acid-induced satiety as a novel therapeutic option for obesity.

Hypotheses: L-arginine and L-phenylalanine reduce food intake in rodents via gut hormone-dependent mechanisms and may represent novel viable therapeutic agents for treatment of obesity. Encapsulating specific amino acids will allow their targeted delivery to the distal small intestine, increasing the release of anorectic gut hormones, and thus reducing food intake.

General aims: The general aims of this thesis are:

1. To characterise the anorectic effects of L-arginine and investigate the mechanisms by which L-arginine modulates food intake.
2. To determine the effects of L-phenylalanine on food intake and gut hormone release in rodents.
3. To examine the effects of specific encapsulated L-amino acids on food intake in rodents.

Chapter II:

The Effect of L-Arginine on Food Intake

2.1 Introduction

2.1.1 L-arginine

L-arginine is a proteinogenic amino acid, first isolated in 1886 from the extract of lupine seedlings, which belong to the legume plant family. However it was not until the early 1930s, and the discovery of urea cycle, that L-arginine was established as a key molecule involved in metabolism and physiology. Arginine is found in almost all kinds of food including meats, nuts and dairy products, though levels are higher in plant based protein than in animal protein (Reyes et al., 1994).

L-arginine has a 3-carbon aliphatic side chain (Fig. 2.1.1). The molecule has a positively charged guanidinium group which gives L-arginine its basic chemical property. The L-arginine cation actively binds negatively charged phosphate anions and it is thus often found in the centre of proteins that bind phosphorylated substrates, where it can play an important role in maintaining the overall charge and consequently the stability of a protein molecule (Toi et al., 1967).
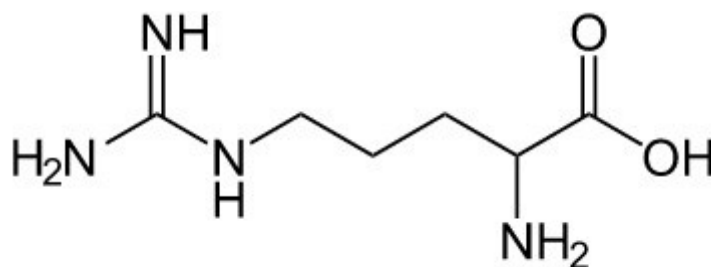
2.1.1 Chemical structure of L-Arginine.
L-arginine consists of a 3-carbon aliphatic side chain and a guanidinium group.

2.1.1.1 L-arginine synthesis

L-arginine is derived from the diet, endogenous synthesis and from protein turnover. In mammals, the levels of endogenous synthesis are usually sufficient to meet body requirements. However, in certain conditions such as inflammation or infection, the levels of

endogenous synthesis may not be sufficient, and L-arginine is therefore classified as a conditionally non-essential amino acid (Barbul, 1986).

In healthy human adults, plasma levels of L-arginine are approximately 80-120 µmol/L (100-200 µM) (Tangphao et al., 1999). The majority of endogenous synthesis occurs as a result of a close collaboration between the intestinal epithelial cells and cells in proximal tubule of the kidney. The biosynthesis of L-arginine can be divided into three major steps which include the synthesis of L-ornithine, L-citrulline, and finally of L-arginine (Fig. 2.1.2) (Reyes et al., 1994).

L-ornithine is synthesised from glutamine or proline, via reactions mediated by the enzymes L-Δ^1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) and proline dehydrogenase (PROHD), respectively. This process only occurs in the small intestine, as both P5CS and PROHD are only expressed in the intestinal mucosa (Windmueller, 1982, Wakabayashi et al., 1994).

The synthesis of L-citrulline from L-ornithine is governed by the action of the enzymes ornithine carbamoyltransferase (OCT) and carbamoylphosphate synthetase 1 (CPS1). Both enzymes are expressed in hepatocytes and epithelial cells in the small intestine, and to a minor extent in the large intestine (Raijman, 1974). The reaction in the liver is considered to be part of the urea cycle. The L-citrulline produced in the small intestine enters general circulation, from where it is taken up by the cells of the proximal tubule of the kidney and converted into L-arginine (Windmueller and Spaeth, 1981, Rabier and Kamoun, 1995).

Synthesis of L-arginine from L-citrulline is mediated by the enzymes argininosuccinate synthase 1 (ASS1) and argininosuccinate lyase (ASL). Unlike OTC and CPS1, ASS1 and ASL are expressed in many cell types, facilitating the synthesis of L-arginine from L-citrulline in different tissues (Wu and Morris, 1998).

L-Glutamine

H_2O

Glutaminase

NH_4^+

2-Oxoglutarate — aspartate aminotransferase — L-Glutamate

Asp — OAA

ATP

P5C synthetase

ADP

L-Glutamyl-γ-phosphate

NADPH + H$^+$

P5C synthetase

NADP$^+$ + P$_i$

L-Glutamyl-γ-semialdehyde

H_2O

H_2O — $\frac{1}{2}O_2$

L-Δ^1-Pyrroline-5-carboxylate

L-Proline — proline oxidase

Glu

OAT

2-oxoglutarate

L-Glutamate

Acetyl-CoA — NAGS

CoA

N-Acetylglutamate

$HCO_3^- + NH_3$ — CP

CPS I

2 ATP — 2 ADP + P$_i$

L-Ornithine

OCT

L-Citrulline

ATP + Asp

ASS

AMP-PP$_i$

L-Argininosuccinate

ASL — Fumarate

L-Arginine

2.1.2 L-Arginine biosynthesis

Diagram illustrating the biochemical pathways involved in the synthesis of L-arginine *in vivo*. ASL: argininosuccinate lyase, Asp: aspartate, ASS: argininosuccinate synthase, CP: carbamoyl phosphate, CPS I: carbamoyl-phosphate synthase I, NAGS: N-acetylglutamate synthase, OAA: oxaloacetate, OAT: ornithine aminotransferase, OCT: ornithine carbamoyltransferase, P5C: L-Δ^1-pyrroline-5-carboxylate. Adapted from (Wu and Morris, 1998).

2.1.1.2 L-arginine metabolism

The metabolism of L-arginine has been greatly investigated over the past 20 years, because it is the source of nitrogen for the nitric oxide pathway. L-arginine synthesis thus provides a regulated source of substrate for nitric oxide (NO) synthesis in a wide range of cells. NO plays an important role in a number of physiological processes, including the regulation of vascular tone (Palmer et al., 1988), neurotransmission (Liu and Barajas, 1993) and immune system function (Albina et al., 1989). Consequently, arginine availability is vital for the normal function within the body. L-arginine is converted to NO by the action of nitric oxide synthase (NOS). L-citrulline is released as a major bi-product of NO synthesis via N(G)-hydroxy-L-arginine (OH-L-Arg), an intermediate product in the biochemical process. This immediate synthesis of L-citrulline within the NO synthesis pathway facilitates the prolonged synthesis of NO in cells in a process termed the citrulline/NO cycle (Wu and Morris, 1998).

Urea generation in the liver requires the breakdown of L-arginine by the action of the arginase enzyme. Arginine synthesis and its subsequent conversion to L-ornithine and urea serves as a mechanism to eliminate nitrogen-containing molecules from the body (Reyes et al., 1994).

In addition to the urea cycle, the synthesis of creatine and creatinine involves the breakdown of L-arginine in the kidney. L-arginine acts as a substrate that is converted to guanidine acetic acid by the addition of L-glycine. The guanidine acetic acid is subsequently used in the liver to produce creatine, which is ultimately taken up by the muscle. Creatine then undergoes further chemical processing to generate creatinine within muscle (Walker, 1979).

L-arginine is also implicated in the synthesis of pyrimidines required for cell and tissue growth, as it mediates the production of orotic acid, the first precursor in this metabolic pathway (Milner and Visek, 1973, Visek, 1992).

2.1.1.3 L-arginine cellular transport

There are several specific amino acid transporter systems involved in the uptake of extracellular L-arginine. The most important transport system implicated in L-arginine transport is the system y^+ (Broer, 2008). This system consists of the cationic amino acid transporter (CAT) proteins CAT1, CAT-2A, CAT-2B and CAT3, in which CAT-2A and CAT-2B are splice variants of the same gene. CAT proteins selectively transport cationic amino acids, including L-arginine, L-lysine and L-ornithine, via a sodium-independent mechanism (Closs et al., 2006). CAT-1 is expressed in almost every tissue, except the liver (Aulak et al., 1996). CAT-2A is predominantly expressed in the liver (Closs et al., 2006), and CAT-3 is exclusively expressed in the brain (Hosokawa et al., 1999).

In addition, L-arginine can be transported via broad spectrum amino acid transporter systems such as system y^+L, b^{0+} and B^{0+} via sodium-dependent mechanisms (Broer, 2008).

In addition to the transport systems themselves, modulators of the transporter activity may also indirectly regulate the uptake of L-arginine. For example, L-arginine uptake is competitively inhibited by the other cationic amino acids such as L-lysine or L-ornithine. In addition, certain NOS inhibitors have been shown to inhibit the cellular uptake of the L-arginine (Bogle et al., 1992).

2.1.2 L-arginine and GPRC6A

The promiscuous L-amino acid receptor GPRC6A preferentially binds basic amino acids including L-arginine (Wellendorph et al., 2005). Indeed, L-arginine is the most potent amino acid activator of the human GPRC6A receptor (Pi et al., 2005).

L-Arginine is a potent insulin secretagogue (Floyd et al., 1966). There is increasing evidence to suggest that L-arginine supplementation in diet improves glycemic control (McKnight et al., 2010). A recent study using GPRC6A $^{exon2(-/-)}$ knockout model demonstrated that L-arginine-induced insulin secretion is mediated by GPRC6A activity (Pi et al., 2012).

However, a study using the GPRC6A$^{exon6(-/-)}$ model reported that L-arginine-induced insulin release is not mediated through GPRC6A (Smajilovic et al., 2013).

Interestingly, a recent study suggested L-arginine stimulates GLP-1 release to improve insulin sensitivity and glucose tolerance in mice (Clemmensen et al., 2013b). Given that GPRC6A is expressed on the surface of gastrointestinal L-cells (Oya et al., 2013), it is plausible that L-arginine-induced GLP-1 release may require GPRC6A activity. However, other studies have suggested that L-arginine stimulates insulin release directly from the beta cells via an electrogenic mechanism (Smith et al., 1997, Thams and Capito, 1999).

L-arginine is implicated in the tissue and wound healing process (Seifter et al., 1978). Evidence suggests that L-arginine enhances the process by increasing the fibroblast proliferation. Furthermore, GPRC6A knockdown in human dermal fibroblasts blocked fibroblast proliferation by L-arginine, suggesting a role for GPRC6A in mediating the effects of L-arginine on fibroblast proliferation (Fujiwara et al., 2014). These data collectively demonstrate a key role for GPRC6A in mediating some of the physiological responses of L-arginine.

2.1.3 Aims and Hypothesis

Hypothesis: L-arginine reduces food intake in rodents by stimulating the release of anorectic gut hormones via the activation of the GPRC6A receptor in the GI tract.

Aims: To investigate

- The effects of L-arginine on food intake and energy expenditure in rodents.

- The role of GPRC6A in mediating the anorectic effects of L-arginine.

- The effects of L-arginine on anorectic gut hormone release.

2.2 Methods

2.2.1 Animals

Male C57BL/6 mice (Harlan, Bicester, Oxon, UK) and GPRC6A knockout (GPRC6a-KO) mice with an average body weight of 18-20g (6-8 weeks old) were individually housed under controlled temperature (21-23ºC) and light cycle (12 hours light, 12 hours dark) with *ad libitum* access to standard chow diet (RM1, Special Diet Services Ltd, Witham, Essex, UK) and water, unless otherwise stated.

The GPRC6a-KO model used in our studies was obtained from the Knockout Mouse Project (KOMP) (Guan et al., 2010). The C57BL/6NTac inbred mouse was used as the genetic background for developing the GPRC6a-KO congenic strain (Taconic, US). The deleted region consisted of 16,596 base pairs, which completely covers the GPRC6a locus. Therefore, this knockout model is different from the knockouts previously described (Pi et al., 2008, Wellendorph et al., 2009). GPRC6a-targeted heterozygous C57B1/6N mice were bred to produce homozygous GPRC6a-KO animals and WT littermate controls. Breeding, maintenance and genotyping of these animals was performed by Dr James Kinsey-Jones.

Male Wistar rats (Charles River, Margate, Kent, UK) weighing 180-220g were individually housed under controlled temperature and light cycle with *ad libitum* access to standard chow diet and water, as described above, unless otherwise stated.

Animals were randomised according to their body weight for all the studies. Animals were handled and acclimatised to the procedures prior to commencement of studies.

All animal procedures were approved and carried out according to the British Home Office Animals Scientific Procedures Act 1986 (Project License: 70/8068).

2.2.2 The effect of oral gavage (OG) administration of L-arginine monohydrochloride (L-Arg.HCl) on food intake in fasted rats during the early light phase

Rats were fasted for 16 hours overnight and were then orally gavaged with water, 8 mmol/kg or 16 mmol/kg L-Arg.HCl (Sigma, Poole, UK) during early hours of light cycle. Animals were returned to their cages following administration and were given pre-weighed standard chow diet. The food intake was measured at 1, 2, 4, 8 and 24 hours following administration.

2.2.3. The effect of OG administration of L-Arg.HCl on food intake in *ad libitum* fed rats during the early dark phase

Ad libitum fed rats were orally gavaged with 8 mmol/kg or 16 mmol/g L-Arg.HCl at the beginning of the dark phase. Animals were returned to cages and food intake was recorded at 1, 2, 4, 8 and 24 hours following administration.

2.2.4 The effect of OG administration of L-Arg.HCl on behaviour in fasted rats during the early light phase

Rats were fasted for 16 hours overnight and were subsequently orally gavaged with either water or 16 mmol/kg L-Arg.HCl. Animals were then returned to their cages and observed by a researcher blinded to the experimental treatment. Each animal was observed for 5 seconds every 15 seconds for a total period of 5 minutes. The observations were carried out during the entire one hour time following administration. Animals were monitored for twelve distinct behavioural observations including feeding, drinking, rearing, locomotion, grooming, pica, bed-making, head-down/hunched, sleeping, tremors, climbing and stationary. These observations were subsequently sub-categorised into six behaviours: feeding (feeding or drinking), locomotion (rearing, locomotion, bed-making and climbing), head-down (head-down, hunched and tremors), grooming, pica and resting (stationary or sleeping) (Ghourab et al., 2011).

2.2.5 The effect of OG administration of L-Arg.HCl on energy expenditure and food intake in fasted mice

Mice were acclimatised to the plexiglass cages for 24 hours to generate stable reference data against which to test the effects of treatments. They were then fasted for 16 hours overnight and were subsequently orally gavaged with water or 24 mmol/kg L-Arg.HCl at 0900 hours (early light phase). Animals were then individually placed in a 24-chamber open-circuit comprehensive laboratory animal monitoring system (CLAMS) (Columbus Instruments, OH, USA) cages and continued to be fasted for the subsequent 8 hours, before food was returned at 1700 hours. Metabolic parameters (VO_2 and VCO_2) were measured by indirect calorimetry and values were normalised with respect to body weight. Respiratory exchange rate (RER) was calculated by determining the ratio between CO_2 produced/O_2 consumed to determine the contribution of carbohydrate and fat to energy expenditure. All the recordings were taken every 24 minutes following administration of the treatments for 24 hours. Ambulatory activity was simultaneously recorded by recording consecutive photo beam breaks (Semjonous et al., 2009).

2.2.6 The role of GPRC6A in mediating the anorectic effect of L-Arg.HCl

2.2.6.1 GPRC6A expression in the GI tract

A cohort of WT Male C57BL/6 mice were fasted for 16 hours overnight to avoid any effects of acute food intake on gene expression. Mice were then decapitated and stomach, duodenum, jejunum, ileum and colon rapidly removed, snap frozen in liquid nitrogen and stored at -80°C for RNA extraction and qPCR. Total RNA was extracted from GI tissues using TRI reagent (Sigma, Poole, UK) in accordance with the manufacturer's instructions. Extracted RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was used at a concentration of 1μg/10μl in triplicates reverse transcription reactions to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Life technologies, Paisley, UK) and according to the

manufacturer's instructions. Real-time quantitative PCR analysis was performed using 1µl of cDNA template in TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Life technologies, Paisley, UK) using the ABI Prism 7900 Sequence Detection System according to the protocols provided by the manufacturer (Life technologies, Paisley, UK). The relative mRNA transcript levels were calculated according to the $2^{-\Delta CT}$ method, with ΔCT being the difference in cycle threshold values between the GPRC6a mRNA (Mm00467618_m1) and the hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA (m00446968_m1) internal control.

2.2.6.2 The effect of OG administration of L-Arg.HCl on food intake in *ad libitum* fed GPRC6a-KO mice in the early dark phase

Two separate cross over studies were performed with 16 mmol/kg and 24 mmol/kg L-Arg.HCl. Mice had *ad libitum* access to food prior to study and were orally gavaged at the beginning of dark phase with either L-Arg.HCl or water. Animals were returned to their cages and a pre-weighed amount of food was provided. Food intake was subsequently measured at 1, 2, 4, 8 and 24 hours post administration. For both studies a total of four cross over arms were performed, with each animal receiving every treatment over the course of the experiment.

2.2.7 The role of gut hormones in mediating the effect of L-Arg.HCl on food intake in rodents

2.2.7.1 Murine colonic crypt isolation

The primary mice colonic crypt isolation technique was based on a method previously established and described by Professor Fiona Gribble, University of Cambridge (Reimann et al., 2008).

Male C57BL/6 mice between 6 and 8 weeks of age were sacrificed by cervical dislocation. The colon was dissected and collected in ice cold Leibovitz-15 (L-15) medium (PAA, UK), opened, rinsed in L-15 and chopped into 1-2 mm pieces. Tissue was then digested in 0.4

mg/ml collagenase XI (Sigma, Poole, UK) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) for an initial 10 minute period. The supernatant was collected and the tissue was digested in fresh DMEM media containing collagenase for 15 minutes. This process was then repeated twice more. The supernatant was centrifuged at 500g for 5 minutes and the pellet resuspended in DMEM. Cell suspensions were filtered through a sterile nylon mesh (pore size 250 µM). Filtered cell suspensions were aliquoted on 24 well plates coated with 1% matrigel (BD Bioscience, Oxford, UK). Plates were incubated overnight at 37°C, 5% CO_2/95% O_2 prior to secretion experiments. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Sigma, Poole, UK) and the adenylyl cyclase activator forskolin (Sigma, Poole, UK), were prepared as 10 mM stocks in dimethyl sulfoxide (DMSO), and a mixture of IBMX and forskolin at a final concentration of 10µM used as positive control in secretion experiments (Reimann et al., 2008).

2.2.7.2 Secretion assays

Secretion assays were performed on primary cultures 24 hours after plating and incubation. All treatments were made up in secretion buffer (refer to Appendix 1). Media was removed and plates were washed twice with secretion buffer. Experimental controls were osmolarity-matched when high concentrations of salt-based treatments were tested using 3X concentrated secretion buffer. Cells were then incubated with 300 µl per well of test reagents for 2 hours at 37°C, 5%CO_2/95% O_2. Following incubation, supernatants were removed and centrifuged at 100 g for 3 minutes to remove cell debris. The resulting supernatant was then stored at -20°C before analysis. Cell lysis buffer (refer to Appendix 1) was added to cells (250 µl/well) which were stored at -80ºC overnight. Plates were then scraped, washed with 250µl of secretion buffer and the lysates stored at -20°C pending analysis. Gut hormones were measured by radioimmunoassay (RIA) and secretion expressed as a fraction of the total peptide (secreted + extracted) measured in each well.

2.2.7.3 GLP-1 Radioimmunoassay

GLP-1 release was measured using a previously established in-house specific and sensitive RIA (Kreymann et al., 1987). The GLP-1 antibody was raised in rabbits against GLP-1 coupled to bovine serum albumin (BSA). The antibody has 100% cross-reactivity with all the amidated forms of GLP-1, but does not cross-react with glycine extended forms (GLP-1$_{1-37}$ and GLP-1$_{7-37}$) or any other known gut hormones. ^{125}I-GLP-1 was prepared by Professor Mohammad Ghatei using the Iodogen method (Wood et al., 1981) and purified by high pressure liquid chromatography (HPLC). The assay was performed in 350µl of 0.06M phosphate buffer (refer to Appendix 1) containing 0.3% BSA and TWEEN20 (Sigma, Poole, UK) (200µl/100ml). The standard curve was constructed by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of GLP-1 at a concentration of 0.125pmol/ml. One hundred µl of supernatant samples and 20 µl of cell lysis samples were assayed. The assay was incubated for four days at 4°C before separation of free and antibody bound GLP-1 using the charcoal absorption (refer to Appendix 1 and 2) technique. Two hundred and fifty µl of dextran coated charcoal (Merck) was added to each tube. Samples were immediately centrifuged at 1500 g in 4°C for 20 minutes. The supernatant (containing the bound-GLP-1) was carefully separated by aspiration and collected in fresh tubes.

After separation, the assays were counted using a gamma scintillation counter (LB2111 Multi Crystal Gamma Counter, Berthold Technologies, Bad Wildbad, Germany) for 240 seconds. Peptide concentrations in each sample were calculated using a non-linear plot (Prism version 6.03, GraphPad Software Inc, CA, USA).

2.2.7.4 PYY Radioimmunoassay

PYY-like immunoreactivity was measured using a previously established in-house specific and sensitive radioimmunoassay (Adrian et al., 1985). The antiserum (Y21) was produced in rabbits against synthetic porcine PYY coupled to BSA by glutaraldehyde. The Y21 antibody has 100% cross-reactivity with both biological active forms of PYY, PYY$_{1-36}$ and PYY$_{3-36}$. The

antibody does not cross-react with other known gut hormones. ^{125}I-PYY was prepared by Professor Mohammad Ghatei using the Iodogen method (Wood et al., 1981) and purified by HPLC. The assay was performed in a total volume of 350µl of 0.06M phosphate buffer (refer to Appendix 1) (pH 7.3) containing 0.3% BSA. The standard curve was generated by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of synthetic PYY at a concentration of 0.5pmol/ml. Supernatants were added at a volume of 150µl and lysates were added at a volume of 40µl. The assay was incubated over three nights at 4°C before separation of the free from antibody-bound label by immunoprecipitation using sheep anti-rabbit antibody (Pharmacia Diagnostics, Uppsala, Sweden). Secondary antibody was added at a volume of 100µl and tubes incubated at room temperature for 1 hour. Following the incubation period, 500µl of 0.01% Triton X-100 (Sigma, UK) and 100 µl of 10% poly ethylene glycol (PEG) (Sigma, Poole, UK) were added and the tubes immediately centrifuged at 1500g at 4°C for 30 minutes. Free and bound radioactivity was measured using gamma scintillation counters as previously described in 2.2.7.3.

2.2.7.5 The effect of OG administration of L-Arg.HCl on plasma GLP-1 and PYY levels in fasted rats in the early light phase

Rats were fasted overnight and received an oral gavage of either water, 12 or 16 mmol/kg L-Arg.HCl and were immediately returned to their cages. Animals were killed by decapitation at 30 or 90 minutes following administration and trunk blood collected in lithium heparin coated tubes (Teklab, County Durham, UK) containing aprotinin (Nordic Pharma, Reading, UK) at approximately 200 kallikrein inhibitor units per ml of blood. Blood was then centrifuged for 10 minutes at 6000g and plasma was separated, aliquoted and stored at -80°C until analysis. On hundred microliters of plasma was assayed by RIA to measure GLP-1 and PYY concentrations as described previously in 2.2.7.3 and 2.2.7.4

2.2.7.6 The role of GLP-1 and Y2 receptors in mediating the effect of L-Arg.HCl on food intake in mice

The anorectic effects of GLP-1 and PYY are mediated via the GLP-1 (Thorens, 1992) and Y2 (Batterham and Bloom, 2003) receptors, respectively. In order to investigate the role of GLP-1 and PYY in mediating the effect of L-Arg.HCl on food intake, both receptors were simultaneously antagonised using the GLP-1 receptor antagonist exendin$_{9-39}$ (Goke et al., 1993) and a selective Y2 receptor antagonist BIIE-0246 (TOCRIS Bioscience, Bristol, UK)(Smith-White et al., 2001).

The efficacy of both antagonists was confirmed by separate feeding studies in which the anorectic effect of exogenous exendin 4, a potent GLP-1 receptor agonist, and of exogenous PYY$_{3-36}$, were blocked by using exendin$_{9-39}$ and BIIE0246, respectively. Mice were fasted overnight and subsequently received an IP injection of saline or 400nmol/kg of exendin$_{9-39}$ (a dose previously shown to block the effects of GLP-1 on food intake) (Williams et al., 2009). This was followed by an immediate IP injection of saline or 1nmol/kg of exendin 4. Mice were returned to their cages with a pre-weighed amount of food provided, and food intake measured at 1 hour following administration.

This protocol was also used in the second study to investigate the ability of BIIE0246 to inhibit the anorectic effect of exogenous PYY$_{3-36.}$ The ability of BIIE0246 to block the anorectic effects of 25nmol/kg of PYY$_{3-36}$ at a previously established dose of 5.26µmol/kg (Ghitza et al., 2007) was confirmed.

This protocol was repeated with co-injection of vehicle or antagonists immediately followed by oral gavage of water or 24 mmol/kg of L-Arg.HCl in fasted mice injected at the early light phase, and in *ad libitum*-fed mice at the beginning of the dark phase.

2.2.8 The role of GPRC6A in mediating L-Arg.HCl-induced gut hormone release from a primary mice colonic L-cells

The WT and GPRC6a-KO primary mice colonic L-cells were isolated and prepared for secretion experiments as described in 2.2.6.1.

Secretion experiments were performed using 100mM L-Arg.HCl. The control for these experiments was secretion buffer that was osmolarity-matched to 100mM L-Arg.HCl osmolarity to account for any possible osmolarity-induced effects on gut hormone release. The osmolarity of the L-Arg.HCl was measured using an osmometer (osmomat 030, Gonotec, Berlin, Germany) and control treatment osmolarity was matched using 3X concentrated secretion buffer. GLP-1 and PYY levels were measured using specific RIAs described above, and secretion data was represented as percentage of total hormone as described in 2.2.6.

2.2.9 The effect of IP administration of L-Arg.HCl on food intake in fasted rats during the early light phase

Rats were fasted for 16 hours overnight and were then intraperitoneally administered with saline, 4 mmol/kg or 8 mmol/kg L-Arg.HCl during the early hours of light cycle. Animals were returned to their cages following administration and were given pre-weighed standard chow diet. The food intake was measured at 1, 2, 4, and 8 hours following administration.

2.2.10 Statistical analysis

All data are expressed as mean ± the standard error of the mean (SEM) except for behavioural study that is expressed as median and interquartile range. The feeding study in GPRC6A-KO mice was analysed using paired T-test. All other feeding studies were analysed using one-way ANOVA and Tukey's *post hoc* test. Behavioural study was analysed using Mann-Whitney test. Energy expenditure and cumulative food intake data were analysed using two-way ANOVA with Bonferroni's *post hoc* test. GPRC6A expression study

was analysed using one-way ANOVA with Tukey's *post hoc* test. Gut hormone studies *in vitro* were analysed using one-way ANOVA with Dunnett's test. Gut hormone release studies in GPRC6A-KO were analysed using multiple t test. Gut hormone studies *in vivo* were analysed using two-way ANOVA with Bonferroni's *post hoc* test. $P < 0.05$ was considered statistically significant. All analysis was carried out using Graphpad Prism software (Prism 6.03, GraphPad Software Inc, CA, USA).

2.3 Results

2.3.1 The effect of OG administration of L-Arg.HCl on food intake in fasted rats during the early light phase

Oral administration of 16 mmol/kg L-Arg.HCl significantly reduced food intake compared to the water control in rats at 0-1 hour period post administration (water: 4.87g ± 0.32g vs. L-Arg.HCl 16 mmol/kg 2.91 ± 0.19g, p<0.001; vs. 8mmol/kg L-Arg.HCl 4.62 ± 0.34g, p<0.01, n=9-10). L-Arg.HCl at the lower dose of 8 mmol/kg had no significant effect on food intake during the 0-1 period compared to water control group (Fig. 2.3.1). There were no significant effects at any other time points investigated.
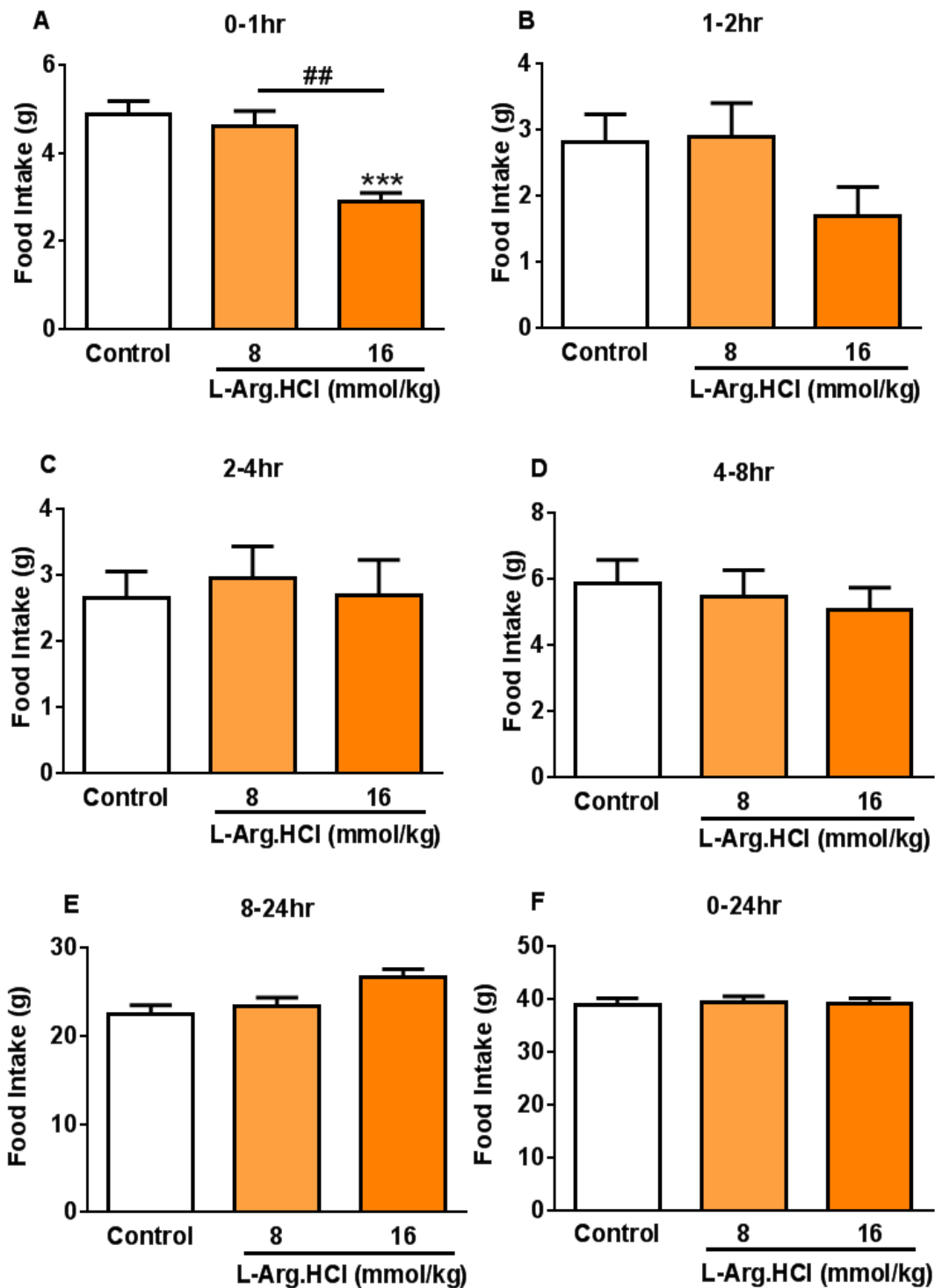
Figure 2.3.1 The effect of oral administration of L-Arg.HCl on food intake in fasted rats during the early light phase.
The effect of OG of water (control), 8, and 16 mmol/kg L-Arg.HCl on food intake in male rats following an overnight fast 0-1 (A), 1-2 (B), 2-4 (C), 4-8 (D), 8-24 (E) and 0-24 (F) hours following administration. Data presented as mean ± SEM. n=9-10., ##p<0.01 vs L-Arg.HCl (8mmol/kg), ***p<0.001 vs water control.

2.3.2 The effect of OG administration of L-Arg.HCl on food intake in *ad libitum* fed rats during the early dark phase

In order to further investigate the anorectic effects of L-arginine, *ad libitum* fed rats were orally gavaged with L-Arg.HCl at the onset of the dark phase which represents the normal physiological feeding period for nocturnal animals. Oral administration of L-Arg.HCl significantly reduced food intake in *ad libitum* fed rats during 0-1 period following administration at both 8 and 16 mmol/kg doses (water: 2.39g ± 0.31g vs. L-Arg.HCl 8 mmol/kg 1.20 ± 0.34g, $p < 0.05$; vs. 16 mmol/kg L-Arg.HCl 0.46 ± 0.16g, $p < 0.001$, n=12-16) (Fig. 2.3.2). Food intake was significantly lower in 16 mmol/kg L-Arg.HCl group compared with 8 mmol/kg L-Arg.HCl at 4-8 hour time interval (8 mmol/kg L-Arg.HCl: 7.00 ± 0.52g vs. 16 mmol/kg L-Arg.HCl: 5.00 ± 0.47, $p < 0.05$, n=12-16). The cumulative food intake at 24 hours following administration was significantly lower in rats administered 16 mmol/kg of L-Arg.HCl compared to the water control group (water: 26.33 ± 0.73g vs. 16 mmol/kg L-Arg.HCl: 22.15 ± 0.84g, $p < 0.01$, n=12-16). There were no significant effects at any other time points investigated.
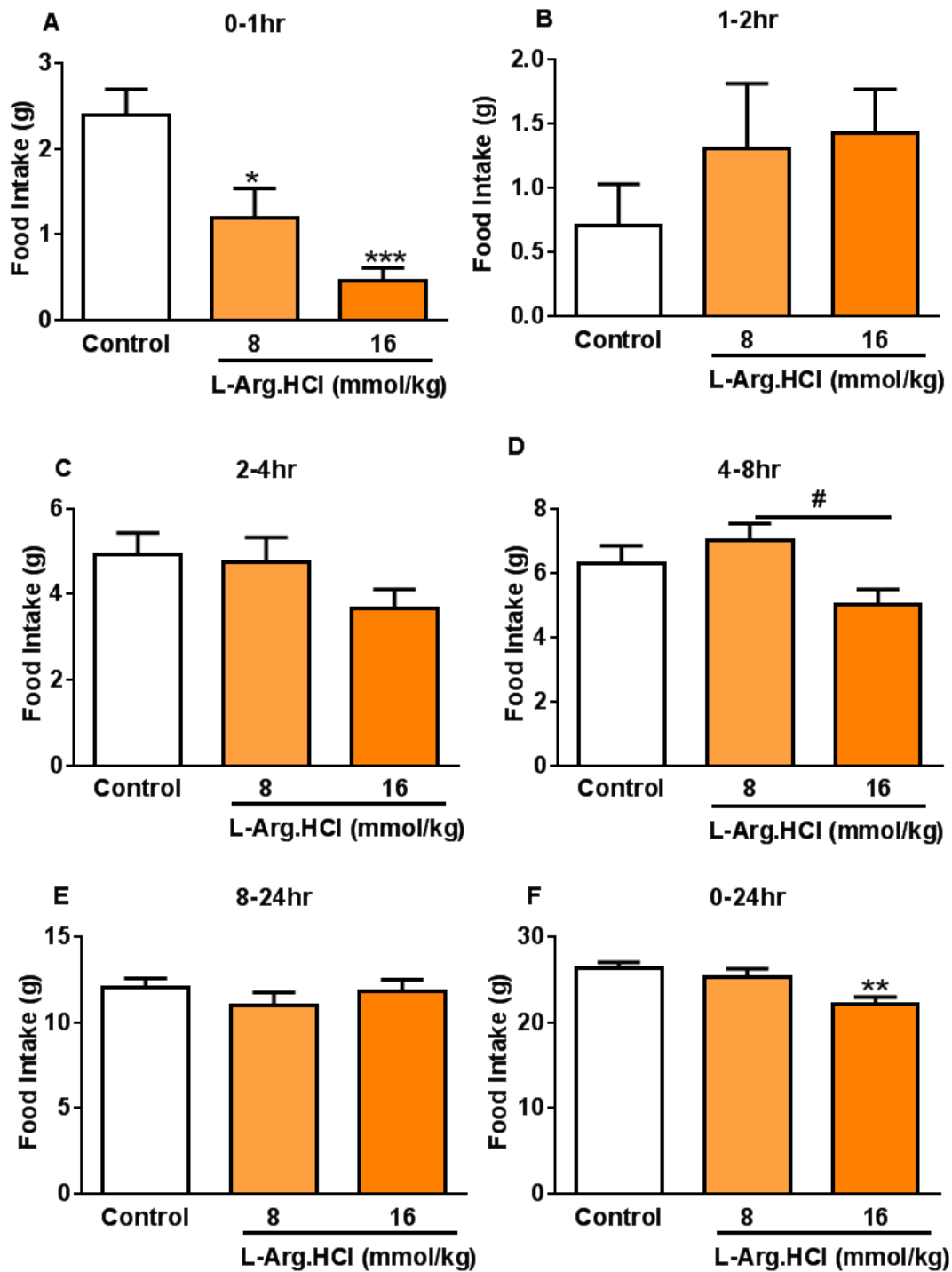
Figure 2.3.2 The effect of oral administration of L-Arg.HCl on food intake in *ad libitum* fed rats during the early dark phase.
The effect of OG of water (control), 8, and 16 mmol/kg L-Arg.HCl on food intake in male rats with *ad libitum* access to food during 0-1 (A), 1-2 (B), 2-4 (C), 4-8 (D), 8-24 (E) and 0-24 (F) hour following administration. Data presented as mean ± SEM. n = 12-16: *p<0.05, **p<0.01, ***p<0.01 vs. water control, #p<0.05 vs. 8 mmol/kg L-Arg.HCl.

2.3.3 The effect of OG administration of L-Arg.HCl on behaviour in fasted rats during the early light phase

Oral gavage administration of 16 mmol/kg L-Arg.HCl had no significant effect on measured behaviours compared to the water control group during 0-1 hour period following administration (Fig. 2.3.3).
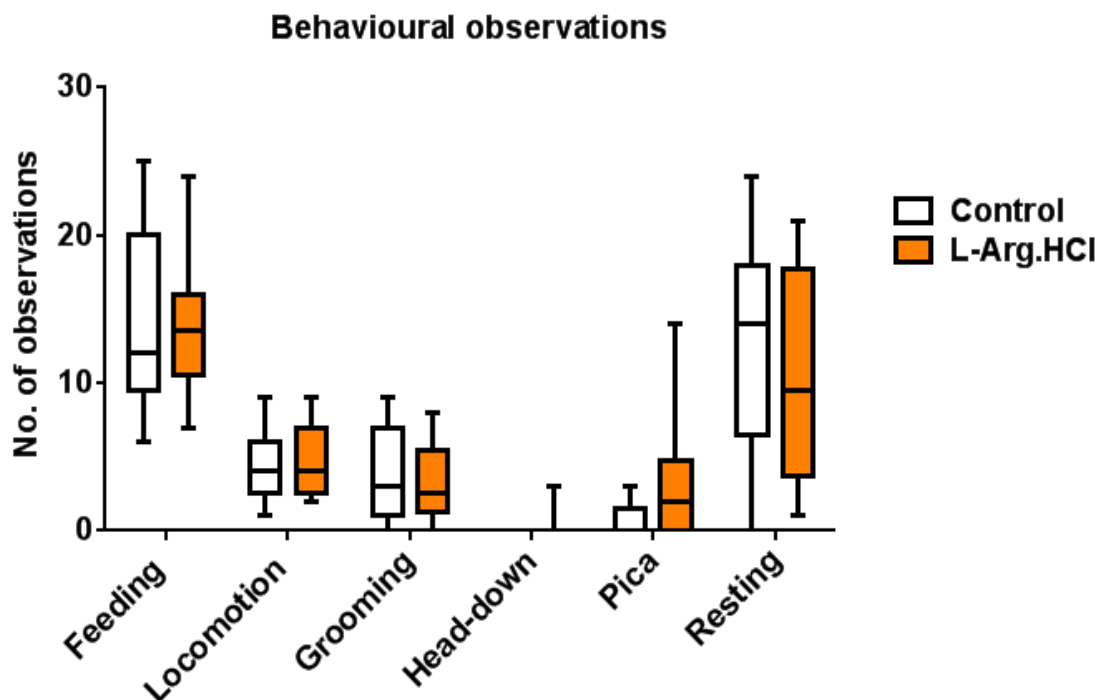
Figure 2.3.3 The effects of oral administration of L-Arg.HCl on behaviour in fasted rats during the light phase.
The effect of OG of water (control) or 16 mmol/kg L-Arg.HCl in overnight fasted male rats on feeding, locomotion, grooming, head down, pica and resting behaviours compared to control group. Data represented as median (interquartile range) for each observation. n = 12-13

2.3.4 The effect of OG administration of L-Arg.HCl on food intake in fasted mice during the early light phase

Oral administration of 16 and 24 mmol/kg of L-Arg.HCl significantly reduced food intake in mice in a dose dependent manner during 0-1 hour interval following administration. (Water: 0.68 ± 0.03g vs. 8mmol/kg L-Arg.HCl 0.71 ± 0.05g; vs. 16 mmol/kg L-Arg.HCl 0.50 ± 0.06g, $p<0.05$; vs. 24 mmol/kg L-Arg.HCl 0.30 ± 0.03g, $p<0.001$, n=8-9). There were no significant differences in food intake in 1-2 and 2-4 periods post administration between treatment groups. L-Arg.HCl 24 mmol/kg significantly reduced food intake compared to the 8 mmol/kg treatment group during the 4-8 and 0-24 hour periods (4-8 hour: 8 mmol/kg L-Arg.HCl 0.78 ± 0.07g vs. 24 mmol/kg L-Arg.HCl 0.48 ± 0.05g, $p<0.05$, n=8-9; 0-24 hour: 8 mmol/kg L-Arg.HCl: 5.33 ± 0.14g vs. 24 mmol/kg 4.66 ± 0.13g, $p<0.01$, n=8-9) (Fig. 2.3.4).
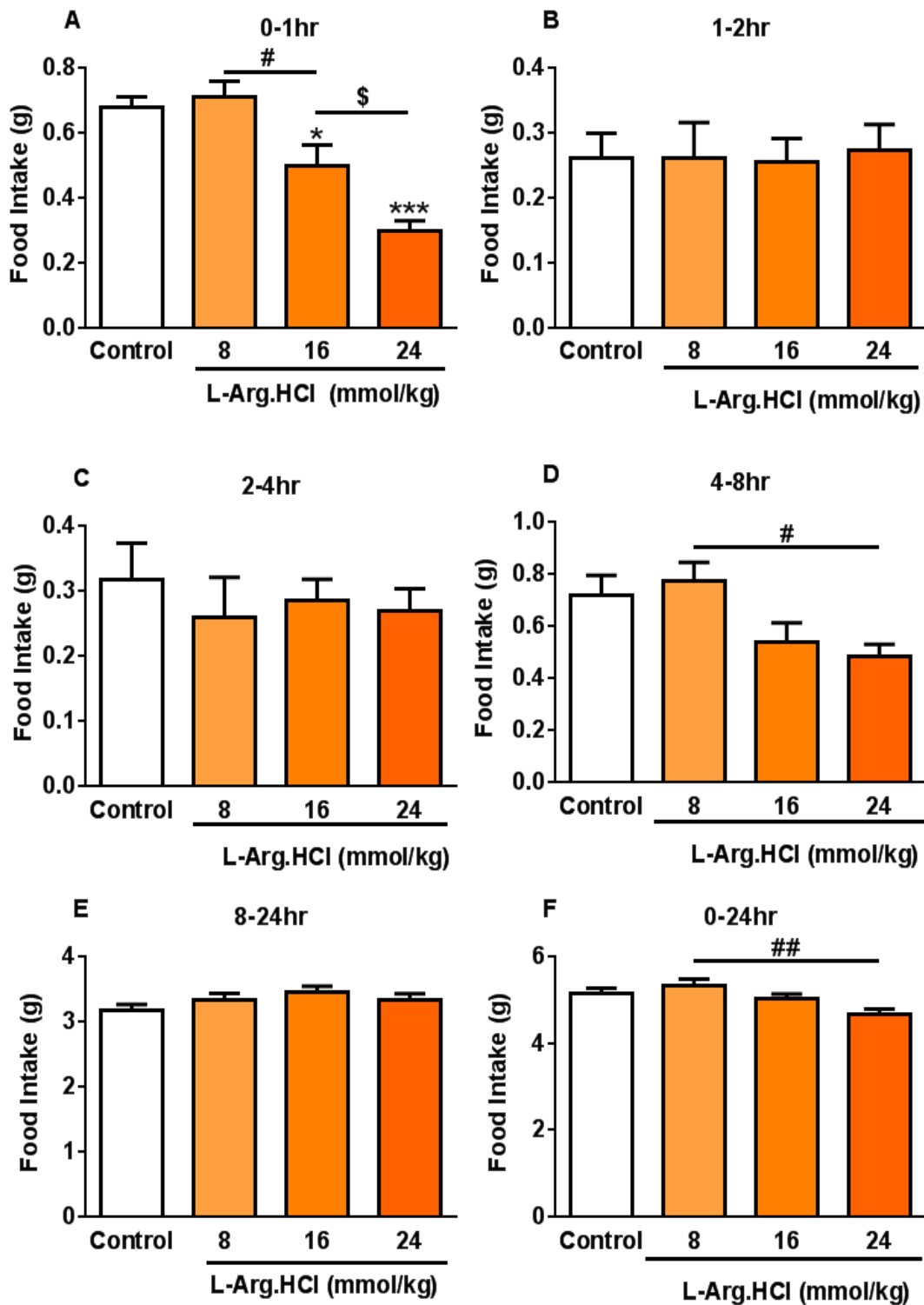
Figure 2.3.4 The effect of oral administration of L-Arg.HCl on food intake in fasted mice during the early light phase.
The effect of OG of water (control), 8, 16 and 24 mmol/kg L-Arg.HCl on food intake in male mice following an overnight fast during 0-1 (A), 1-2 (B), 2-4 (C), 4-8 (D), 8-24 (E) and 0-24 (F) hour following administration. Data presented as mean ± SEM. n=8-9. *p<0.05, ***p<0.001 vs. water control; #p<0.05, ##p<0.01 vs. 8mmol/kg L-Arg.HCl; $p<0.05 vs. 16 mmol/kg L-Arg.HCl.

2.3.5 The effect of OG administration of L-Arg.HCl on energy expenditure and food intake in fasted mice

To investigate the effect of L-Arg.HCl on energy expenditure, mice were placed in CLAMS metabolic cages as described in section 2.2.5. Oral administration of 24 mmol/kg L-Arg.HCl had no significant effect on oxygen consumption (VO_2) (time and treatment interaction p = 0.9981, time factor p<0.001, treatment factor p<0.05, two way ANOVA) or carbon dioxide production (VCO_2) (time and treatment interaction p = 0.9953, time factor p <0.001, treatment factor p = 0.3397, two way ANOVA) in mice during the 8 hours following administration. Returning food caused similar increases in VO_2 and VCO_2 in both treatment groups (Fig. 2.3.5 A & B). RER was significantly lower following return of the food in L-Arg.HCl treated group compared to the water control (Fig. 2.3.5 C). L-Arg.HCl treated animals ate less compared to the water control group when their food was returned 8 hours post administration. This effect was significant at every time point recorded throughout the dark phase period (Figure 2.3.5 D).
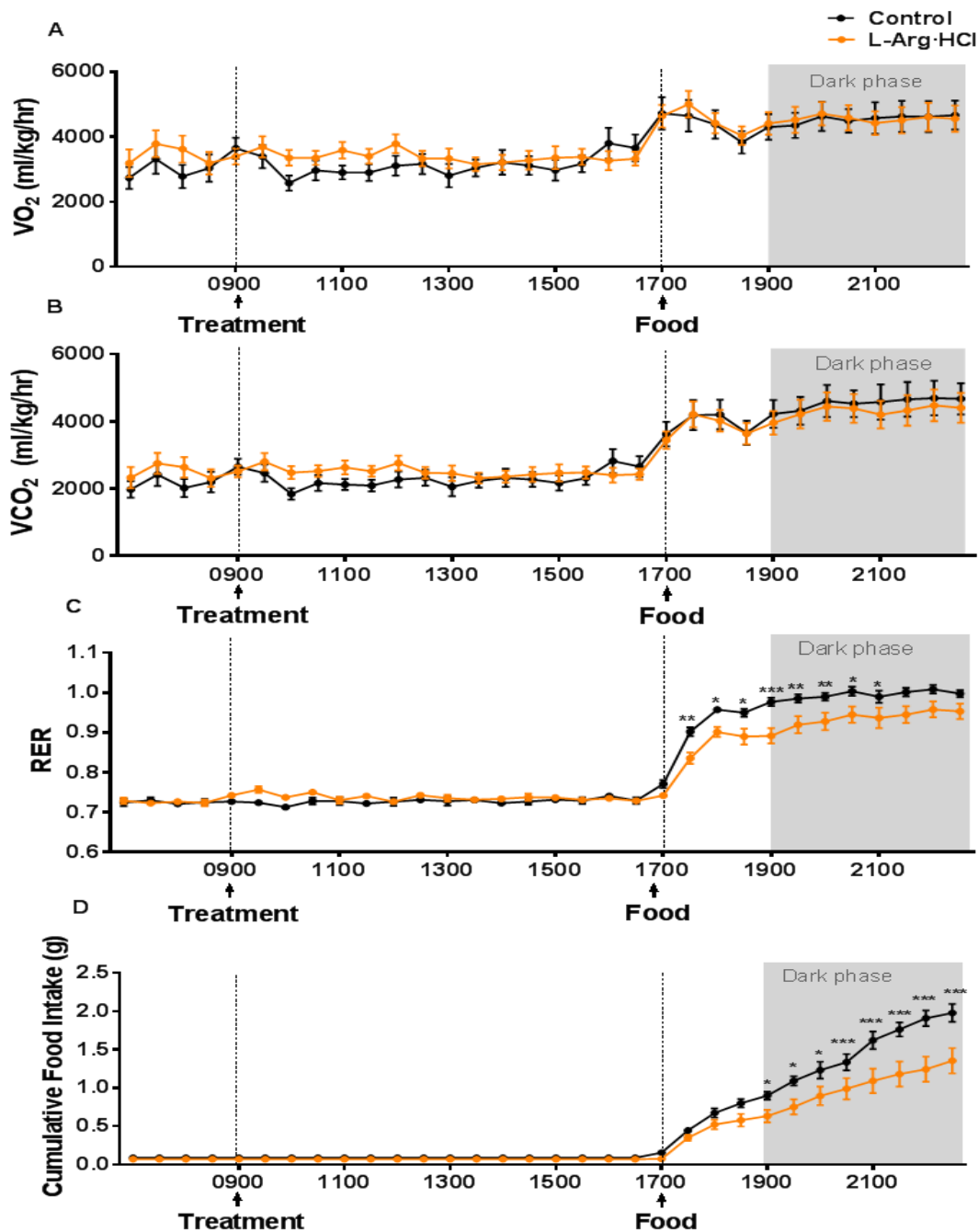
Figure 2.3.5 The effect of OG administration of L-Arg.HCl on energy expenditure and food intake in fasted mice.
The effect of OG administration of water (control) or 24 mmol/kg L-Arg.HCl on oxygen consumption (A), carbon dioxide production (B), respiratory exchange rate (C) and food intake (D) in mice injected at early light phase and placed in CLAMS cages. The oral gavage was performed at 09:00 and food was returned at 17:00 as indicated by dotted line. Recordings were taken over a period of 24 hours and at subsequent 24 minute intervals following administration. Shaded area represents the dark phase from 19:00. Data presented as mean ± SEM. n=12 per group. *p<0.05, **p<0.01, ***p<0.001 vs water control.

2.3.6 The role of GPRC6A in mediating the effect of L-Arg.HCl on food intake

2.3.6.1 The expression of GPRC6a in the GI tract

GPRC6a expression was detected in all levels of the GI tract investigated. The highest expression was observed in the jejunum and colon (Fig. 2.3.6)
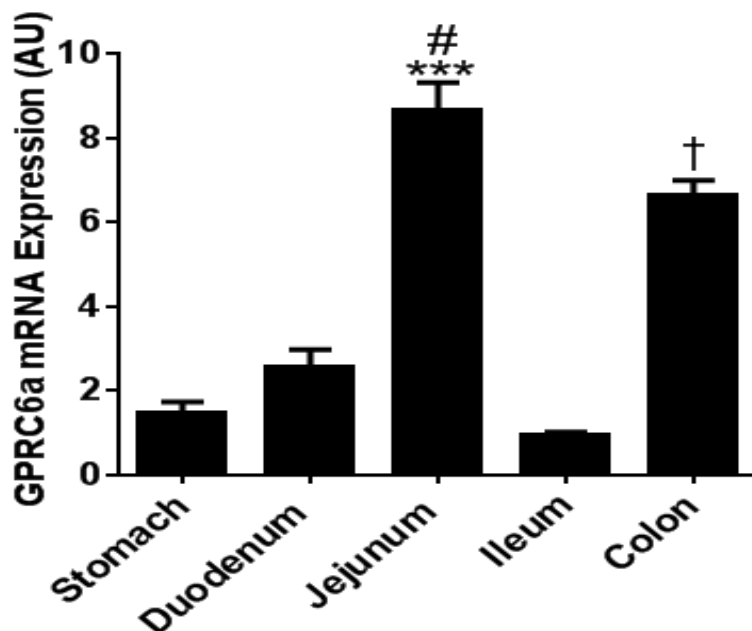
Figure 2.3.6 GPRC6a expression in the mouse GI tract.
Relative expression of GPRC6a mRNA in different regions of GI tract of overnight fasted male mice. Data is presented as mean ± SEM. ***p<0.001 vs. stomach, duodenum and ileum. #P<0.01 vs colon. †P<0.001 vs stomach, duodenum and ileum. n= 7.

2.3.6.2 The role of GPRC6A in mediating the effect of L-Arg.HCl on food intake

Oral administration of 16 mmol/kg L-Arg.HCl significantly reduced food intake in both WT and GPRC6a-KO mice to a similar magnitude at 0-1 hour period following administration (WT, Water: 0.33 ± 0.06g vs. L-Arg.HCl: 0.14g ± 0.04g. $p < 0.05$; GPRC6a-KO, Water: 0.40 ± 0.04g vs. L-Arg.HCl: 0.06 ± 0.02g. $p < 0.01$, n=4 cross over) (Fig. 2.3.7A). Similarly, oral gavage of 24 mmol/kg L-Arg.HCl significantly reduced food intake during the first hour post administration in both WT and GPRC6a-KO (WT, Water: 0.26 ± 0.09g vs. L-Arg.HCl: 0.02g ± 0.03g. $p < 0.05$; GPRC6a-KO, Water: 0.30 ± 0.02g vs. L-Arg.HCl: 0.02 ± 0.03g. $p < 0.01$, n=4, cross over) (Fig. 2.3.7B).
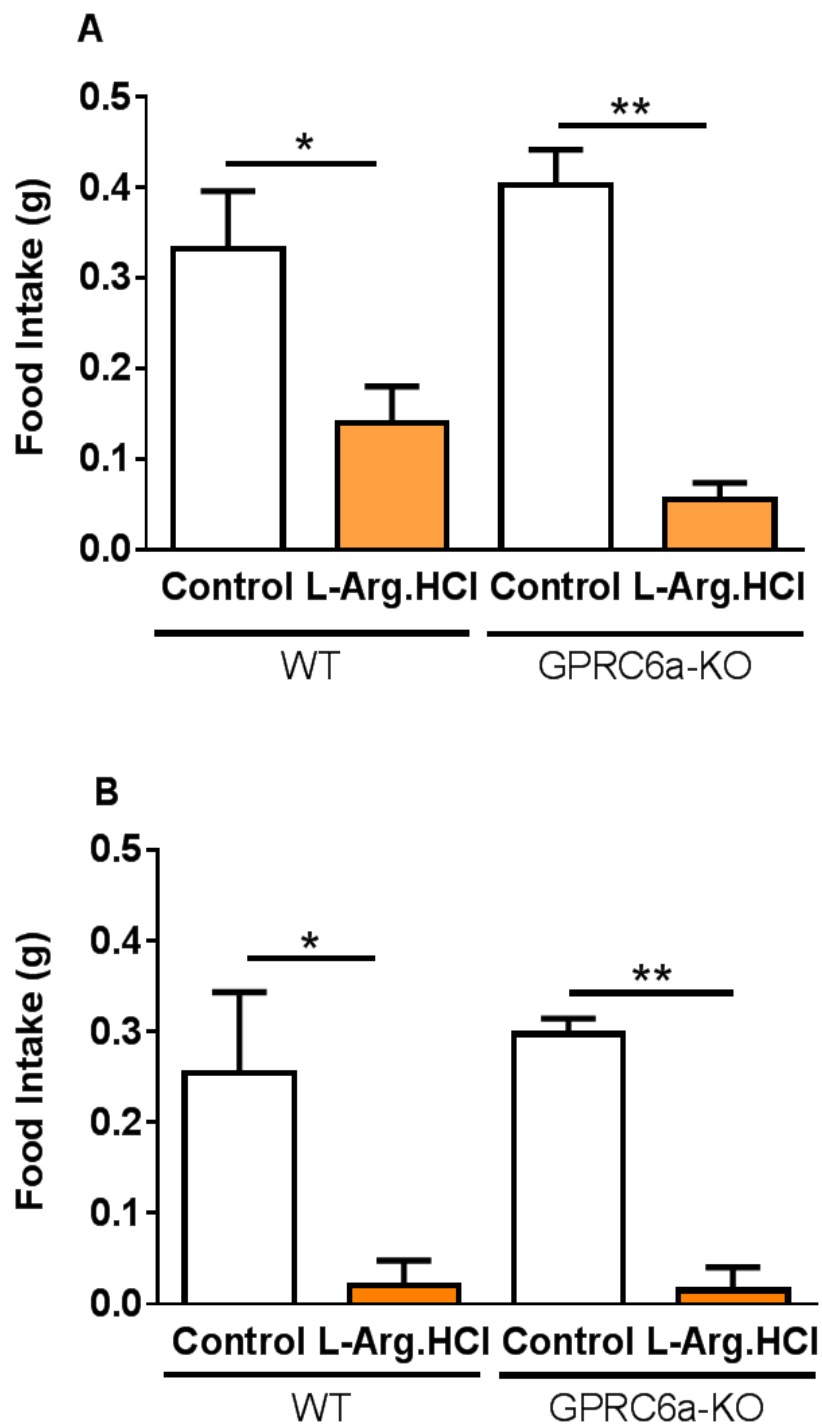
Figure 2.3.7 The effect of L-Arg.HCl on food intake in *ad libitum* fed mice lacking GPRC6a in the early dark phase.
The effect of oral gavage administration of water (control) or 16 mmol/kg L-Arg.HCl (A) and water or 24 mmol/kg L-Arg.HCl (B) on 0-1 hour food intake in WT and GPRC6a-KO mice with *ad libitum* access to food injected at the beginning of dark phase. Data expressed as mean ± SEM. *p<0.05, **p<0.01 vs. water control. n = 4, cross over for both (A) and (B).

2.3.7 The role of gut hormones in mediating the effect of L-Arg.HCl on food intake

2.3.7.1 The effect of L-Arg.HCl on GLP-1 and PYY release from primary murine colonic L-cells

L-Arg.HCl stimulated GLP-1 release from primary L-cells culture (control: 2.00 ± 0.40% vs. 1mM L-Arg.HCl: 1.97 ± 0.65%, vs. 10 mM L-Arg.HCl: 4.83 ± 1.55%, vs. 100 mM L-Arg.HCl: 8.28 ± 2.10%. $p < 0.05$, n=3 plates in triplicate) (Fig 2.3.8 A). A concentration of 100 mM L-Arg.HCl significantly stimulated PYY release from the primary L-cells culture (Control: 3.80 ± 0.50% vs. 100mM L-Arg.HCl: 24.87 ± 6.33%, $p < 0.001$, n=3 plates in triplicate) (Fig. 2.3.8 B).

Incubating cells with IBMX-forskolin mixture caused a significant release of both GLP-1 (Control: 2.00 ± 0.40% vs. IBMX-forskolin: 11.37 ± 2.03%. $p < 0.01$, n=3 plates in triplicate) and PYY (Control: 3.80 ± 0.50% vs. IBMX-forskolin: 43.78 ± 1.49%, $p < 0.001$, n=3 plates in triplicate).
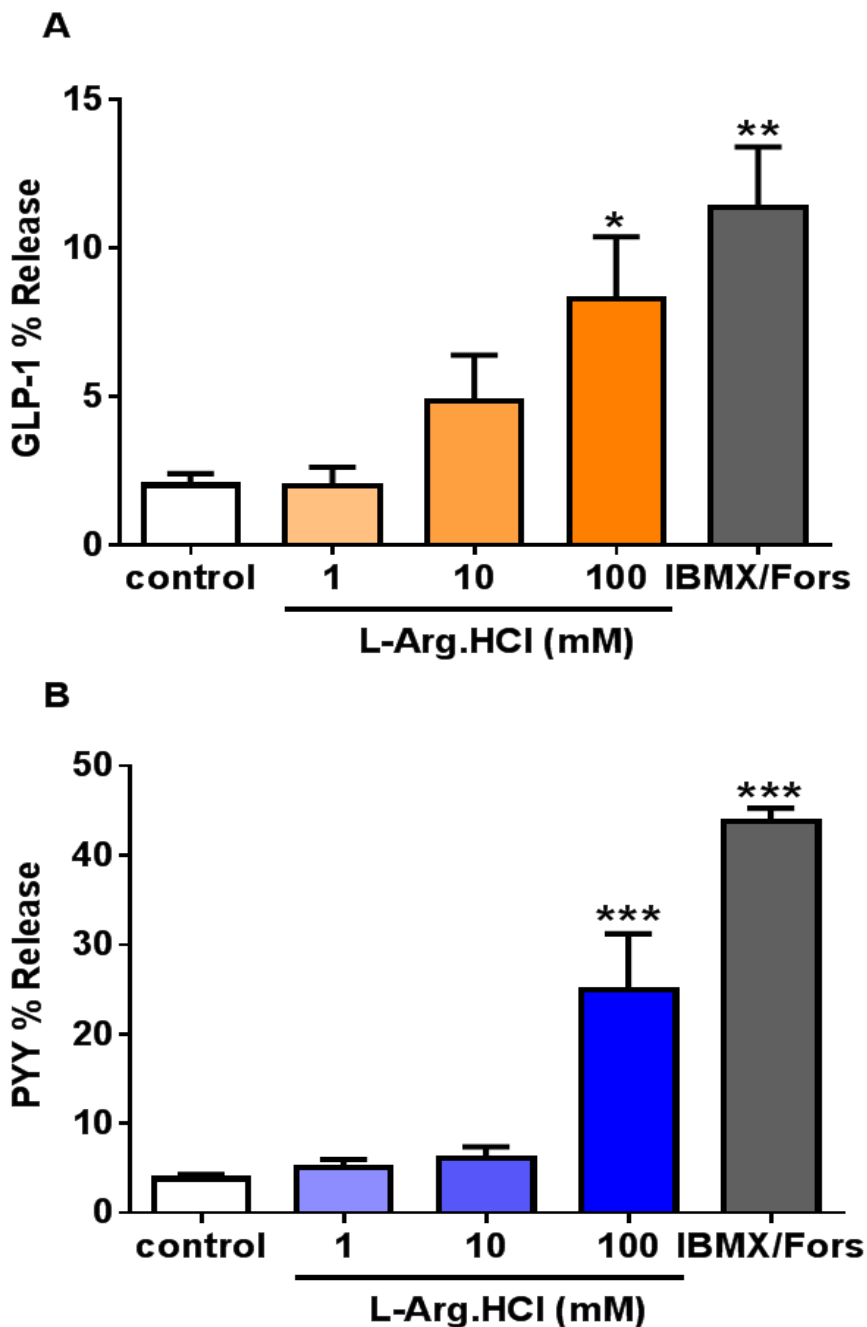
Figure 2.3.8 The effect of L-Arg.HCl on GLP-1 and PYY release from primary murine colonic epithelium.
The effect of L-Arg.HCl on GLP-1 (A) and PYY (B) release from primary mice colonic L-cells incubated with 1, 10 and 100 mM L-Arg.HCl for 2 hours. IBMX-forskolin mix (10 μM, each) was used as positive control. The release is shown as percentage of total hormone contained for each well in the experiment. Data presented as mean ± SEM. n = 3 plates in triplicate from 9 mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control.

2.3.7.2 The role of GPRC6A in GLP-1 and PYY release from mice primary colonic L-cells

Exposure to 100mM L-Arg.HCl significantly stimulated the release of GLP-1 from primary WT L-cells (Control: 2.08 ± 0.17% vs. L-Arg.HCl: 10.02 ± 2.73%. $p<0.05$, n=2 plates in triplicate) (Fig 2.3.9 A). GLP-1 levels were also elevated in GPRC6a-KO primary L-cells treated with L-Arg.HCl (Control: 3.96 ± 0.75% vs. L-Arg.HCl: 6.89 ± 1.81%). However this effect did not reach statistical significance (Fig 2.3.9 A).

Similarly, treatment of WT L-cells with 100mM L-Arg.HCl significantly stimulated PYY release in both WT L-cells (Control: 5.01 ± 0.49% vs. L-Arg.HCl: 13.76 ± 1.86%. $p<0.001$, n=2 plates in triplicate) and GPRC6a-KO L-cells (Control: 6.99 ± 1.10% vs. L-Arg.HCl: 12.78 ± 1.25%. $p<0.05$, n=2 plates in triplicate) (Fig. 2.3.9 B).
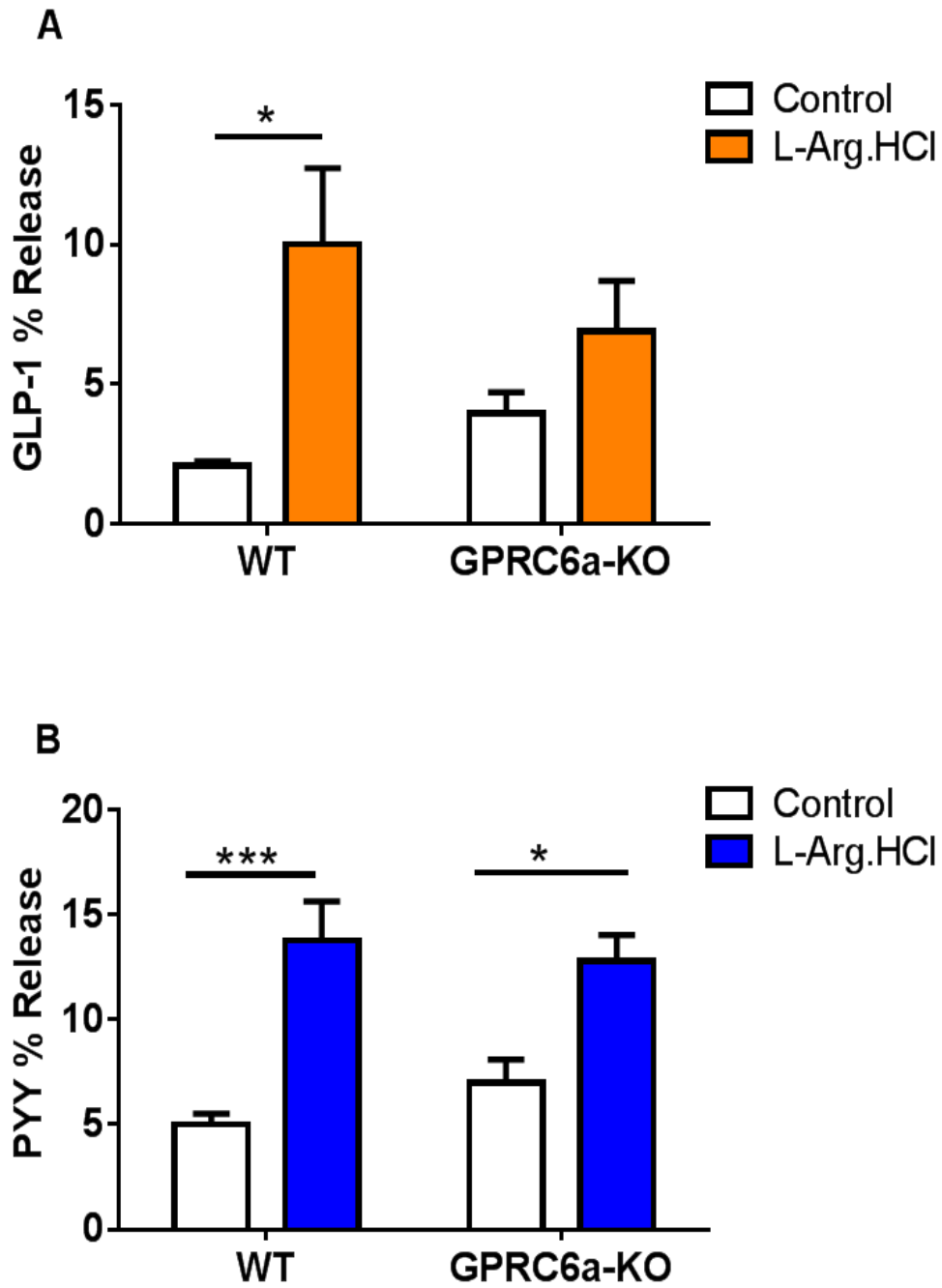
Figure 2.3.9 The effect of GPRC6A on GLP-1 and PYY release from primary murine colonic epithelium. The effect of L-Arg.HCl on GLP-1 (A) and PYY (B) release from WT and GPRC6A-KO primary mice colonic L-cells incubated with 100 mM L-Arg.HCl for 2 hours. The release is shown as percentage of total hormone contained for each well in the experiment. Data presented as mean ± SEM. n = 2 plates in triplicate, from 6 mice. *p<0.05, ***p<0.001 vs. control.

2.3.7.3 The effect of OG administration of L-Arg.HCl on plasma GLP-1 and PYY levels in rats

Oral administration of 16 mmol/kg L-Arg.HCl significantly elevated the plasma GLP-1 levels in rats at both 30 minutes (Water: 1.90 ± 0.31 pmol/l vs. 16 mmol/kg L-Arg.HCl 5.60 ± 0.57 pmol/l, $p<0.001$, n=6-8) and 90 minutes (Water: 2.64 ± 0.19 pmol/l vs. 16 mmol/kg L-Arg.HCl 6.56 ± 0.51 pmol/l, $p<0.001$, n=6-8) following administration compared to water control. Similarly, a lower dose of 12 mmol/kg L-Arg.HCl significantly increased plasma GLP-1 levels at 90 minutes following administration (Water: 2.64 ± 0.19 pmol/l vs. 12 mmol/kg L-Arg.HCl 4.32 ± 0.54 pmol/l, $p<0.05$, n=6-8) (Fig 2.3.10 A). PYY plasma levels were elevated following administration of L-Arg.HCl, but these effects did not achieve statistical significance (Fig. 2.3.10 B).
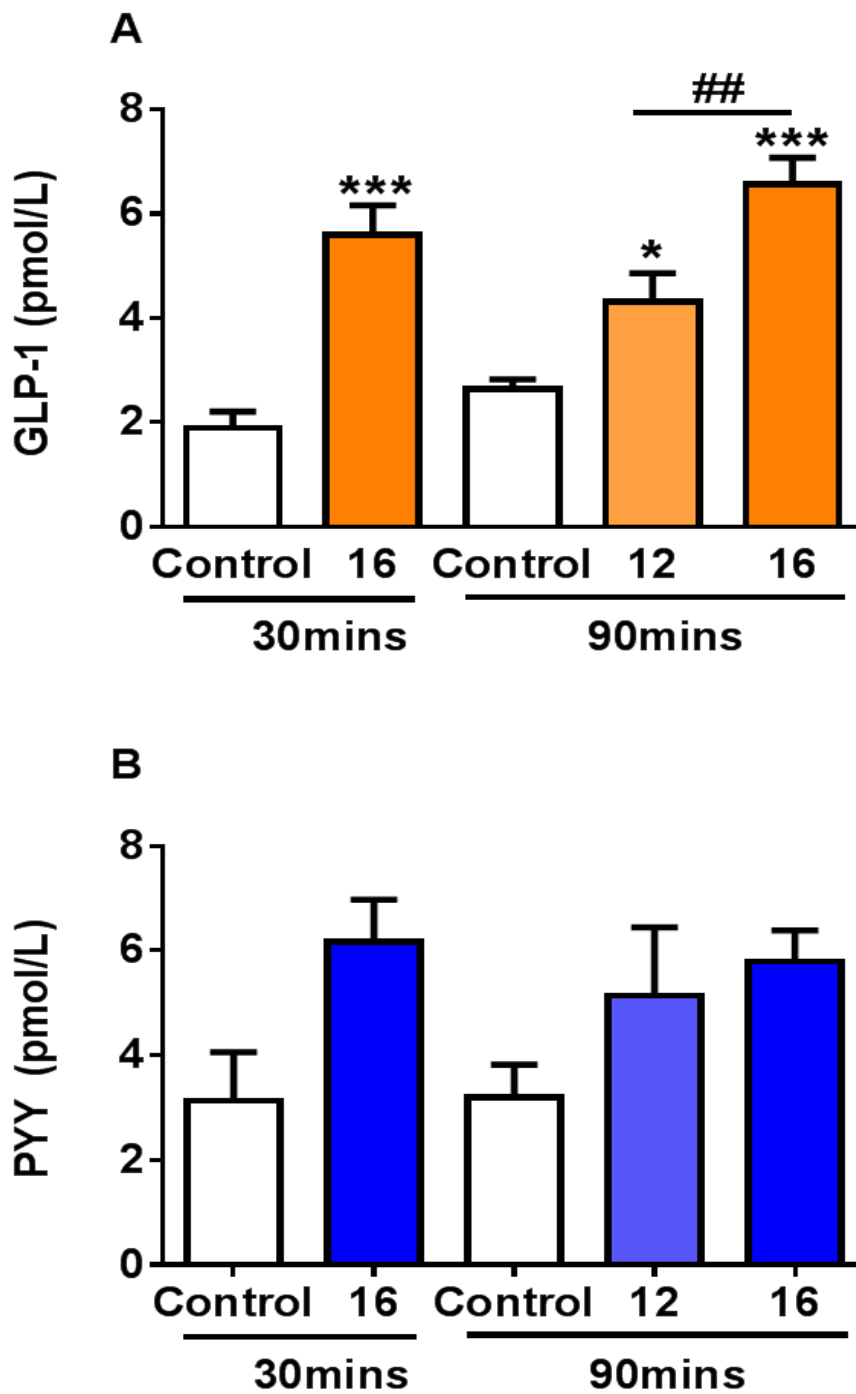
Figure 2.3.10 The effect of OG administration of L-Arg.HCl on GLP-1 and PYY release in fasted rats in the early light phase.
The effect of OG administration of water (control), 12 or 16 mmol/kg L-Arg.HCl on GLP-1 (A) and PYY (B) in overnight fasted male rats at 30 and 90 minutes following administration. Data presented as mean ± SEM. n = 6-8. *$p<0.05$, ***$p<0.001$ vs. water control, ##$p<0.01$ vs. 12 mmol/kg L-Arg.HCl.

2.3.7.4 The role of GLP-1 and Y2 receptors in mediating the effect of L-Arg.HCl on food intake in mice

IP administration of 1nmol.kg exendin 4 significantly reduced food intake in the first hour following administration (saline: 0.37 ± 0.06g vs.exendin 4: 0.12 ± 0.02g. P<0.01, n=10). IP injection of 400nmol/kg of exendin $_{9-39}$ significantly attenuated the effect of exendin 4 on food intake (exendin 4: 0.12 ± 0.02 vs. exendin 4 + exendin $_{9-39}$: 0.37 ± 0.04g. p<0.01, n=10) (Fig. 2.3.11 A).

IP administration of 25nmol.kg PYY$_{3-36}$ significantly reduced food intake in the first hour following administration (Vehicle: 0.54 ± 0.05g vs. PYY$_{3-36}$: 0.19 ± 0.03g. P<0.001, n=10). IP injection of 5.26µmol/kg of BIIE-0246 significantly attenuated the effect of exogenous PYY$_{3-36}$ on food intake (PYY $_{3-36}$: 0.19 ± 0.03 vs. PYY$_{3-36}$ + BIIE-0246: 0.41 ± 0.04g. p<0.01, n=10) (Fig. 2.3.11 B).

Oral gavage of 24 mmol/kg L-Arg.HCl significantly reduced food intake both in fasted mice injected at the light phase (Vehicle: 0.72 ± 0.04g vs. L-Arg.HCl: 0.45 ± 0.06g. p<0.05, n=10) (Fig 2.3.11 C) and fed mice injected at the beginning of dark phase (Vehicle: 0.30 ± 0.03g vs. L-Arg.HCl: 0.08 ± 0.02g. p<0.001, n=10) (Fig 2.3.11 D). Oral gavage of L-Arg.HCl following an IP administration of a mixture of 5.26µmol/kg BIIE0246 and 400nmol/kg exendin$_{9-39}$ also significantly reduced food intake in both fasted mice injected at light phase (Vehicle: 0.72 ± 0.04g vs. L-Arg.HCl + antagonists: 0.37 ± 0.06g. p<0.001, n=10) (Figure 2.3.11 C) and fed mice injected during dark phase (Vehicle: 0.30 ± 0.03g vs. L-Arg.HCl + antagonists: 0.07 ± 0.03g. p<0.001, n=10) (Fig 2.3.11 D). GLP-1 and Y2 receptor antagonism had no significant effect on the anorectic actions of L-Arg.HCl.
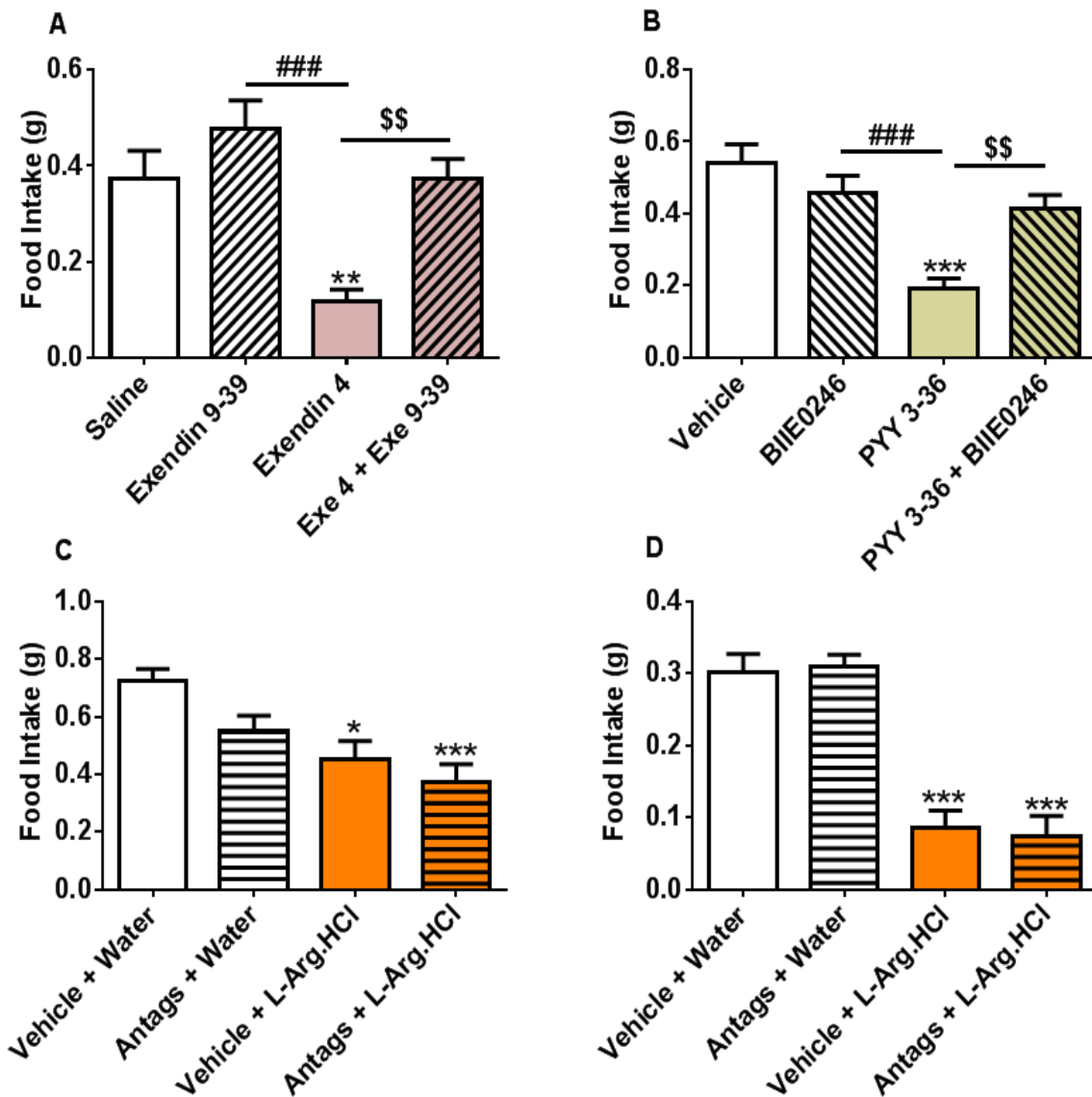
Figure 2.3.11 The role of GLP-1 and Y2 receptors in mediating the effect of L-Arg.HCl on food intake in mice.

The effect of IP administration of 400 nmol/kg exendin$_{9\text{-}39}$ on the anorectic effect of 1nmol/kg exogenous exendin 4 in fasted mice at 0-1 hour post administration (n = 10) (A). The effect of IP administration of 5.26 µmol/kg BIIE0246 on the anorectic effect of 25 nmol/kg PYY$_{3\text{-}36}$ in fasted mice at 0-1 hour post administration (n = 10). The effect of IP administration of a mixture of 400 nmol/kg exendin$_{9\text{-}39}$ and 5.26 µmol/kg BIIE0246 on the anorectic effect of orally gavaged 24 mmol/kg L-Arg.HCl in fasted mice during early light phase (n = 10) (C) and *ad libitum* fed mice during dark phase (n = 10) (D) in the 0-1 hour period post administration. Data presented as mean ± SEM. n=10 per group. (A): **$p < 0.01$ vs saline control, ###$p < 0.001$ vs. Exendin $_{9\text{-}39}$, \$\$$p < 0.01$ vs. Exendin 4; (B): ***$p < 0.001$ vs vehicle control, ###$p < 0.001$ vs. BIIE0246, \$\$$p < 0.01$ vs. PYY $_{3\text{-}36}$; (C) and (D): *$p < 0.05$, ***$p < 0.001$ vs. Vehicle control group.

2.3.8 The effect of IP administration of L-Arg.HCl on food intake in fasted rats during the early light phase

IP administration of 4 and 8 mmol/kg L-Arg.HCl significantly reduced food intake in rats during 0-1 hour period following administration compared to the saline control group (saline: 5.36 ± 0.43g vs. 4 mmol/kg L-Arg.HCl 3.87 ± 037g, $p<0.05$; vs. 8 mmol/kg L-Arg.HCl 3.56 ± 0.18g, $p<0.01$, n=9). There were no significant effects at any other time points investigated (Fig. 2.3.12).
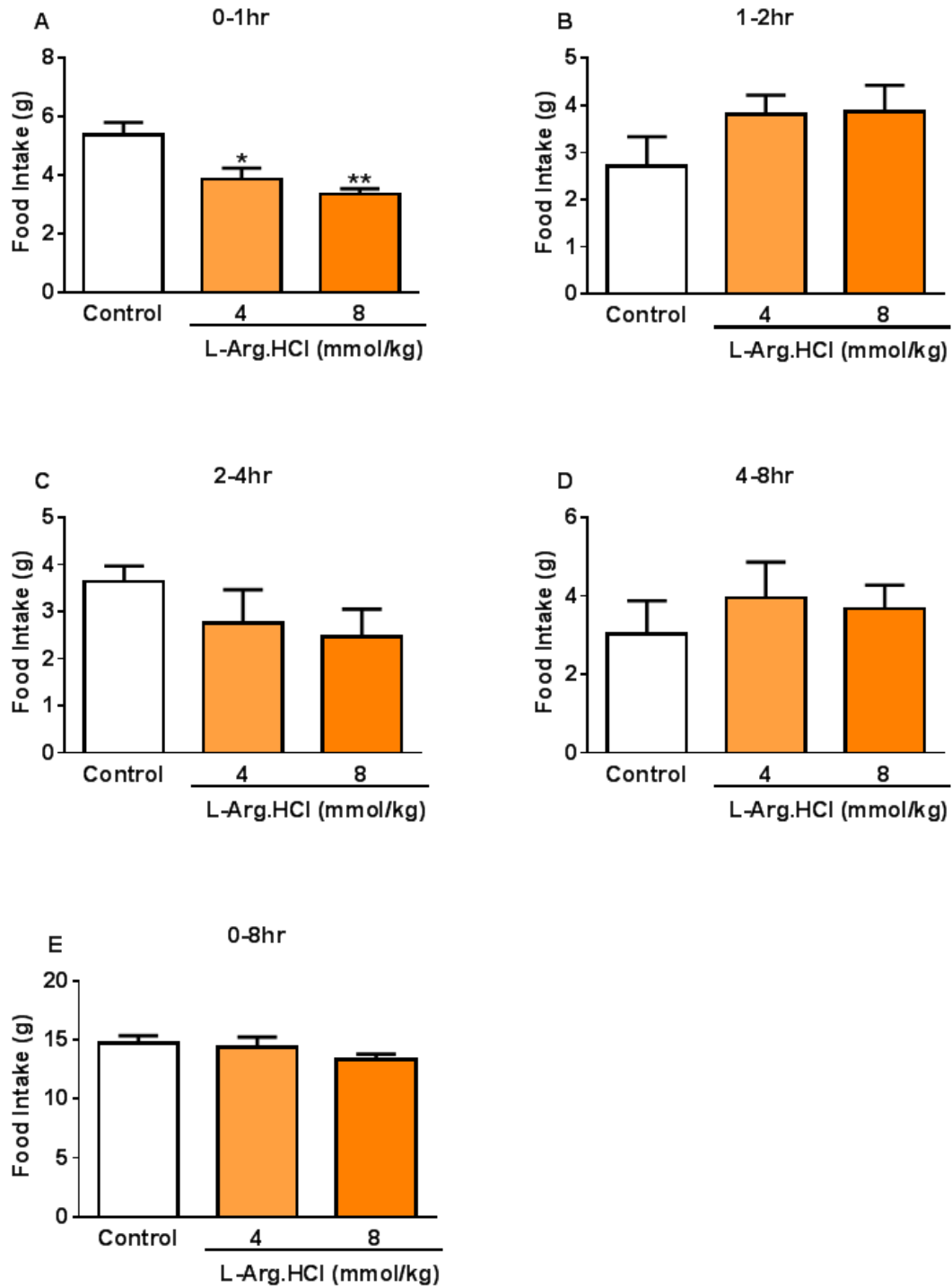
Figure 2.3.12 The effect of IP administration of L-Arg.HCl on food intake in fasted rats during the early light phase. The effect of IP administration of saline (control), 4, and 8 mmol/kg L-Arg.HCl on food intake in male rats following an overnight fast during 0-1 (A), 1-2 (B), 2-4 (C), 4-8 (D) and 0-8 (E) hour following administration. Data presented as mean ± SEM. n=9 *p<0.05, **p<0.01 vs saline control.

2.4 Discussion

High protein diets are associated with satiety and promote weight management. It is suggested that the anorectic effect of such diets may in part be due to the amino acid composition of the protein ingested (Veldhorst et al., 2008). The work in this chapter investigated the anorectic effects of L-arginine in rodents and the mechanism by which these effects are mediated. The pilot data previously generated in our laboratory demonstrated that amongst individual amino acids tested, L-arginine potently reduced food intake in rats. Therefore the role of L-arginine in satiety and appetite regulation was further explored in the experiments described in this chapter, demonstrating that L-arginine reduces food intake in both mice and rats, and stimulates the release of the anorectic gut hormones GLP-1 and PYY. However, the effects of L-arginine on GLP-1 and PYY release are unlikely to mediate the anorectic effects of L-arginine.

L-arginine contains a guanidinium cation side chain that makes L-arginine extremely basic in solution. Therefore, in order to avoid any pH dependent effect in our studies, the neutral L-Arg.HCl salt was used.

Oral administration of L-Arg.HCl significantly reduced food intake in rats. The effect was observed in fasted animals administered L-Arg.HCl during the light phase, and in *ad libitum* fed rats injected during the dark phase. This was important as it demonstated L-Arg.HCl had an anorectic effect in the dark phase, representing the physiological feeding time for nocturnal rats. Rats seemed to be more sensitive to the effect of L-Arg.HCl during the dark phase, as the anorectic effect of L-Arg.HCl with the same dose as tested in light phase resulted in an exaggerated reduction in food intake in rats during 0-1 hour period post administration. This effect was independent of any obvious behavioural side effects. The anorectic effect of L-arginine in rats has been shown by another group (Jordi et al., 2013). Jordi et al demonstrated a significant reduction of food intake in rats following OG of 6.7 mmol/kg L-arginine. In our dose finding studies, a dose of 4 or 6 mmol/kg had no effect on food intake and a dose of 8 mmol/kg was required to significantly reduce food intake in rats.

However, Jordi et al used L-arginine rather than an L-arginine salt in their studies, and it is possible that the basic nature of the L-arginine solution administered may have contributed to the outcome of their study.

In order to further investigate the anorectic potential of L-arginine, its effects in mice were investigated. Similar to rats, oral gavage of L-Arg.HCl resulted in a dose dependent reduction of food intake in mice. However, higher isomolar doses were required in order to produce similar magnitude of response as observed in rats. This might be expected, as mice require higher doses per body weight due to their high metabolic and clearance rate compared to the rats. However, it is unclear whether such differences would be expected in an agent hypothesised to act in the gut lumen, rather than in systemic circulation. A dose of 24 mmol/kg L-Arg.HCl potently reduced food intake in mice during the first hour following administration.

Evidence suggests that L-amino acids may be sensed by the promiscuous G-protein coupled receptors GPRC6A, CaSR and T1R1/T1R3. Basic amino acids including L-arginine are potent activators of GPRC6A (Clemmensen et al., 2014). GPRC6A is highly expressed in the GI tract and is involved in a number of important physiological pathways (Clemmensen et al., 2014, Wellendorph and Brauner-Osborne, 2004). In order to further examine the mechanisms mediating anorectic effect of L-arginine, the role of GPRC6A was examined using GPRC6a-KO mice. Oral administration of L-Arg.HCl significantly reduced food intake in both WT and GPRC6a-KO mice. The effect of L-Arg.HCl was examined at both 24 mmol/kg, which was previously shown to reduce food intake in mice, and at a lower dose of 16 mmol/kg, with the aim of trying to reduce the possibility of non-specific and presumably non-GPRC6A mediated mechanisms. In addition, GPRC6A ablation did not appear to completely block L-Arg.HCl induced GLP-1 and PYY release from a primary cultured murine colonic epithelium, though the effect on GLP-1 release did appear to be attenuated. Small interfering RNA (siRNA) induced depletion of endogenous GPRC6A abolished the GLP-1 release stimulated by L-ornithine from GLUTag cell line (Oya et al., 2013). These data

collectively suggest that GPRC6A is not necessary for the anorectic effects of L-Arg.HCl. However, the involvement of other amino acid-sensing receptors cannot be ruled out. L-arginine activates both T1R1-T1R3 and CaSR receptors, albeit to a lesser extent than GPRC6A (Wellendorph et al., 2009a), and therefore it is possible that the effects observed are mediated via these receptors. It has been demonstrated that T1R1-T1R3 responds to a range of amino acids, including L-arginine, and that the L-arginine response is enhanced in presence of inosine-5'-monophosphate (IMP), a known T1R1-T1R3 enhancer (Nelson et al., 2002). In addition L-arginine induced GLP-1 and PYY release was significantly dampened in presence of CaSR antagonist from isolated rat small intestinal loops, suggesting that the release is in part mediated by CaSR (Mace et al., 2012).

There may be other mechanisms involved in mediating the effects of L-Arg.HCl. Amino acid transporter systems may be involved in amino acid sensing in the gut. Recent evidence suggests that amino acid transporters have receptor-like properties, sensing the cellular amino acid availability. A recent study demonstrated that neutral amino acid transporter SNAT2, has transceptor activity and has been implicated in glutamine stimulated GLP-1 release from primary intestinal L-cells cultures (Tolhurst et al., 2011).

Evidence suggests that L-arginine stimulates the release of insulin from pancreatic beta-cells by causing membrane depolarization. Furthermore, this effect is not mediated by calcium or ATP sensitive potassium channels. It seems that L-arginine elevates calcium concentrations and hence causes membrane depolarization as a consequence of its electrogenic transport in the beta-cells via specific amino acid transporters (Smith et al., 1997).

There is evidence to suggest that the vagus nerve responds to nutrient load and is involved in protein-induced satiety (Darcel et al., 2005, Faipoux et al., 2008). Proteins and amino acids have shown to activate neurons within NTS via visceral vagus mediated signals. In addition, GLP-1R, Y2R and CCK receptor are expressed on vagal afferents and it is purposed that vagal afferents play a key role in gut-brain mediated responses in satiety and

appetite regulation (Tome et al., 2009). Works performed in our laboratory examined the role of gut hormones in mediating the effect of L-Arg.HCl on food intake. The anorectic effect of L-Arg.HCl appears not be mediated via the vagus as vagotomy had no effect on the anorectic property of L-Arg.HCl in rats (unpublished data).

L-arginine can influence hormone release from other endocrine tissues. It stimulates insulin secretion from the pancreatic islets (Adeghate et al., 2001) and growth hormone from the pituitary (Villalobos et al., 1997). In addition, oral administration of L-arginine stimulated insulin release via a GLP-1-dependent mechanism (Clemmensen et al., 2013b). I therefore investigated the role of anorectic gut hormones in mediating the effect of L-Arg.HCl on food intake. L-Arg.HCl stimulated the release of both GLP-1 and PYY from primary cultured murine colonic epithelium. Previous work performed in our laboratory confirmed that the concentrations of L-Arg.HCl tested had no cytotoxic effects on cultured cells. Furthermore, oral administration of L-Arg.HCl significantly elevated circulating GLP-1 levels in rats. Following establishing the effect of L-Arg.HCl in stimulating GLP-1 and PYY release, it was hypothesized that the anorectic effect of L-Arg.HCl may be mediated by its effect on gut hormone release. In order to investigate this hypothesis, the effect of co-antagonism of GLP-1 and Y2 receptor on food intake in mice was examined. GLP-1 and Y2 receptors were simultaneously blocked using available synthetic pharmacological antagonists at doses previously used to block the anorectic effects of exogenous exendin 4 and PYY_{3-36}. The co-antagonism of GLP-1 and Y2 receptor had no effect on the anorectic action of L-Arg.HCl, suggesting that the effects of L-Arg.HCl are not mediated via gut hormone release.

In addition, IP administration of L-Arg.HCl significantly reduced food intake in rats, suggesting a potential post absorptive mechanism is involved in mediating its anorectic effect. Further work is required to establish whether the anorectic effect of L-Arg.HCl is centrally mediated. A recent study by Jordi et al suggested that the anorectic effect of L-arginine is mediated centrally via AP. OG of L-arginine resulted in an increase in c-foc

positive cells in AP. Additionally, the anorectic effect of L-arginine was abolished in animals that had undergone AP lesioning surgery (Jordi et al., 2013).

I found that acute administration of L-Arg.HCl had no significant effect on oxygen consumption and carbon dioxide production in mice. It has been previously shown that dietary supplementation with L-arginine for 10 weeks increases energy expenditure in mice placed on a low protein diet (Clemmensen et al., 2012). Chronic L-arginine supplementation leads to increased skeletal muscle mass and reduced fat mass in obese and lean animals (Jobgen et al., 2009, Tan et al., 2009). These changes in body composition may result in an increased basal metabolic rate (Lazzer et al., 2010). However, the effects of L-arginine on rodents with a deficient protein intake might be expected to be different to those in rodents with a normal protein intake.

In addition, this study in the CLAMS demonstrated that oral gavage of 24 mmol/kg L-Arg.HCl significantly reduced food intake and appetite in mice when food was returned 8 hours post administration. This is interesting as it demonstrates a prolonged anorectic effect, that is not necessarily present if food is returned immediately, and suggests that there is some kind of mechanism for retaining the anorectic signalling initiated by L-arginine if it is not immediately acted upon. It also suggests that L-arginine can have long term effects on food intake in specific circumstances, which are more likely to be exploitable by weight loss promoting agents.

In summary, the findings presented in this chapter demonstrate the anorectic property of L-Arg.HCl in rodents. The exact mechanisms by which L-Arg.HCl exert its effect is still unclear. However, our data suggests that it is unlikely to be via GPRC6A. We also examined the role of anorectic gut hormones, and although observed an increase in gut hormone circulatory profile following administration, the effect is unlikely to be mediated by gut hormones. Further work is required to determine the mechanisms by which L-Arg.HCl reduces food

intake and whether delivery of L-Arg.HCl to specific regions of the GI tract may facilitate its utility as a potential anti-obesity agent.

Chapter III:

The Effect of L-Phenylalanine on Food Intake

3.1 Introduction

3.1.1 L-Phenylalanine

L-Phenylalanine (L-Phe) is a proteinogenic and essential amino acid that has a hydrophobic benzyl side chain that results in its classification as a non-polar amino acid (Fig. 3.1.1) (Young and Pellett, 1987). Phenylalanine is naturally found in breast milk of mammals. Other major sources include dietary intake from meat, dairy and eggs, and the endogenous recycling of amino acid stores in the body. L-Phe is utilised in protein synthesis, the oxidation of tyrosine, and as a substrate in other biochemical pathways (Williams et al., 2008).
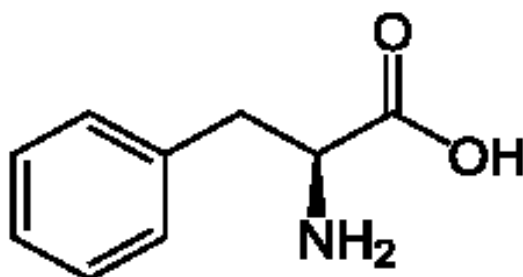
3.1.1 The chemical structure of L-phenylalanine
L-Phenylalanine is an essential non-polar amino acid which is consisted of a hydrophobic benzyl side chain.

Conversion of L-Phe to L-tyrosine is catalysed by the enzyme phenylalanine hydroxylase (PAH), and predominantly occurs in the liver (Lichter-Konecki et al., 1999). This conversion is biologically important: deficiencies of PAH result in a rare congenital condition called phenylketonuria (PKU), in which phenylalanine accumulates within the body, and which can result in brain damage (Williams et al., 2008).

PAH requires tetrahydrobiopterin (BH4) and oxygen as cofactors to breakdown the benzene side chain and ultimately convert L-Phe to L-tyrosine (Fig. 3.1.2). L-tyrosine is further metabolized to L-DOPA (L-3,4-dihydroxyphenylalanine), pyruvate, fumarate and phenol. L-

DOPA is used as a substrate in pathways leading to the synthesis of neurotransmitters including dopamine, noradrenaline and adrenaline (Wiggins et al., 2015).
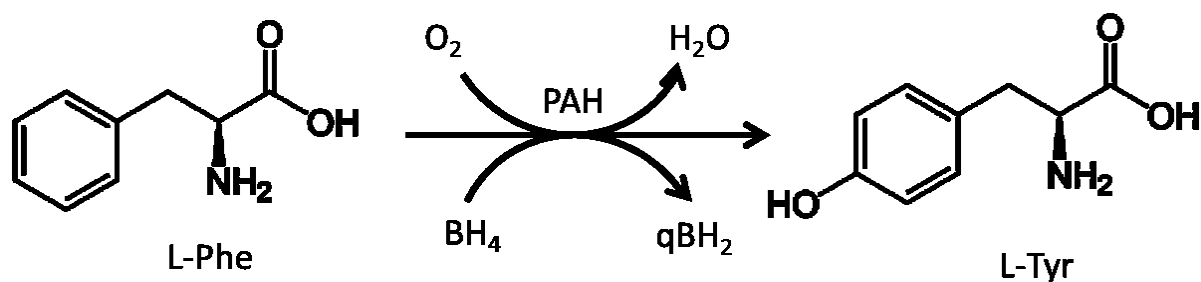
Figure 3.1.2 L-Phenylalanine metabolism in the body.
L-Phe is converted to L-tyrosine in a reaction that requires the activity of PAH, BH_4 and O_2. BH_4: tetrahydrobiopterin, qBH_2: quinonoid dihydrobiopterin.

3.1.2 CaSR

The CaSR is named for its well characterised role in the regulation of calcium homeostasis, but has a role in a number of cellular processes including gene expression, proliferation, differentiation and hormone secretion (Riccardi and Brown, 2010, Riccardi and Kemp, 2012, Alfadda et al., 2014). Calcium is the major activating ligand of the CaSR. However, physiologically, the CaSR responds to pH, and allosterically binds other divalent cations, di- and tri-peptides, aromatic L-amino acids, and less potently, aliphatic amino acids (Brown and MacLeod, 2001, Conigrave et al., 2002). The CaSR is widely expressed in various tissues throughout the body, where it occurs as a homodimer on the cell surface (Bai et al., 1998), and signals through both $G\alpha_q$ and $G\alpha_i$ or $G\alpha_s$ in a cell type specific manner (Handlogten et al., 2001).

CaSR is highly expressed in the gut and pancreas, and has been implicated in regulation of energy homeostasis (Alfadda et al., 2014). It is expressed in the exocrine pancreatic acinar and ductal cells, as well as on pancreatic alpha and beta cells, where it has shown to regulate the secretion of insulin and glucagon (Bruce et al., 1999, Squires et al., 2000). In the GI tract, CaSR has been reported to be expressed in enteroendocrine G-cells (Ray et al.,

1997), ghrelin-expressing cells (Engelstoft et al., 2013), I-cells (Liou et al., 2011), D-cells (Haid et al., 2012) and L-cells (Diakogiannaki et al., 2013).

3.1.3 CaSR and the GI tract

3.1.3.1 Stomach

Several studies have reported that the CasR is expressed in the stomach. It was first identified in the amphibian *Necturus maculosus* gastric mucosa and localised to the basolateral membrane of epithelial cells in the antrum and gastrin-secreting G-cells in the fundus (Cima et al., 1997). CaSR expression was later identified in the basolateral and, to a lesser extent, the apical membrane of human gastric mucosa epithelial cells (Ray et al., 1997). CaSR expression has been confirmed on both the basolateral and apical membrane on the G-cells in humans (Buchan et al., 2001), but only on the basolateral membrane of parietal cells in rats stomach (Cheng et al., 1999). Expression of CaSR on both the basolateral and apical membrane in G-cells would allow the receptor to respond to ligands present in both luminal content and circulation, whereas parietal cells would presumably only be able to respond to circulating ligands. Such ligands might include L-amino acids absorbed from the intestine, or released from various tissues (Conigrave and Brown, 2006).

CaSR activation stimulates the release of gastrin from the G-cells in the stomach. In addition, stimulation of the receptor on the basolateral membrane of the parietal cells increases acid secretion via activation of H^+, K^+-ATPase. Conversely, inhibition of the receptor is associated with a reduction in gastric acid secretion (Geibel et al., 2001). Furthermore, L-amino acids including L-Phe have been shown to stimulate gastric acid secretion in isolated rat stomach *ex vivo* preparations (Busque et al., 2005).

3.1.3.2 Small and large intestines

CaSR is expressed throughout the small intestine. It is localised on the basal membrane of epithelial cells of small intestinal villi and crypts. However, it is only expressed on the apical surface of the small intestinal epithelial cells (Chattopadhyay et al., 1998). It is expressed in

the duodenum, jejunum and ileum (Gama et al., 1997, Symonds et al., 2015), and on the basolateral and apical surfaces of crypt epithelial cells in the colon (Chattopadhyay et al., 1998, Cheng et al., 2002).

Within the colon, CaSR plays an important role in water and salt transport (Alfadda et al., 2014). In addition, CaSR expression may be associated with reduced risk of colon cancer. The receptor is highly expressed in healthy colon, but is absent in specimen from patients with colon cancer (Rogers et al., 2012).

The expression of CaSR on hormone secreting enteroendocrine cells has led to suggestions that it may regulate the release of gut hormones. As previously mentioned, aromatic amino acids are potent allosteric activators of the receptor. The aromatic amino acids L-Phe and L-tryptophan stimulate CCK secretion from isolated I-cells (Wang et al., 2011a). Similarly, L-Phe stimulates the release of CCK from STC-1 cells in a calcium-dependent manner (Hira et al., 2008). In addition, specific L-amino acids including L-Phe, L-tryptophan, L-arginine, L-glutamine and L-asparagine have been shown to stimulate the release of the energy and glucose homeostasis-regulating gut hormones PYY, GLP-1 and GIP from isolated rat small intestine through a CaSR-dependent mechanism (Mace et al., 2012). CaSR may thus mediate L-amino acid-induced hormone secretion in the GI tract to regulate food intake and metabolism.

3.1.4 Hypothesis and aims

Hypothesis: L-Phe reduces food intake in rodents by modulating the release of appetite-regulating gut hormones, and this effect is mediated via activation of the CaSR in the GI tract.

Aims: To investigate:

- The anorectic effect of L-Phe in rodents.
- The effect of L-Phe on energy expenditure in rats.

- The role of CaSR in mediating the anorectic effects of L-Phe

- The effect of L-Phe on gut hormone release *in vitro* and *in vivo*.

- The effect of chronic administration of L-Phe on body weight and food intake in diet-induced obese (DIO) mice.

3.2 Methods

3.2.1 Animals

Male C57BL/6 mice (Harlan, Bicester, Oxon, UK) with an average body weight of 18-20g (6-8 weeks old) and male Wistar rats (Charles River, Margate, Kent, UK) weighing 180-220g were individually housed under controlled temperature (21-23ºC) and light cycle (12 hours light, 12 hours dark) with *ad libitum* access to standard chow diet (RM1, Special Diet Services Ltd, Witham, Essex, UK) and water, unless otherwise stated.

All animals were randomised according to their body weight for all the studies. Animals were handled and acclimatised to the procedures prior to commencement of studies.

All animal procedures were approved and carried out according to the British Home Office Animals Scientific Procedures Act 1986 (project License: 70/8068).

3.2.2 The effect of OG administration of L-Phe on food intake in fasted rats during the early light phase

Rats were fasted overnight for 16 hours and were subsequently orally gavaged with water or 3 mmol/kg L-Phe (Sigma, Poole, UK) during early hours of light cycle. Animals were immediately returned to their cages and were given pre-weighed standard chow diet. Food intake was monitored at 1, 2, 4, 8 and 24 hours following administration.

3.2.3 The effect of OG administration of L-Phe on food intake in fasted mice during the early light phase

Mice were fasted overnight for 16 hours and were subsequently orally gavaged with vehicle (10% TWEEN20, water) or 12 mmol/Kg L-Phe. Mice were returned to their cages and were provided with pre-weighed standard chow. Food intake was monitored at 1, 2, 4, 8 and 24 hours following administration.

3.2.4 The effect of OG administration of L-Phe on food intake in *ad libitum* fed mice during the early dark phase

Ad libitum fed mice were orally gavaged with vehicle (10% TWEEN20, water), 6 or 12 mmol/kg L-Phe (Sigma, UK) at the beginning of dark phase. Animals were returned to their cages and food intake was recorded at 1, 2, 4, 8 and 24 hours post administration.

3.2.5 The effect of OG administration of L-Phe on food intake in *ad libitum* fed DIO mice during the early dark phase

Mice, 6-8 weeks old, were given *ad libitum* access to 60% high fat diet (HFD) (Research Diets, New Brunswick, USA) for 8 weeks. Animals were *ad libitum* fed prior to the study and were orally gavaged with vehicle (10% TWEEN20, water), 6 or 12 mmol/kg L-Phe at the beginning of dark phase. Animals were returned to their cages and food intake was recorded at 1, 2, 4, 8 and 24 hours post administration.

3.2.6 The effect of OG administration of L-Phe on food intake and energy expenditure in fasted rats during the early light phase

Rats were fasted for 16 hours overnight and were subsequently orally gavaged with water or 3 mmol/kg L-Phe at 0900 hours (early light phase). Animals were then individually placed in CLAMS cages. Food was provided immediately following administration. Energy expenditure and food intake were measured using CLAMS cages as previously described in section 2.2.5.

3.2.7 The role of gut hormones in mediating the anorectic effect of L-Phe

3.2.7.1 The effect of L-Phe on GLP-1 and PYY release from a murine primary L-cell culture

The mice colonic culture and secretion experiments were performed according to a previously described method (refer to 2.2.6.1 and 2.2.6.2). L-cells were incubated with 1, 10,

50 and 100mM of L-Phe for 2 hours; GLP-1 and PYY release measured using specific in-house GLP-1 and PYY RIAs (refer to 2.2.6.3 and 2.2.6.4).

3.2.7.2 The effect of L-Phe on GLP-1 release from STC-1 cells

3.2.7.2.1 Culture of STC-1 cells

STC-1 cells were maintained and grown in DMEM (with 4.5g/L D-glucose, L-glutamine and pyruvate) (Gibco, Life Technologies, CA, USA) + 10% foetal bovine serum + 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured at 37°C in 95% O_2/5% CO_2 and were passaged every 2–3 days. All studies were performed in cells on typically 20-50 passages. Prior to seeding or passage, cells were detached by addition of versene (refer to appendix I) followed by 0.05% trypsin (Gibco, Life technologies, CA, USA). Cells were then centrifuged for 5 minutes at 300g and the resulting pellet resuspended in fresh media and transferred into new containers accordingly (Hira et al., 2008).

3.2.7.2.2 Secretion assays

STC-1 cells were plated in 24 well plates at a seeding density of 5 x 10^5 cells per well. Cells were then incubated for 24 hours to reach 90% confluency. The secretion experiments were performed according to a previously described method (refer to 2.2.6.2).

3.2.7.2.3 Lactate dehydrogenase (LDH) cytotoxicity assay

A Cytoscan LDH cytotoxicity assay (G-Biosciences, MO. USA) was used to test STC-1 cell health following treatment of cells with various concentrations of L-Phe. The assay measures the levels of lactose dehydrogenase, a stable cystosolic enzyme which is released upon cell death and lysis, via a coupled enzymatic reaction utilizing a red coloured reagent, formazan. The assay was performed according to the manufacturer's instruction. STC-1 cells were treated with 30, 50 and 100mM L-phe and 500mM potassium hydroxide (KOH) (positive control) for a period of 2 hours, and absorbance at 490nm was measured using a micro-plate reader (BioTek Model No. ELx808, VT, USA). The results were expressed as

percentage cytoxicity relative to the maximal LDH release from the lysed wells according to the manufacture's instruction and the following formula (Broussas et al., 2013):

$$\% \, Cytotoxicity = \frac{Experimental \, OD_{490} - Spontaneous \, OD_{490}}{Maximum \, LDH \, release \, OD_{490}} \times 100$$

3.2.7.3 The effect of OG administration of L-Phe on gut hormone release in fasted rats during the early light phase

Rats were fasted overnight and received an oral gavage of either water or 3 mmol/kg L-Phe and were immediately returned to their cages. Animals were killed by decapitation at 30 minutes following administration. For GLP-1 and PYY analysis, trunk blood was collected in lithium heparin coated tubes (Teklab, County Durham, UK) containing aprotinin (Nordic Pharma, Reading, UK) at 200 kallikrein inhibitor units per ml of blood. Blood was then centrifuged for 10 minutes at 6000g and plasma was separated, aliquoted and stored at -80°C until analysis. One hundred microliters of plasma was used in RIA assays to measure GLP-1 and PYY concentrations as described previously in 2.2.6.

Acylated ghrelin analysis was carried out using a commercial rat/mouse active ghrelin ELISA kit (Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Trunk blood was collected in ethylenediaminetetraacetic acid (EDTA) coated tubes (SARSTEDT, Nümbrecht, Germany) containing 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma, Poole, UK) (1mg/ml, final concentration). Blood was then centrifuged for 10 minutes at 6000g and plasma was separated and transferred to a fresh tube containing 0.05M final concentration of hydrochloric acid, before being stored at -80°C until analysis.

3.2.8 The role of CaSR in mediating the anorectic effect of L-Phe

3.2.8.1 CaSR expression in the GI tract

A cohort of WT Male C57BL/6 mice fed RM1 standard chow diet (RM1 diet; Special Diet Services Ltd., Witham, Essex, UK) were fasted for 16 hours overnight to avoid any effects of acute food intake on gene expression. Mice were then decapitated and stomach, duodenum, jejunum, ileum and colon rapidly removed, snap frozen in liquid nitrogen and stored at -80°C for RNA extraction and qPCR. Total RNA was extracted from GI tissues using TRI reagent (Sigma, Poole, UK) in accordance with the manufacturer's instructions. Extracted RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was used at a concentration of 1µg/10µl in triplicates reverse transcription reactions to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Life technologies, Paisley, UK) and according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed using 1µl of cDNA template in TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Life technologies, Paisley, UK) using the ABI Prism 7900 Sequence Detection System according to the protocols provided by the manufacturer (Life technologies, Paisley, UK). The relative mRNA transcript levels were calculated according to the $2^{-\Delta CT}$ method, with ΔCT being the difference in cycle threshold values between CaSR mRNA (mCG130161), and the hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA (m00446968_m1) internal control.

3.2.8.2 The effect of a CaSR agonist on food intake in fasted rats during the early light phase

Rats were fasted overnight for 16 hours and then received an oral gavage of vehicle (1.8% DMSO, water), or 1.1 or 11.2 mg/kg of R568.HCl (TOCRIS Bioscience, Bristol, UK), a synthetic CaSR agonist, during the early light phase. R568.HCl pharmacological doses used in these studies were previously shown not to effect plasma calcium or parathyroid hormone levels (Murakami et al., 2000). Rats were returned to their cages following administration

and were provided with pre-weighed standard chow. Food intake was monitored at 1, 2, 4, 8 and 24 hours following administration.

3.2.8.3 The effect of a CaSR antagonist on the anorectic effects of L-Phe in rats

3.2.8.3.1 Ileal cannulated rats

Previous data suggested that L-Phe might influence food intake by acting on the CaSR in the small intestine. PYY and GLP-1 are relatively highly co-expressed in the ileum, and CaSR is also expressed on L cells in this region (Symonds et al., 2015). Given the difficulty in matching the absorption characteristics and pharmacokinetics of orally administered L-Phe and a CaSR antagonist, it was decided to examine the effects of blocking the effects of L-Phe on food intake following direct administration into the ileum. Male Wistar rats weighing 250-300g were implanted with a catheter into the ileum, approximately 5cm from the ileocaecal junction. The catheter was tunnelled to the scapular region and secured in place using a vascular access harness (Instech Laboratories Inc, PA, USA). The surgery was performed by Charles River (UK) and animals were transferred to Imperial College prior to experimentation. The catheter was flushed and locked with 0.5ml 0.9% saline (Fresenius Kabi Ltd, Manor Pk, UK). The catheter was accessible via an access port using a 22G stainless steel blunt needle (Covidien, MN, USA). Animals were maintained under conditions previously described in 3.2.1.

3.2.8.3.2 The effect of CaSR antagonism on the anorectic effects of L-Phe following direct administration into the ileum of *ad libitum* fed rats in the early dark phase

Rats with *ad libitum* access to food were injected directly into the ileum via the catheter access port at the beginning of dark cycle. Rats were injected with vehicle (0.5% DMSO, 0.9% saline), 1µM of the CaSR antagonist NPS2143.HCl (TOCRIS Bioscience, Bristol, UK), 10mM L-Phe or 10mM L-Phe in combination with 1µM NPS2143.HCl, in a total injection volume of 0.5ml. Animals were immediately returned to their cages and food intake was measured at 30 minutes, 1, 2, 4, 8 and 24 hours post administration.

3.2.8.4 The effect of CaSR antagonism on GLP-1 release from STC-1 cells

STC-1 cells were seeded and incubated as described above (section 3.2.7.2) before treatment with vehicle control, 10µM of the CaSR antagonist NPS2143.HCl, 50 mM L-Phe, or 50mM L-Phe with 10µM NPS2143.HCl for 2 hours. GLP-1 release was measured using an in-house GLP-1 RIA.

3.2.9 The effects of chronic administration of L-Phe in DIO mice

Male C57BL/6 mice 6-8 weeks of age were grouped-housed (n = 5/cage) and were given *ad libitum* access to water and 60% HFD (Research Diets, New Brunswick, USA) for a period of 8 weeks. Animals were then individually housed and were given one week to acclimatise to the new housing condition prior to start of experiments.

Mice were orally gavaged with vehicle (10% TWEEN20, water) or 12 mmol/kg L-Phe twice daily throughout the dark phase at 1900 hours and then 0100 hours for 4 nights. The gavage number was then increased to three times for the 3 subsequent nights, injecting at 1900 hours, 2300 hours and 0300 hours.

3.2.9.1 The effect of repeated OG administration of L-Phe on food intake and body weight in mice

Body weight was recorded at day 0 prior to administration and again at 24 hours and onset of dark phase. Food intake was measured daily at the beginning of dark phase and at one hour following the first daily gavage.

3.2.9.2 The effect of repeated OG administration of L-Phe on plasma leptin levels in mice

Mice were decapitated and trunk blood was collected in heparin coated tubes. The plasma was separated following centrifugation of blood samples as described previously. Leptin levels were determined using a mouse leptin ELISA kit (Millipore, Darmstadt, Germany). The assay was performed according to the manufacturer's instructions.

3.2.9.3 The effect of repeated OG administration of L-Phe on plasma gut hormone levels in mice

Acidified plasma samples were used to measure acylated ghrelin levels in plasma. Plasma collection and ghrelin measurements were performed using mouse/rat active ghrelin ELISA kit (Millipore, Darmstadt, Germany) and according to manufacturer's instruction as previousley described in section 3.2.7.3. GLP-1 and PYY plasma levels were measured using RIA as previously described in section 3.2.7.3.

3.2.10 Statistical analysis

All data are expressed as mean ± SEM. All feeding studies were analysed using T-test or one-way ANOVA with Tukey's *post hoc* test where appropriate. Energy expenditure and cumulative food intake data were analysed using two-way ANOVA with Bonferroni's *post hoc* test. All *in vitro* studies were analysed using one-way ANOVA with Dunnett's test with exception of secretion study using CaSR antagonist in STC-1 cells where one-way ANOVA and Tukey's *post hoc* test were applied. Gut hormone studies *in vivo* were analysed using T-test. CasR expression study was analysed using one-way ANOVA with Tukey's *post hoc* test. Chronic studies where analysed using two-ANOVA with Sidak's multiple comparison test. P<0.05 was considered statistically significant. All analysis was carried out using Graphpad Prism software (Prism 6.03, GraphPad Software Inc, CA, USA).

3.3 Results

3.3.1 The effect of OG administration of L-Phe on food intake in fasted rats during the early light phase

Oral gavage of 3 mmol/kg L-Phe significantly decreased food intake in rats compared to the water control at 0-1 hour interval following administration (water: 6.87 ± 0.39g vs. L-Phe 3 mmol/kg: 4.78 ± 0.33g, $p<0.001$, n=12-13) (Fig. 3.1.1 A). The food intake was significantly higher in L-Phe treated rats at 1-2 hour post administration (control: 2.46 ± 0.41g vs. L-phe 3 mmol/kg: 4.28 ± 0.38g, $p<0.01$, n=12-13) (Fig. 3.1.1 B). There was a significant reduction in food intake in the L-Phe treated cohort at the 2-4 hour time interval compared to water cohort (control: 3.12 ± 0.61g vs. L-Phe 3 mmol/kg: 1.29 ± 0.44g, $p<0.05$, n=12-13) (Fig. 3.3.1 C). The cumulative food intake at 4 hours post administration was significantly lower in rats treated with L-Phe (control 12.45 ± 0.66g vs. L-phe 3 mmol/kg: 10.35 ± 0.48g, $p<0.05$, n=12-13) (Fig. 3.3.1 D). This was followed by a significant increase in food intake in L-Phe group at 4-8 hour time period post administration (control 4.08 ± 0.64g vs. L-phe 3 mmol/kg: 7.12 ± 0.65g, $p<0.01$, n=12-13) (Fig. 3.3.1 E). There were no significant differences in food intake between groups at any other time point at which it was measured.
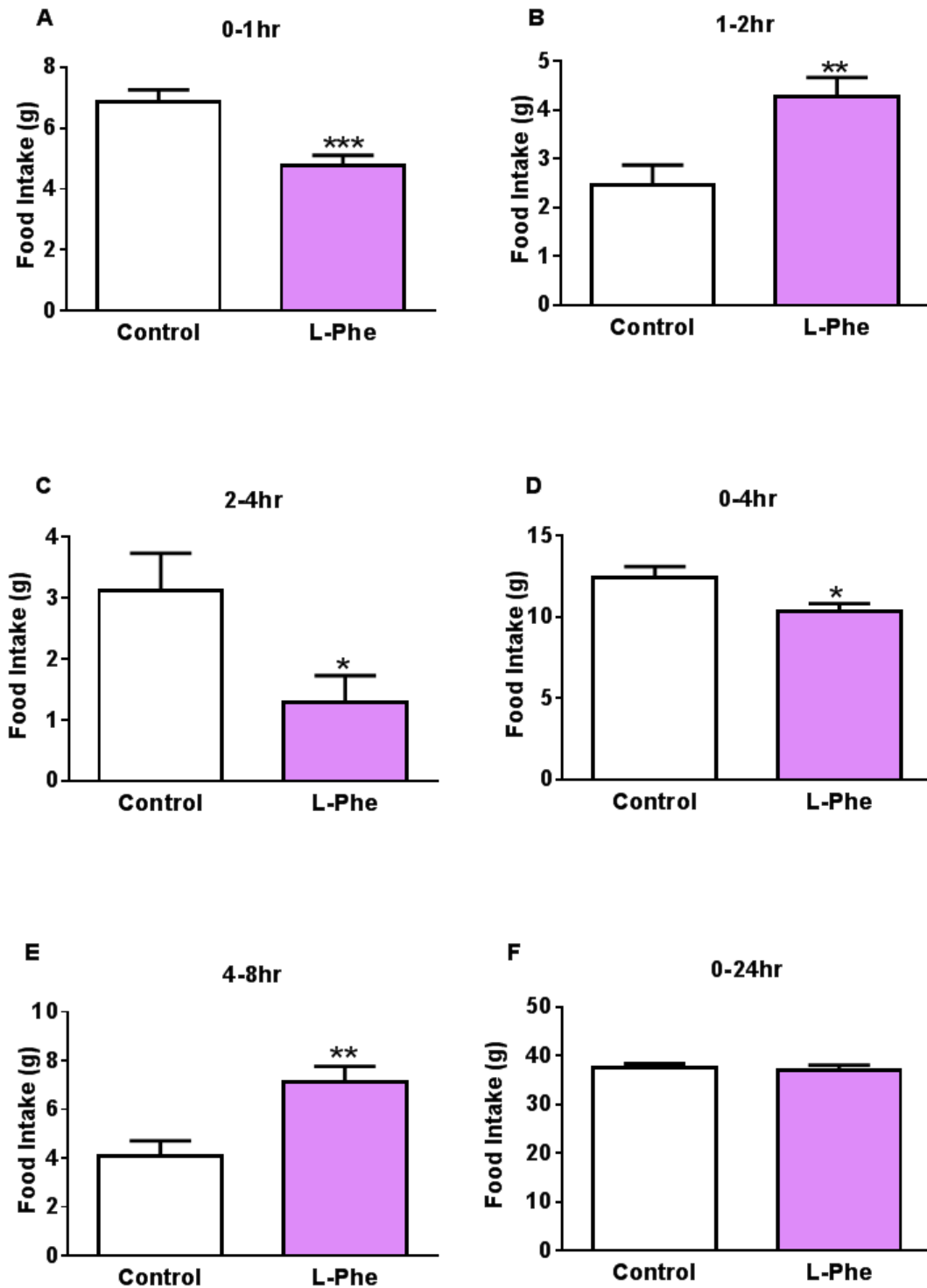
Figure 3.3.1 The effect of OG administration of L-Phe on food intake in fasted rats during the early light phase.
The effect of OG of control (water) or 3 mmol/kg L-Phe on food intake in rats following an overnight fast during 0-1 (A), 1-2 (B), 2-4 (C), 0-4 (D), 4-8 (E) and 0-24 (F) hour following administration. Data presented as mean ± SEM. n=12-13. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs water control.

3.3.2 The effect of OG administration of L-Phe on food intake in fasted mice during the early light phase

Oral gavage of 12 mmol/kg L-Phe in fasted mice significantly reduced food intake at 0-1 hour following administration compared to the vehicle (10% TWEEN20, water) group (Vehicle: 0.79 ± 0.072g vs. 12 mmol/kg L-Phe: 0.21 ± 0.066g, p<0.001, n=5 per group) (Fig. 3.3.2 A). The effect remained significantly different at 0-2 hour time interval (Vehicle: 0.92 ± 0.070g vs. 12 mmol/kg L-Phe: 0.35 ± 0.12g, p<0.01, n=5 per group) (Fig. 3.3.2 C). There were no significant differences in food intake between groups at any other time point at which it was measured.

Figure 3.3.2 The effect of OG administration of L-Phe on food intake in fasted mice during the early light phase.
The effect of oral gavage of vehicle (10% TWEEN20, water) or 12 mmol/kg L-Phe on food intake in overnight fasted mice during the early hours of light phase at 0-1 (A), 1-2 (B), 0-2 (C), 4-8 (D), 8-24 (E) and 0-24 (F) hours post administration. Data presented as mean ± SEM. n= 5 per group. **p<0.01, ***p<0.001 vs vehicle control.

3.3.3 The effect of OG administration of L-Phe on food intake in *ad libitum* fed mice during the early dark phase

Oral gavage of 6 mmol/kg L-phe significantly reduced food intake in mice at 0-1 post administration (Vehicle: 0.35 ± 0.04g vs. 6 mmol/kg L-Phe: 0.22 ± 0.03g, $p<0.05$ vs. 12 mmol/kg L-Phe: 0.23 ± 0.04g, n=10 per group) (Fig. 3.3.3 A). Food intake was significantly lower in animals treated with 12 mmol/kg at 1-2 hour time period post administration (Vehicle: 0.38 ± 0.03g vs. 12 mmpl/kg L-Phe: 0.20 ± 0.04g $p<0.01$; 6 mmol/kg L-Phe: 0.34 ± 0.04g vs 12 mmpl/kg L-Phe: 0.20 ± 0.04g $p<0.05$, n=10 per group) (Fig. 3.3.3 B) leading to a significant reduction in cumulative food intake at 2 hours in mice treated with higher dose of 12 mmol/kg compared to the vehicle group (Vehicle: 0.73 ± 0.05g vs. 12 mmol/kg L-Phe: 0.43 ± 0.07g, $p<0.01$, n=10 per group) (Fig. 3.3.3 C). There were no significant differences in food intake between groups at any other time point at which it was measured.
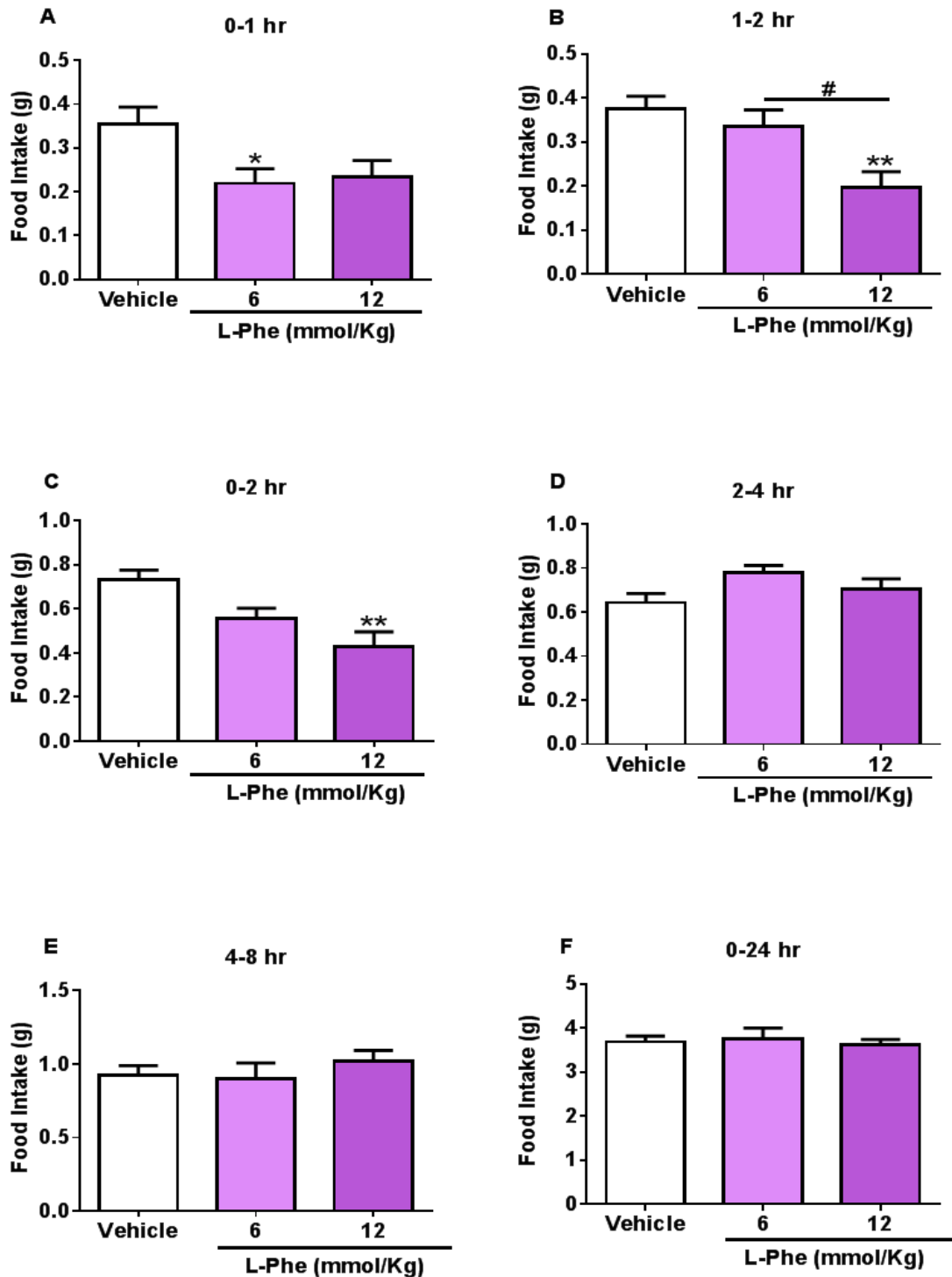
Figure 3.3.3 The effect of OG administration of L-Phe on food intake in *ad libitum* fed mice during the early dark phase.
The effect of OG administration of vehicle (10% TWEEN20, water), 6 mmol/kg or 12 mmol/kg L-Phe on food intake in mice with *ad libitum* access to food injected at the beginning of dark phase at 0-1 (A), 1-2 (B), 0-2 (C), 2-4 (D), 4-8 (E) and 0-24 (F) hour post administration. Data expressed as mean ± SEM. n = 10 per group. *$p<0.05$, **$p<0.01$ vs vehicle; #<0.05 vs 6mmol/kg L-Phe.

3.3.4 The effect of OG administration of L-Phe on food intake in *ad libitum* fed DIO mice during the early dark phase

Oral gavage of 12 mmol/kg L-Phe reduced food intake in DIO mice during 0-1 hour period post administration (Vehicle: 0.19 ± 0.05g vs. 6 mmol/kg L-Phe: 0.12 ± 0.03g, $p > 0.05$ vs. 12 mmol/kg L-Phe: 0.07 ± 0.02g, $p < 0.01$, n=10 per group) (Fig. 3.3.4 A). There were no significant differences in food intake between groups at any other time point at which it was measured. Cumulative food intake remained lower in the 12 mmol/kg L-Phe group both at 0-2 and 0-4 hour post administration, but this effect did not reach statistical significance (Fig. 3.3.4 C and Fig. 3.3.4 D).
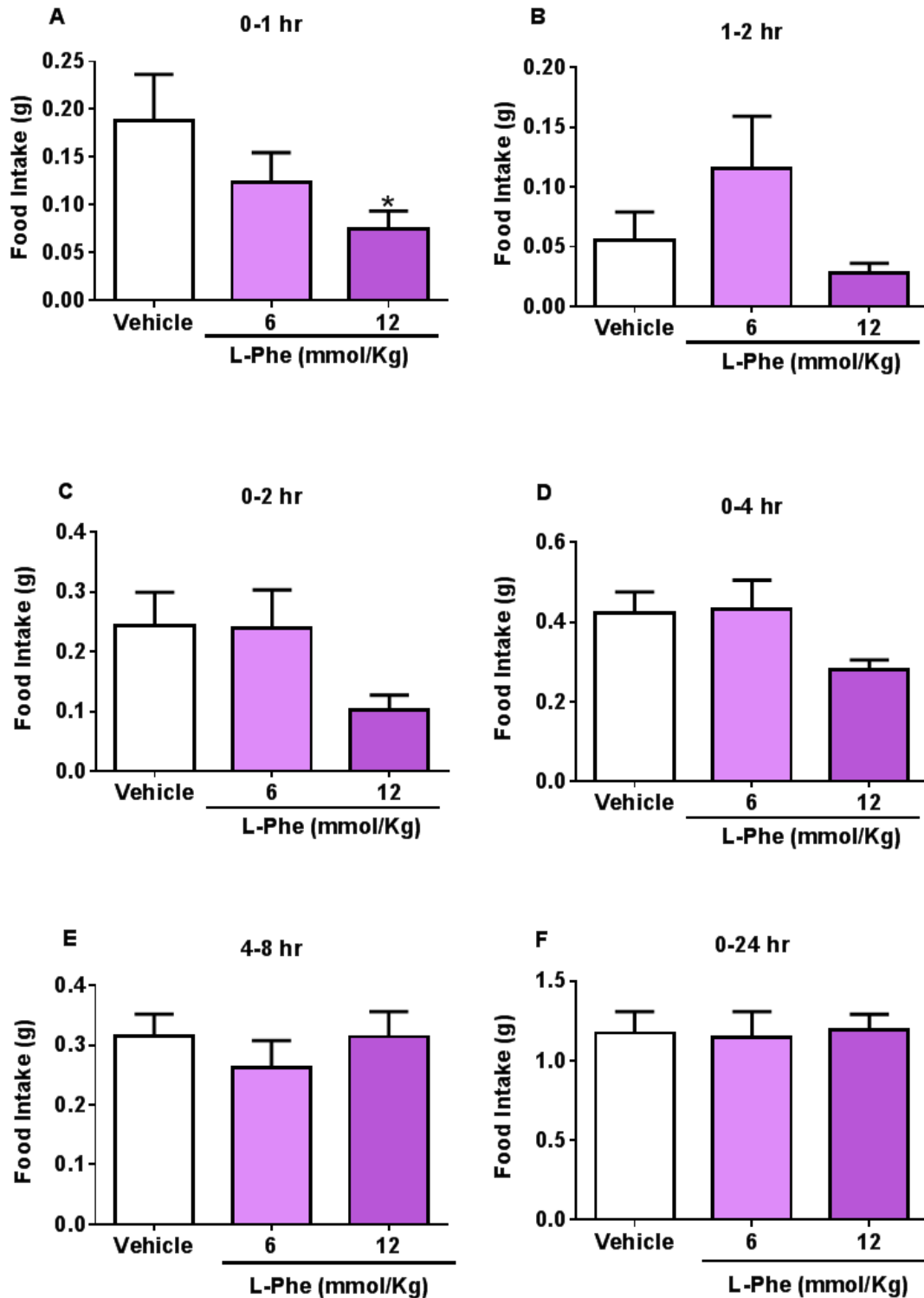
Figure 3.3.4 The effect of OG of L-Phe on food intake in *ad libitum* fed DIO mice during the early dark phase.
The effect of OG administration of vehicle (10% TWEEN20, water), 6 mmol/kg or 12 mmol/kg L-Phe on food intake in DIO mice with *ad libitum* access to 60% HFD injected at the beginning of dark phase at 0-1 (A), 1-2 (B), 0-2 (C), 0-4 (D), 4-8 (E) and 0-24 (F) hour post administration. Data expressed as mean ± SEM. n = 10 per group. *p<0.05 vs vehicle.

3.3.5 The role of gut hormones in mediating the anorectic effect of L-Phe

3.3.5.1 The effect of L-Phe on GLP-1 and PYY release from a murine primary L-cell culture

L-Phe significantly stimulated the release of GLP-1 from the primary L-cell culture (control: 4.54 ± 0.87 pmol/L vs. 1mM L-Phe: 7.21 ± 1.28 pmol/L vs. 10mM L-Phe: 10.46 ± 2.02 pmol/L, $p<0.05$. vs. 100 mM L-Phe: 12.27 ± 2.28 pmol/L, $p<0.01$, n=2 plates in triplicates) (Fig. 3.3.5 A).

Similarly, in a separate secretion experiment, L-Phe significantly stimulated the release of PYY at 50mM (control: 15.24 ± 4.28 pmol/L vs. 50 mM L-Phe: 46.46 ± 9.92 pmol/L, $p<0.05$, n=2 plates in duplicates) and 100 mM concentrations (control: 15.24 ± 4.28 pmol/L vs. 100 mM L-Phe: 43.45 ± 6.00 pmol/L, $p<0.05$, n=2 plates in duplicates) (Fig. 3.3.5 B).
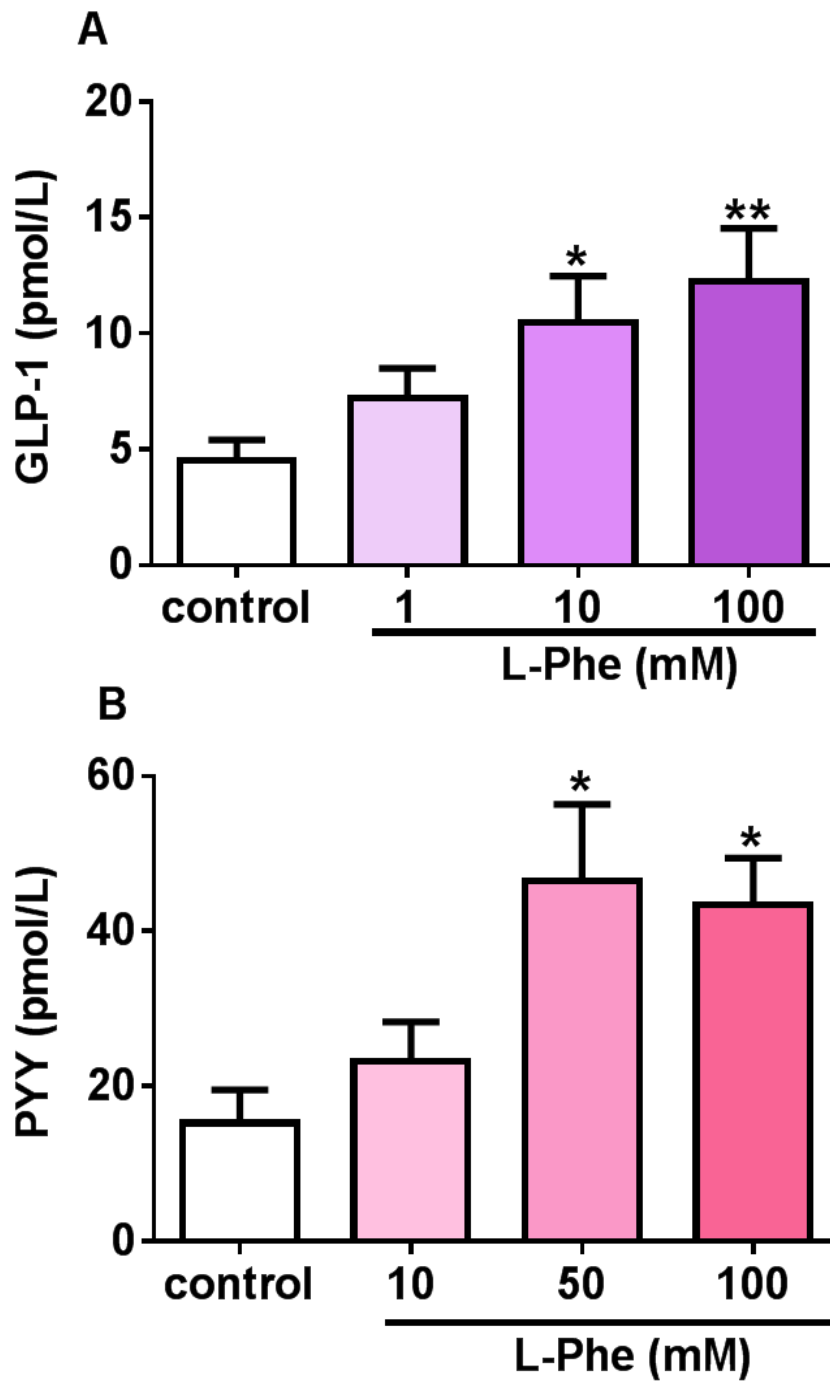
Figure 3.3.5 The effect of L-Phe on GLP-1 and PYY release from primary mice colonic L-cells culture.
The effect of L-Phe on GLP-1 release from primary mice colonic L-cells incubated with 1, 10 and 100 mM L-Phe for 2 hours. n = 2 in triplicate plates from 6 mice (A). The effect of L-Phe on PYY release from primary mice colonic L-cells incubated with 10, 50 and 100 mM L-Phe for 2 hours. n = 2 in duplicates from 4 mice. Data presented as mean ± SEM. *p<0.05, **p<0.01 vs control

3.3.5.2 The effect of L-Phe on GLP-1 release from STC-1 cells

L-Phe significantly stimulated the release of GLP-1 from STC-1 cells. Thirty mM L-Phe increased GLP-1 release by approximately 9 fold compared to the baseline control (control: 1.51 ± 0.68 pmol/L vs. 30 mM L-Phe: 9.47 ± 1.75 pmol/L, $p<0.01$, n=3 plates in triplicates). Similarly 50 and 100mM L-Phe significantly stimulated GLP-1 release compared to the baseline control (control: 1.51 ± 0.68 pmol/L vs. 50 mM L-Phe: 11.88 ± 2.08 pmol/L, $p<0.001$. vs. 100 mM L-Phe: 18.89 ± 1.35 pmol/L, $p<0.001$, n=3 plates in triplicates) (Fig. 3.3.6).
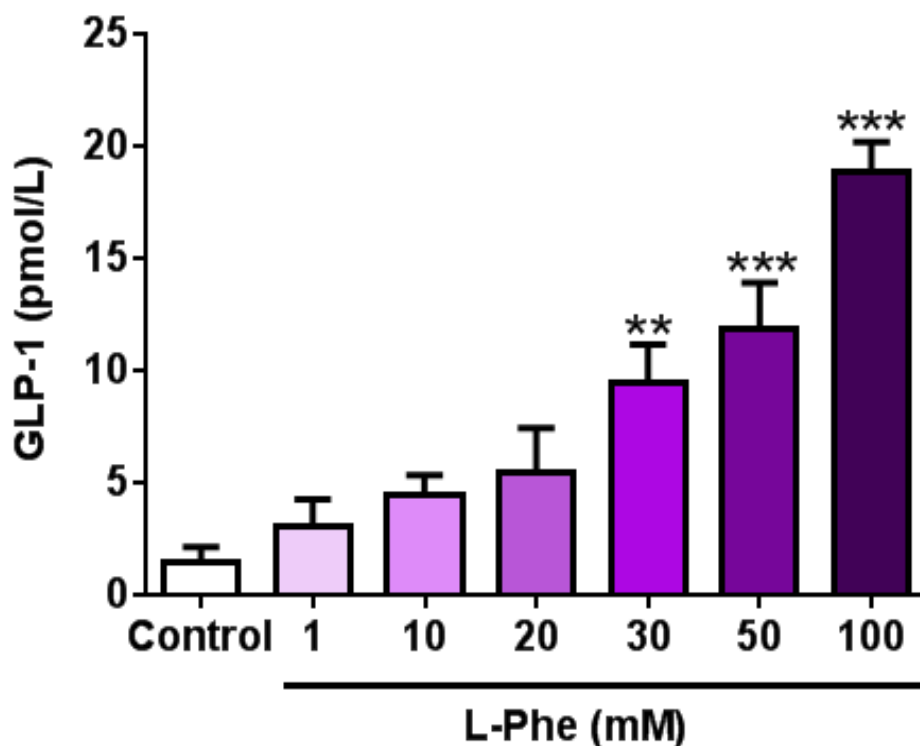
Figure 3.3.6 The effect of L-Phe on GLP-1 release from STC-1 cells.
The effect of 1, 10, 20, 30, 50 and 100 mM L-Phe on GLP-1 release from STC-1 cells. Cells were incubated with each treatment for 2 hours. n = 3 plates in triplicates. Data presented as mean ± SEM. *$p<0.05$, **$p<0.01$, p***<0.001 vs control.

3.3.5.2.1 The effect of L-Phe on cytotoxicity in STC-1 cells

The effect of L-Phe on cell health was determined using an LDH assay. LDH release following incubating the cells with increasing doses of L-Phe was measured and expressed as a percentage of maximum LDH release in lysate wells. Potassium chloride (KCl) at a dose of 500mM was used as positive control, and resulted in a significant increase in percentage cytotoxicity ($35.40 \pm 6.78\%$, $p<0.01$, n=4-8 wells) compared to the spontaneous cytotoxicity in the control group. L-Phe had no significant effect on cytotoxicity at any concentration tested (30 mM: $0.35 \pm 0.84\%$, 50 mM: $1.04 \pm 0.77\%$ and 100 mM: $3.23 \pm 0.74\%$) (Fig. 3.3.7).
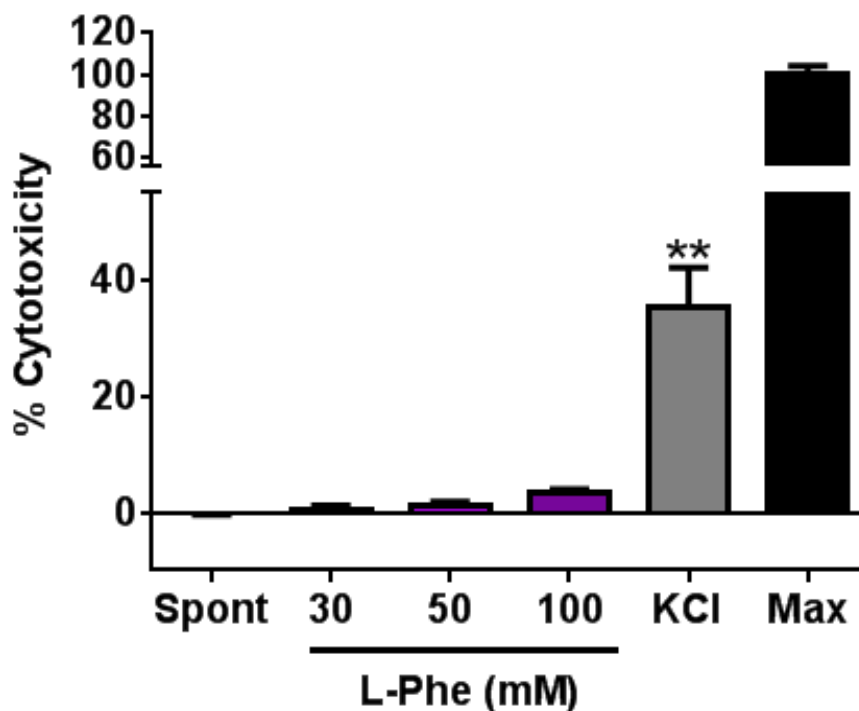
Figure 3.3.7 The effect of L-Phe on cytotoxicity in STC-1 cells.
The effect of 30, 50, 100 mM L-Phe and 500 mM KCl on cytotoxicity in STC-1 cells following 2 hours incubation. Cytotoxicity is expressed relative to spontaneous cytotoxicity (Spont) and as a percentage of maximum cytotoxicity from lysed wells (Max). Data expressed as mean ± SEM, n = 4 wells for L-Phe doses and n = 8 wells for KCl. **p<0.01 vs Spont.

3.3.5.3 The effect of OG administration of L-Phe on gut hormone release in fasted rats in the early light phase

Oral gavage of 3 mmol/kg of L-Phe significantly increased plasma GLP-1 levels (control: 24.48 ± 1.86 pmol/L vs. 3mmol/kg L-Phe: 36.94 ± 2.94 pmol/L, $p < 0.01$, n=11 per group) (Fig. 3.3.8 A) but not PYY levels 30 minutes post administration (Fig 3.3.8 B). Plasma acylated ghrelin levels were significantly lower following oral L-Phe administration compared to the water control group (control: 424.8 ± 55.89 pg/ml vs. 3mmol/kg L-Phe: 278.0 ± 32.10 pg/ml, $p < 0.05$, n=11 per group) (Fig. 3.3.8 C).
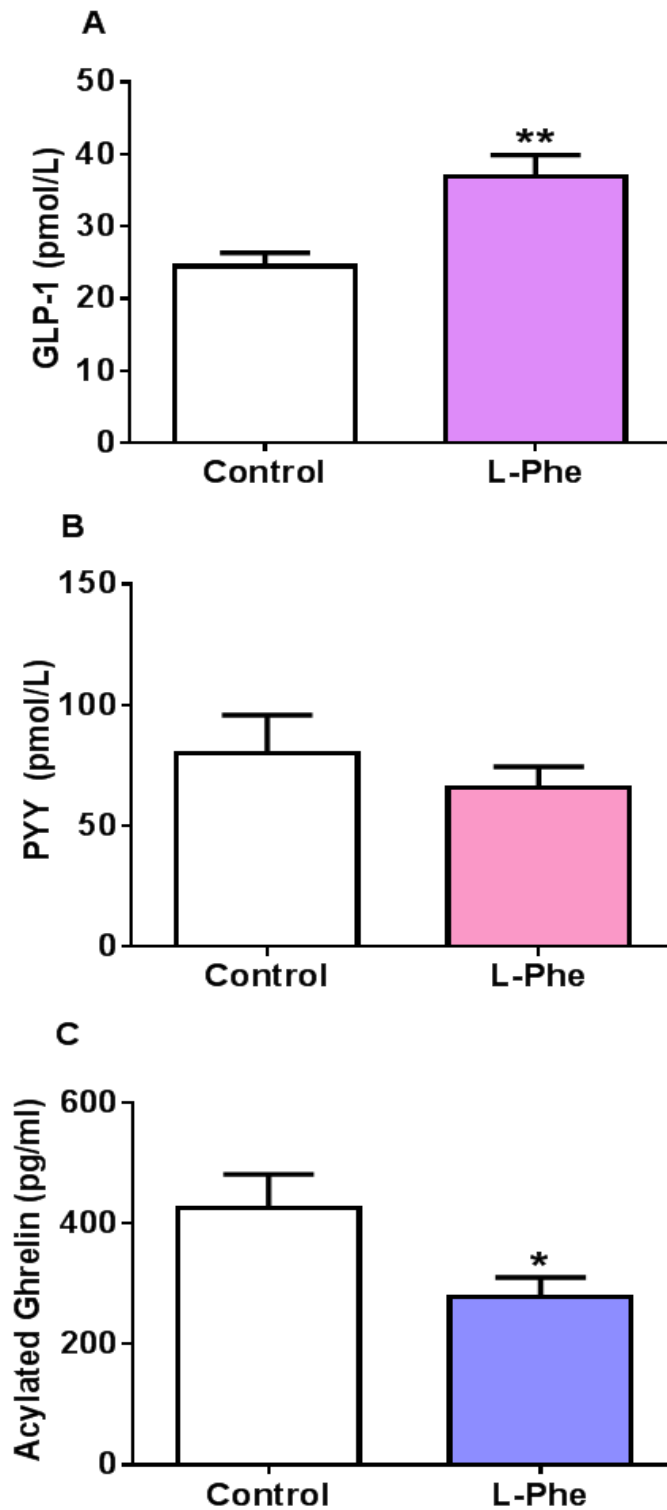
Figure 3.3.8 The effect of L-Phe on gut hormone release in fasted rats in the early light phase.
The effect of oral gavage of water (control) or 3 mmol/kg L-Phe on plasma GLP-1 (A), PYY (B) and acylated ghrelin (C) levels 30 minutes after administration in rats following an overnight fast. n = 11. Data expressed as mean ± SEM. *$p<0.05$, **$p<0.01$ vs control.

3.3.6 The role of CaSR in mediating the anorectic effect of L-Phe

3.3.6.1 CaSR expression in the GI tract

CaSR expression was detected in all regions of the GI tract investigated. There was no significant difference in the expression levels between different regions (Fig. 3.3.9).
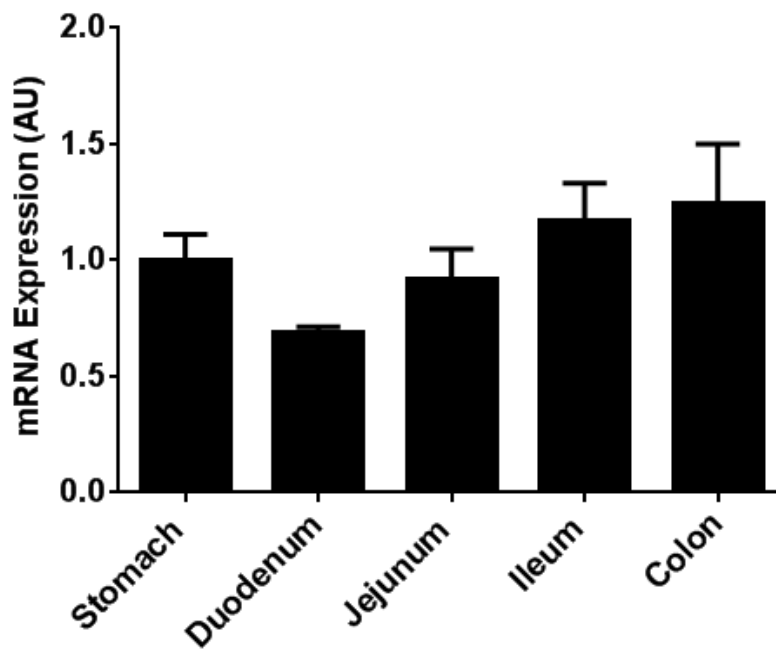
Figure 3.3.9 Expression of CaSR in the mouse GI tract.
Relative expression of CaSR mRNA in different regions of the GI tract in overnight fasted male mice. The expression levels are relative to stomach expression. Data is presented as mean ± SEM. n= 4-6 per region.

3.3.6.2 The effect of OG administration of a CaSR agonist on food intake in fasted rats during the early light phase

Oral administration of 1.1 mg/kg of R568.HCl, a synthetic CaSR agonist, significantly reduced food intake compared to the vehicle (vehicle: 8.33 ± 0.47g vs. 1.1 mg/kg R568.HCl: 6.48 ± 0.61g, p<0.05, n=8-9) (Fig. 3.3.10 A). The food intake was also reduced with higher dose of 11.2 mg/kg of the agonist during 0-1 hour time interval compared to the vehicle, but this effect did not reach statistical significance (p>0.05). There were no significant differences in food intake between groups at any other time point at which it was measured.
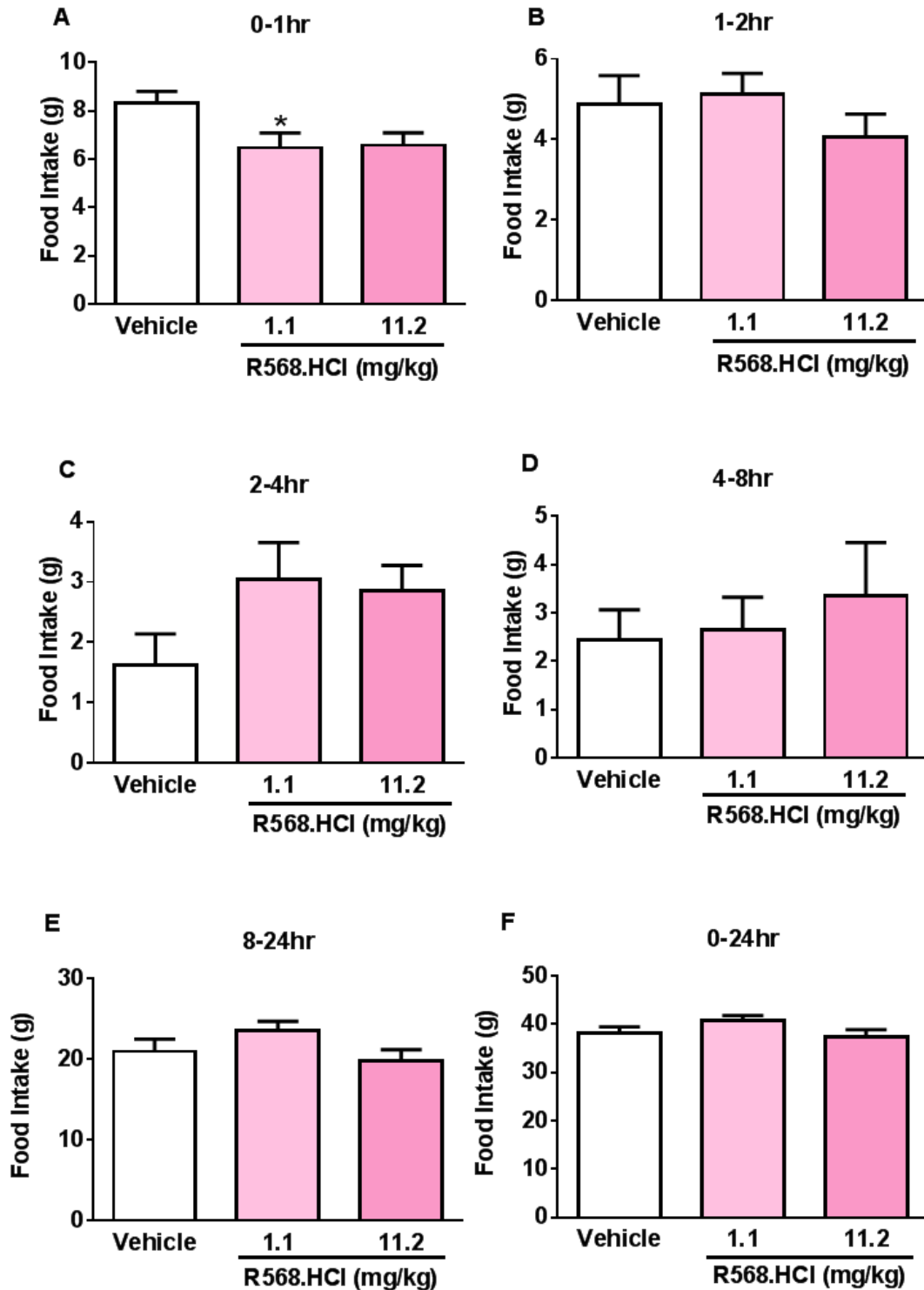
Figure 3.3.10 The effect of OG administration of R568.HCl on food intake in fasted rats during the early light phase.
The effect of OG of vehicle (1.85% DMSO in water), 1.1 mg/kg or 11.2 mg/kg of R568.HCl on food intake in rats following an overnight fast during 0-1 (A), 1-2 (B), 2-4 (C), 4-8 (D), 8-24 (E) and 0-24 (F) hour following administration. Data presented as mean ± SEM. n=8-9. *p<0.05 vs vehicle.

3.3.6.3 The effect of ileal administration of L-Phe on food intake in *ad libitum* fed rats in the early dark phase

There was a strong trend for ileal administration of 10 mM L-Phe in rats to reduce food intake in the first 30 minutes (Vehicle: 1.59 ± 0.55g vs. 10mM L-Phe: 0.59 ± 0.38g, $p = 0.06$, n=9 per group) and 0-1 hour period (Vehicle: 2.39 ± 0.50g vs. 10mM L-Phe: 1.06 ± 0.55g, $p = 0.08$, n=9 per group) following administration, but this effect did not reach statistical significance (Fig. 3.3.11 A and Fig 3.3.11 C). The co-administration of L-Phe with NPS2143.HCl, a CasR antagonist, appeared to attenuate this effect of L-Phe on food intake during 0-30 minutes (10mM L-Phe: 0.59 ± 0.38g vs. L-Phe + 1µM NPS2143.HCl: 1.53 ± 0.30g, $p = 0.052$, n=9 per group) and 0-1 hour (10mM L-Phe: 1.06 ± 0.55g vs. L-Phe + 1µM NPS2143.HCl: 2.80 ± 0.36g, $p = 0.057$, n=9 per group) post administration (Fig. 3.3.11 A and Fig 3.3.11 C). There were no significant differences in food intake between groups at any other time point at which it was measured.

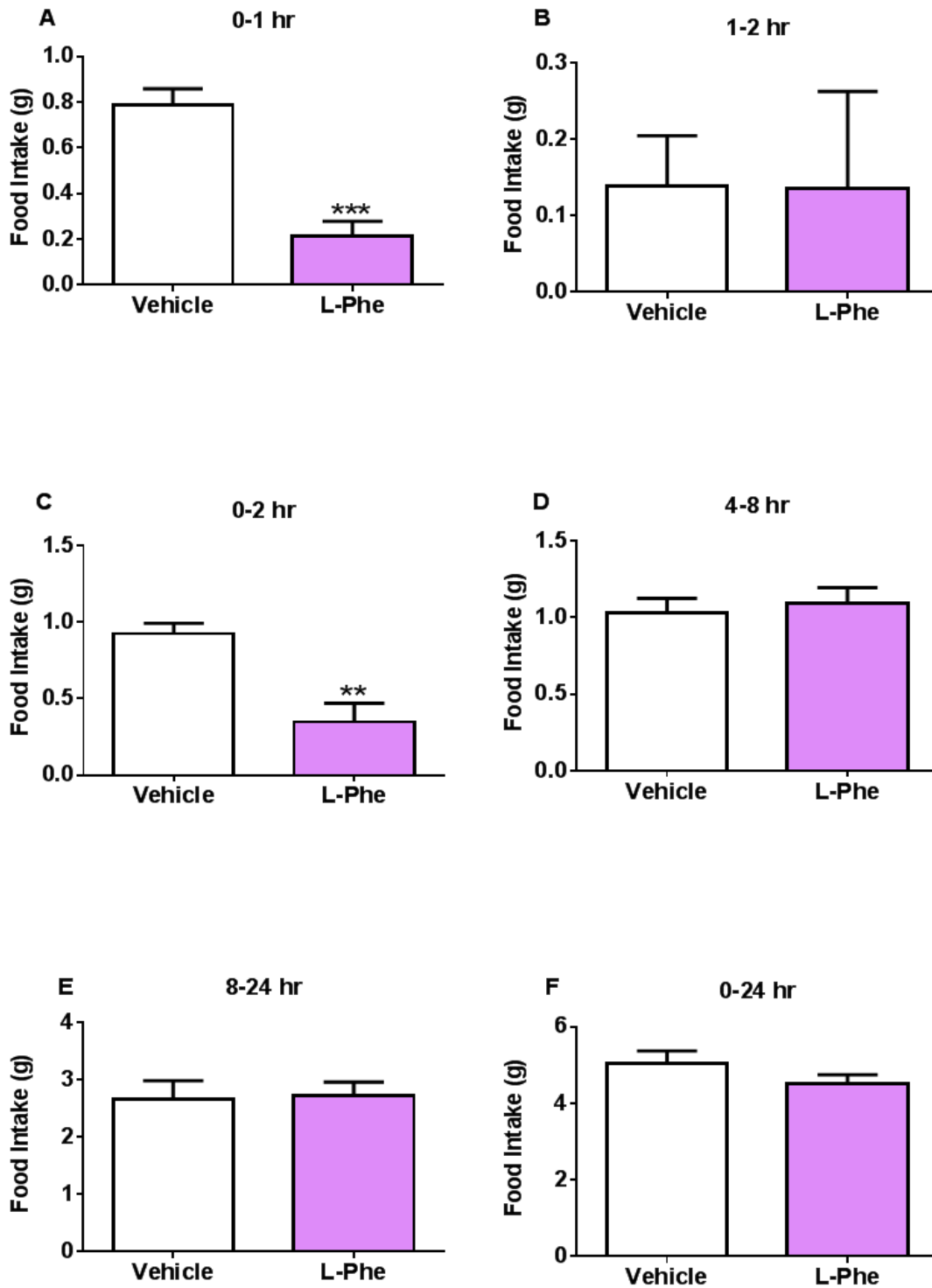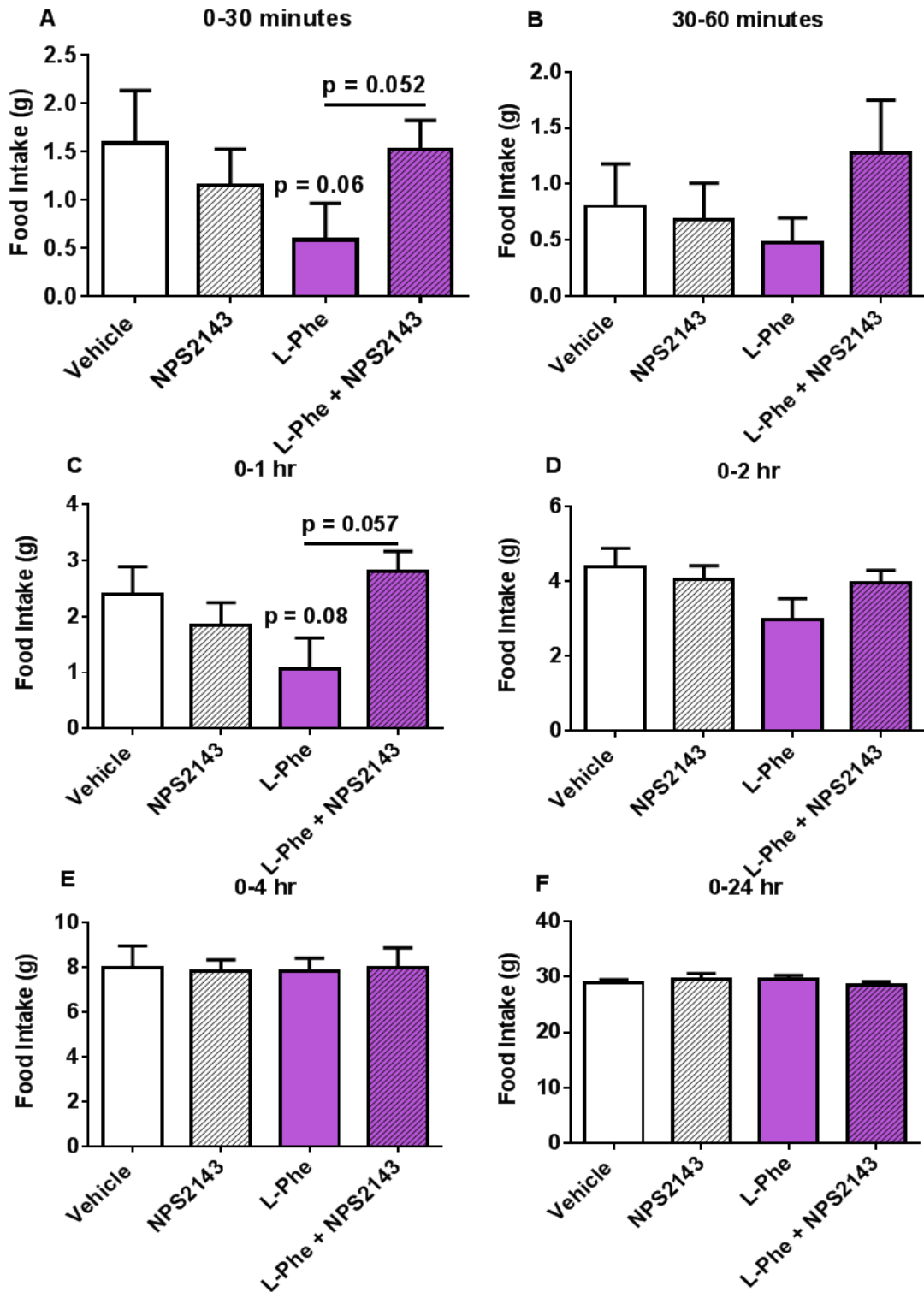**Figure 3.3.11 The effect ileal administration of L-Phe on food intake in rats.** The effect of ileal administration of vehicle (0.25 DMSO, 0.9% saline), 1 µM NPS.2143.HCl, 10 mM L-Phe or 10 mM L-Phe and 1 µM NPS2143.HCl on food intake in rats with *ad libitum* access to food injected at the beginning of dark phase at 0-30 (A), 30-60 (B) minutes, 0-1 (C), 0-2 (D), 0-4 (E) and 0-24 (F) hour post administration. Data expressed as mean ± SEM. n = 9 per group, crossover.

3.3.6.4 The effect of a CaSR antagonist on the L-Phe-induced GLP-1 release from STC-1 cells

L-Phe at a dose of 50mM significantly stimulated the release of GLP-1 from STC-1 cell-line (control: 0.47% ± 0.05% vs. 50 mM L-Phe: 2.53% ± 0.21%, p<0.001, n=8 plates). The effect of 50mM L-Phe on GLP-1 release was significantly attenuated in presence of 10µM NPS2143.HCl, a synthetic antagonist to CasR (50 mM L-Phe: 2.53% ± 0.21% vs. 50mM L-Phe + 10 µM NPS2143.HCl: 1.77% ± 0.18%, p<0.01, n=8 plates). Treatment of cells with 10µM NPS2143.HCl alone, had no significant effect on GLP-1 release (Fig. 3.3.12).
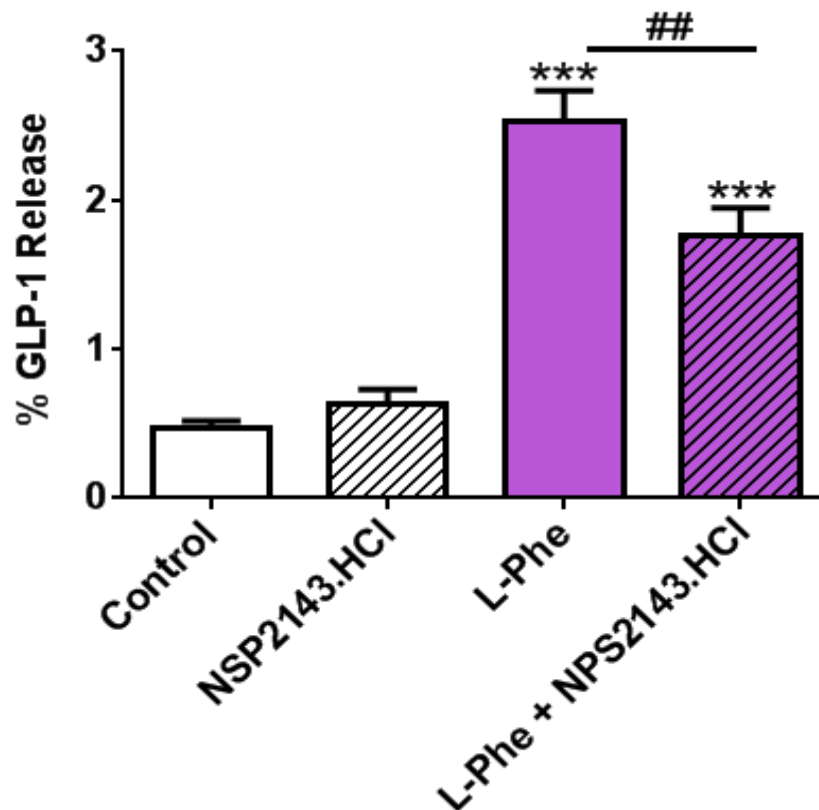
Figure 3.3.12 The effect of a CaSR antagonist on L-Phe-induced GLP-1 release from STC-1 cells.
The effect of 50mM L-Phe in presence or absence of 10µM NPS2143.HCl on GLP-1 release from STC-1 cells following 2 hours incubation. Data expressed as mean ± SEM n = 8 plates. **p<0.01, ***p<0.001 vs. control; ##<0.01 vs. L-Phe.

3.3.7 The effect of OG administration of L-Phe on food intake and energy expenditure in fasted rats in the early light phase

To investigate the effect of L-Phe on energy expenditure, rats were placed in CLAMS metabolic cages as described in section 2.2.5. Oral administration of 3 mmol/kg L-Phe reduced food intake in mice, however the effect did not reach statistical significance (Fig 3.3.13 A). Oral gavage of 3 mmol/kg L-Phe had no significant effect on VO_2, VCO_2 and RER in mice during the 8 hours following administration (Fig. 3.3.13 B-D).

Oral gavage of 3 mmol/kg L-Phe had no significant effect on mice activity as demonstrated by measuring the total animal motion at X-axis (XTOT) (Fig. 3.3.13 E).
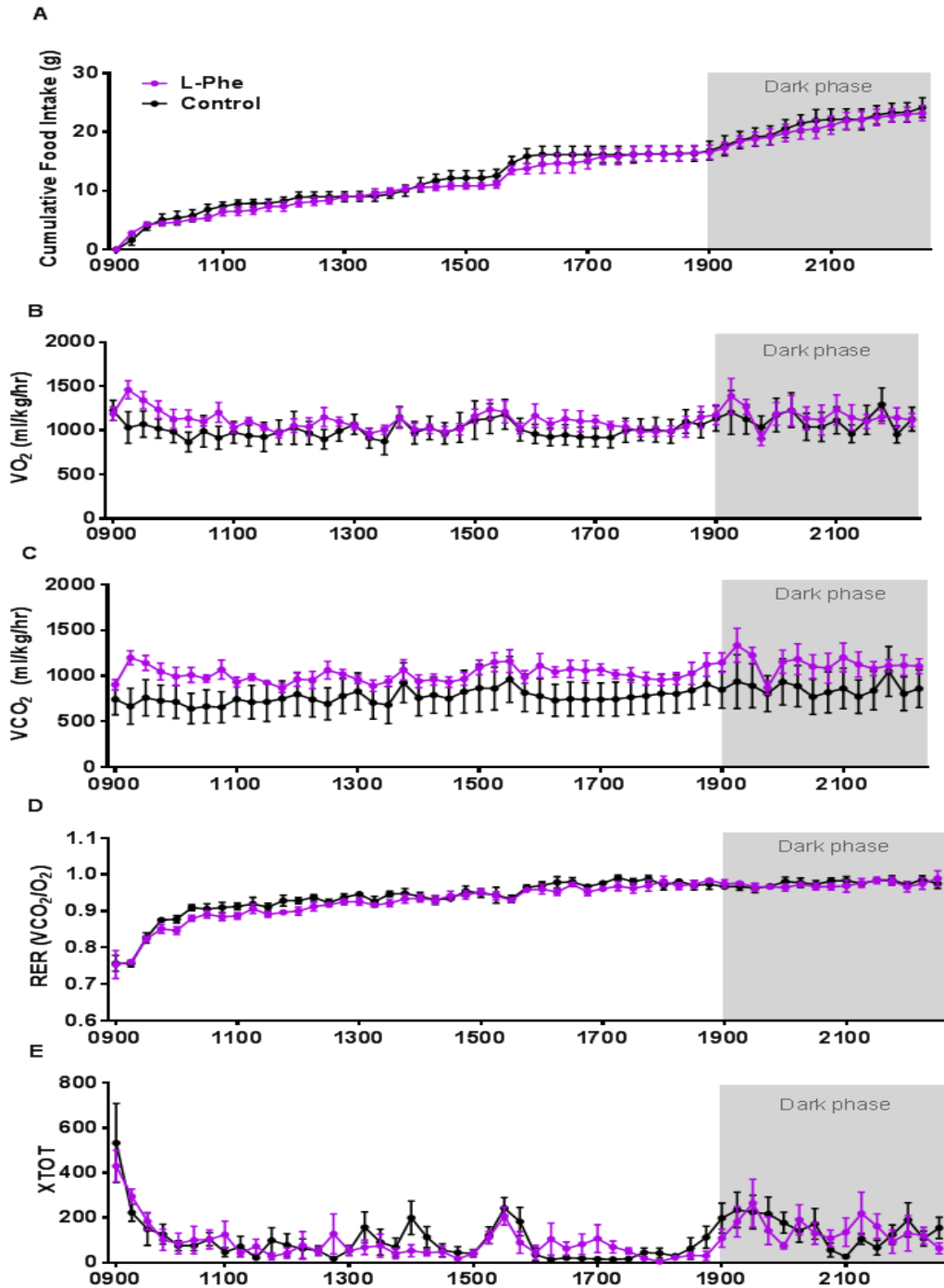
Figure 3.3.13 The effect of OG administration of L-Phe on food intake and energy expenditure in fasted rats.

The effect of OG administration of water (control) or 3 mmol/kg L-Phe on cumulative food intake (A), oxygen consumption (B), carbon dioxide production (C), respiratory exchange rate (D) and activity (XTOT) (E) in rats injected at early light phase and placed in CLAMS cages. The oral gavage was performed at 09:00 and food was returned immediately. Recordings were taken over a period of 12 hours and at subsequent 12 minute intervals following administration. Shaded area represents the dark phase from 19:00. Data presented as mean ± SEM. n=6 per group.

3.3.8 The effect of repeated OG administration of L-Phe in DIO mice

3.3.8.1 The effect of repeated OG administration of L-Phe on food intake and body weight in DIO mice

Oral administration of 12 mmol/kg L-Phe acutely reduced food intake in mice during 0-1 hour time point following administration at day 0 of chronic administration (Vehicle: 0.13 ± 0.03g vs. 12mmol/kg L-Phe: 0.05g ± 0.01g, $p<0.05$, n=10 per group) (Fig. 3.3.14 A).

Repeated twice daily oral administration of 12 mmol/kg L-Phe over 4 days had no significant effect on food intake or body weight (Fig. 3.3.14 B and C). However, 3-times daily administration of 12 mmol/kg L-Phe significantly reduced food intake in mice. The cumulative food intake was statistically different on day 6 (Vehicle: 13.02 ± 0.49g vs. 12 mmol/kg L-Phe: 10.69 ± 0.66g, $p<0.001$, n=10 per group) and day 7 (Vehicle: 14.89 ± 0.58g vs. 12 mmol/kg L-Phe: 11.90 ± 0.71g, $p<0.001$, n=10 per group). This effect was reflected in changes in body weight, with a significant decrease in body weight change at day 5 (Vehicle: -0.96 ± 0.24g vs. 12 mmol/kg L-Phe: -1.91 ± 0.36g, $p<0.05$, n=10 per group), day 6 (Vehicle: -1.28 ± 0.22g vs. 12 mmol/kg L-Phe: -3.00 ± 0.35g, $p<0.001$, n=10 per group), and day 7 (Vehicle: -1.69 ± 0.32g vs. 12 mmol/kg L-Phe: -3.43 ± 0.37g, $p<0.001$, n=10 per group) (Fig. 3.3.14).
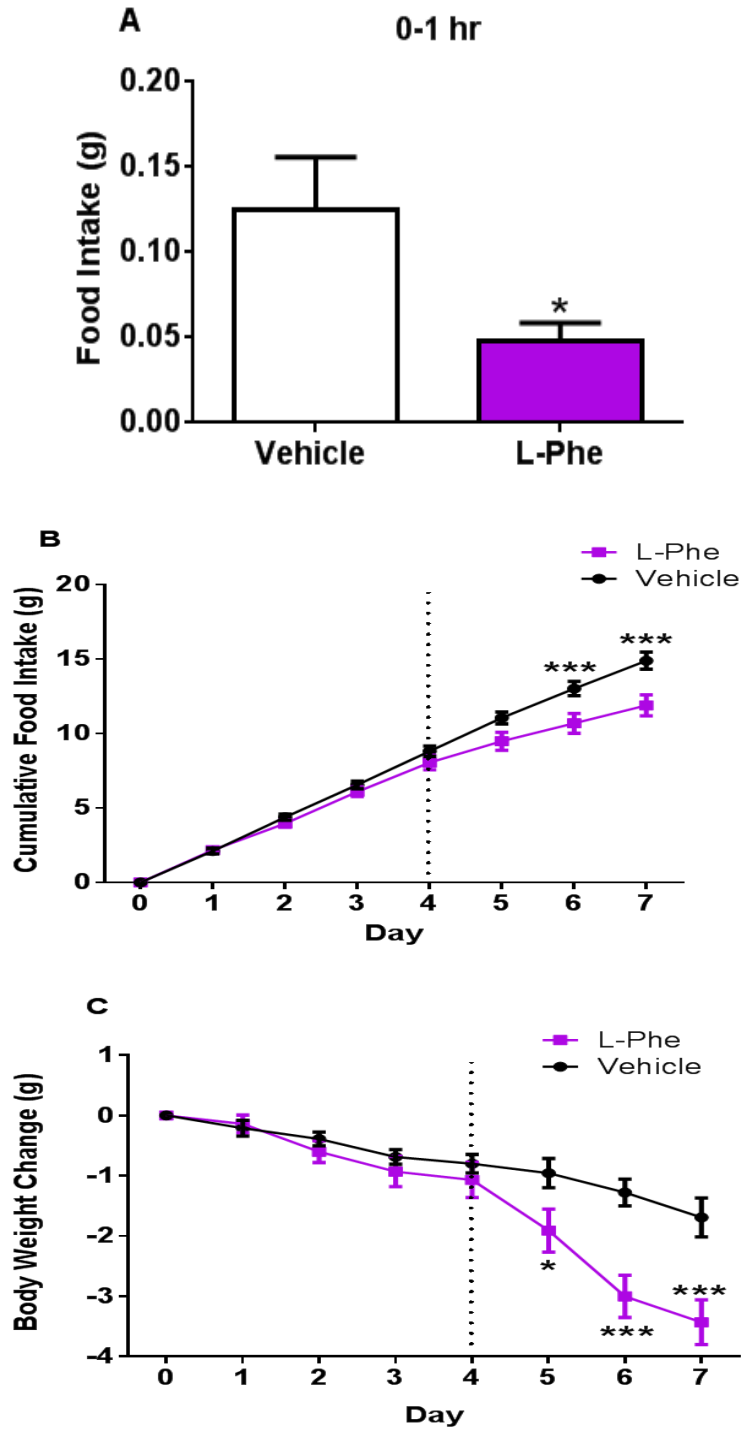
Figure 3.3.14 The effect of repeated OG administration of L-Phe on food intake and body weight in DIO mice.
The effect of oral gavage of vehicle (10% TWEEN20, water) or 12 mmol/kg L-Phe on 0-1 hour food intake at the beginning of dark phase on day 0 of repeated administration (A) and the effect on cumulative food intake (B) and body weight change (C) following twice daily OG during days 0 to 3 and three times daily OG during 4 to 7 days of chronic study. Dotted line highlights the start of three times daily injection. Data expressed as mean ± SEM, n = 10 per group. *p<0.05, ***p<0.001 vs. vehicle.

3.3.8.2 The effect of repeated administration of L-Phe on plasma leptin levels in DIO mice.

Repeated oral gavage of 12 mmol/kg L-Phe significantly reduced plasma leptin levels in DIO mice compared to the vehicle controls (Vehicle: 27.91 ± 2.46 ng/ml vs. 12 mmol/kg L-Phe: 18.05 ± 2.70 ng/ml, $p < 0.05$, n=10 per group) (Fig. 3.3.15).
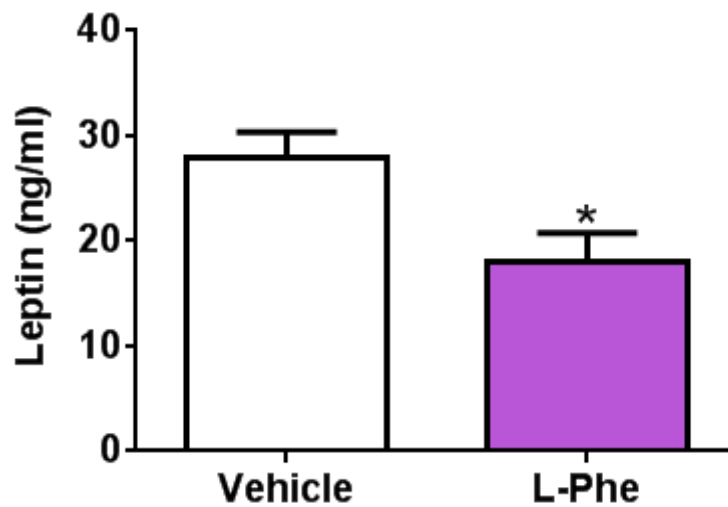
Figure 3.3.15 The effect repeated administration of L-Phe on plasma leptin levels in DIO mice.
The effect of repeated administration of vehicle (10% TWEEN20, water) or 12 mmol/kg L-Phe on leptin plasma levels following a 7-day chronic administration study. Data expressed as mean ± SEM, n = 10 per group. *$p < 0.05$ vs vehicle.

3.3.8.3 The effect of repeated administration of L-Phe on circulating gut hormone levels in DIO mice

Oral gavage of 12 mmol/kg L-Phe chronically for 7 days resulted in significantly elevated plasma acylated ghrelin levels compared to the vehicle treated animals (Vehicle: 367.1 ± 30.77pg/ml vs. L-Phe: 519.5 ± 50.25pg/ml, $p < 0.05$, n=10 per group) (Fig. 3.3.16 A). There was also a trend towards an increase in plasma GLP-1 and PYY levels compared to the vehicle, but these changes were not statistically significant (Fig. 3.3.16)
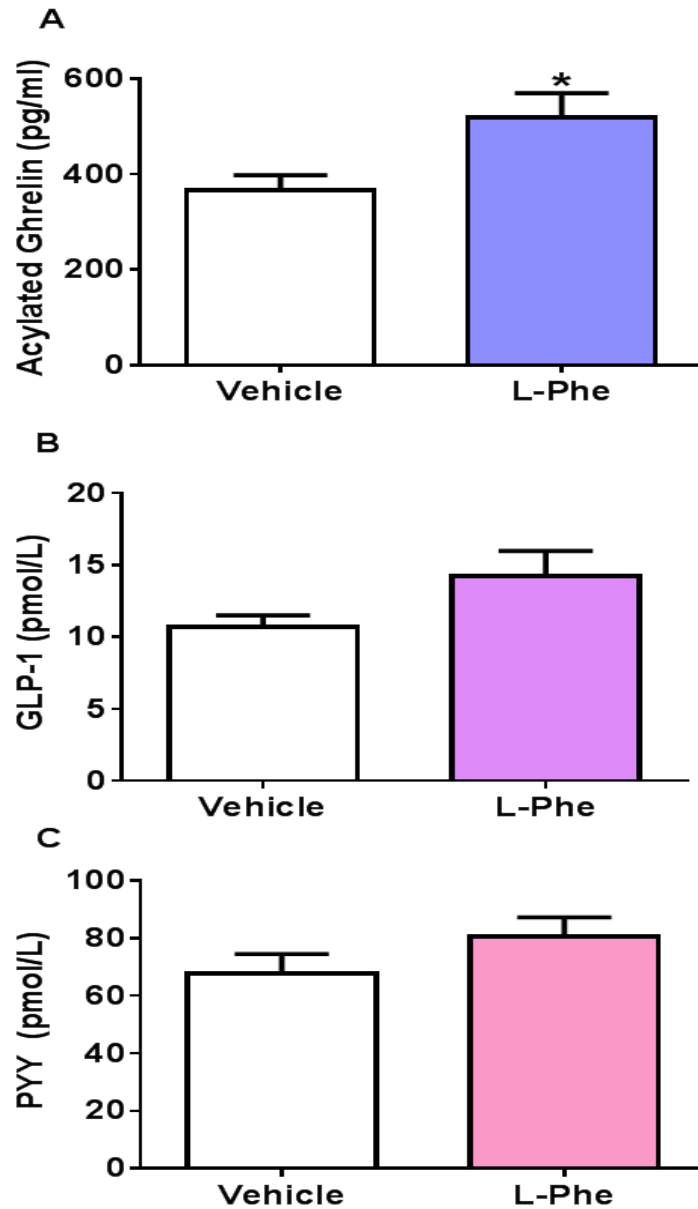
Figure 3.3.16 The effect of repeated OG administration of L-Phe on circulating gut hormone levels in DIO mice.
The effect of 7 days repeated oral gavage of vehicle (10% TWEEN20, water) or 12 mmol/kg L-Phe on plasma acylated ghrelin (A), GLP-1 (B) and PYY (C). n = 10. Data expressed as mean ± SEM. *$p < 0.05$ vs. vehicle.

3.4 Discussion

The studies described in this chapter investigated the effects of L-Phe on food intake in rodents, and the mechanisms by which these effects are mediated. The findings demonstrate that L-Phe can reduce food intake in both mice and rats. In addition, they suggest that the effect may be mediated via CaSR and changes in gut hormone release, though further studies are required to confirm these findings.

As previously mentioned, individual amino acids have different anorectic properties and current evidence suggests that the satiety associated with high protein diets are due to the amino acid constituents rather than protein *per se* (Veldhorst et al., 2008). Studies investigating the anorectic effect of L-Phe are limited. This may in part be due to poor solubility of the amino acid in aqueous solutions suitable for administration in rodents. Jordi and colleagues examined the effect of oral gavage of a range of individual amino acids including L-Phe on food intake in rats. They found no significant effect on food intake following OG of 6.7 mmol/kg L-Phe compared to water control (Jordi et al., 2013). Similarly, another study reported that OG or subcutaneous administration of L-Phe at doses up to 720mg/kg (4.5 mmol/kg) had no significant effect on food intake in rats. However in the same study, IP administration of 90 mg/kg L-Phe (approximately 0.6 mmol/kg) significantly reduced food intake in rats. The authors concluded that the route of administration influences the anorectic effect of L-Phe (Bialik et al., 1989). In contrast to previously published work, the work presented here demonstrates that oral administration of 3 mmol/kg L-Phe can significantly reduce food intake in rats. This effect was seen in both fasted rats administered L-Phe in the early light phase, and *ad-libitum* fed animals administered L-Phe in the early dark phase. Similarly, OG administration of L-Phe significantly reduced food intake in mice. However, mice required a relatively higher dose (12 mmol/kg) of L-Phe to achieve a comparable reduction in food intake, an effect which was reproducible in both light and dark phase. As previously mentioned, this might be expected, as mice often require a higher dose per body weight to achieve the same effects compared to rats due to differences

in metabolic rate between the species. However, this is likely to be a greater contributory factor for an agent acting systemically than for one acting in the GI tract.

Aromatic amino acids are potent allosteric activators of the promiscuous L-specific amino acid G-protein coupled receptor, CaSR (Smajilovic et al., 2014). In order to investigate the role of CaSR in mediating the anorectic effect of L-Phe, synthetic inhibitors and a synthetic activator of the receptor were used studies in rodents. Oral administration of the CaSR agonist R568.HCl significantly reduced food intake in rats. Calcium is the natural ligand to the receptor and thus any pharmacological changes can directly affect the levels of calcium. Therefore, doses of agonist used in the feeding study were chosen carefully to avoid any direct effect on calcium homeostasis. Previous published research has demonstrated that oral administration of R568.HCl at doses used in present study had no effect on plasma calcium or parathyroid hormone levels in rats (Murakami et al., 2000). There seemed little difference in the anorectic effects of oral administration of R568.HCl at a dose of 1.1 mg/kg or a tenfold higher dose of 11.2 mg/kg (though the effects of the higher dose did not achieve statistical significance). This might suggest that the lower dose results in the maximum effect of CaSR agonism in the gut on food intake, at least in the first hour after administration. Interestingly, the suppression of appetite following OG of R568.HCl was similar to the effect of 3 mmol/kg L-Phe on food intake in rats. Greater effects on food intake were seen with higher doses of L-Phe in mice, which might reflect the involvement of other systems, or that, for example, R568.HCl is quickly absorbed but that L-Phe can exert its effects further down the GI tract.

Direct ileal administration of 10mM L-Phe in conscious rats appeared to reduce food intake in rats injected in the dark phase, an effect that appeared to be attenuated by co-administration of the CaSR antagonist NPS.2143.HCl. Mace and colleagues had previously used 10 mM L-Phe to perfuse isolated rat small intestinal preparations (Mace et al., 2012). These observations suggest a direct role for CaSR in mediating the anorectic effects of L-Phe in the gut.

The effect of L-Phe on gut hormone release was investigated. It was hypothesized that L-Phe stimulates gut hormone release via a CaSR-dependent mechanism, as recent evidence has suggested L-Phe stimulates release of CCK from I-cells (Wang et al., 2011a, Ballinger and Clark, 1994). CCK regulates the release of bile and digestive enzymes from the gall bladder and pancreas respectively. It also has anorectic properties and has been shown to act as satiety hormone in various species (Liddle et al., 1985). Various studies have demonstrated the effect of L-Phe on CCK release in STC-1 cells (Hira et al., 2008) and isolated intestinal I-cells preps (Mace et al., 2012). Furthermore, evidence suggests that L-Phe-induced CCK release is mediated by the CaSR (Hira et al., 2008, Brennan et al., 2014).

Oral administration of L-Phe significantly elevated circulating GLP-1 levels in rats. However, PYY levels remained unchanged. In addition, oral L-Phe reduced acylated ghrelin levels in rats. It has been previously shown that L-cystiene reduces food intake in rats, an effect that may in part be due to reduction in acylated ghrelin levels observed following oral administration (McGavigan et al., 2015). L-Phe has been previously shown to stimulate GLP-1 release from murine primary colonic L-cells (Tolhurst et al., 2011). In the current studies, L-Phe stimulated the release of GLP-1 and also stimulated the release of PYY from a murine primary colonic L-cell culture, and of GLP-1 from STC-1 cells. LDH cytotoxicity assays suggested that L-Phe-induced GLP-1 release from STC-1 cells is not secondary to cell toxicity. These findings suggest that L-Phe may mediate its anorectic effect by altering the release of appetite-modulating GI hormones.

The expression of CaSR has been confirmed in different regions of the GI tract. A recent study demonstrated that CaSR is enriched in ghrelin cells of the stomach and that CaSR mediates the release of ghrelin. Calcium chloride and R568.HCl inhibit ghrelin release from primary gastric mucosal cells. However, activation of CaSR in the stomach seems to have a complex dual inhibitory and stimulatory role, as R568.HCl can inhibit and stimulate ghrelin release dependent upon Ca^{2+} concentrations (Engelstoft et al., 2013).

CaSR is enriched in GLP-1-expressing cells in the upper and lower small intestine and the colon (Diakogiannaki et al., 2013). Mace and colleagues demonstrated that L-Phe stimulated GLP-1 and PYY release from isolated rat small intestinal loops via a CaSR dependent mechanism (Mace et al., 2012). The CaSR inhibitor calhex231 blocked the L-Phe-stimulated release of GLP-1 and PYY, whereas R568.HCl augmented the release. In accord with these observations, treating cells with the CaSR antagonist NPS2143 attenuated L-Phe-induced GLP-1 release from STC-1 cells. However treatment of cells with the antagonist resulted in only a partial reduction in GLP-1 signal suggesting that there may be other overlapping mechanisms involved in L-Phe-induced GLP-1 release. L-Phe has activity at T1R1-T1R3 receptor (Wellendorph et al., 2009a). A study by Dale and colleagues demonstrated that L-Phe stimulates CCK release from STC-1 cells via a mechanism that requires the activity of T1R1-T1R3 receptor (Daly et al., 2013). In addition, L-Phe uptake is facilitated by the presence of specific amino acid transporter systems as previously described in section 1.8.4. These findings suggest that L-Phe mediates its effects on gut hormone release at least in part via the CaSR.

CaSR knockout mouse models have been used to investigate the role of CaSR in physiology. However, these models are not easy to use to assess the role of CaSR in energy homeostasis because of their complex phenotypes. The global *CaSR* knockout mouse is characterised by elevated Ca^{2+} and parathyroid hormone levels, bone abnormalities, stunted growth and premature death (Ho et al., 1995, Chang et al., 2008), making it difficult to distinguish specific effects on energy homeostasis from the ill health caused by disrupted calcium homeostasis. There is a gut-specific CaSR knockout model in which *CaSR* is conditionally deleted from the GI epithelium. However, this resulted in hyper-proliferation of colonic epithelial cells and changes in crypt structure in those mice (Rey et al., 2012), which might be expected to mask specific effects of CaSR on gut hormone release and appetite.

L-Phe reduces food intake acutely in both mice and rats. In order to investigate the chronic effect of L-Phe on body weight, a diet induced mice model of obesity was used. We first examined the effect of acute L-Phe administration on appetite in DIO mice. Similar to lean mice, DIO mice had significantly lower food intake following acute oral gavage of 12 mmol/kg L-Phe. Initially, mice were orally gavaged twice daily and the food intake and body weight was monitored for 4 days. Although, oral administration of L-Phe reduced food intake acutely, the repeated administration did not significantly influence body weight. However, increasing the number of oral gavages to three per night resulted in a significant reduction in food intake and subsequently body weight in mice. Consistent with the weight loss, plasma leptin levels were significantly lower in L-Phe treated animals, suggesting the weight loss reflects some loss of fat. These findings suggest L-Phe administration may have potential in the treatment of obesity.

Further work is required to investigate the weight loss effect in DIO mice. Although it resulted in a reduction in circulating leptin levels, body compostion studies are required to confirm the loss of fat rather than lean mass in chronically L-phenylalanine-treated animals.

In order to investigate the effect of chronic L-Phe administration on gut hormone profiles, DIO mice were sacrificed at the end of chronic study and plasma was collected. There was no significant difference in GLP-1 and PYY levels between L-Phe and vehicle treated animals. This may reflect that the animals had not received L-Phe for 24 hours; any effect on these hormones may be acute following each administration rather than cumulative. Despite the earlier observation that acute oral administration of L-Phe reduced acylayted ghrelin signal in rats, chronic L-Phe administration resulted in a significant increase in circulating ghrelin levels. Ghrelin plasma levels are reduced in obese individuals and elevated following weight loss, an effect which is thought to represent a feedback loop intended to reduce food intake (Tschop et al., 2001, Cummings et al., 2002). However, the increase in ghrelin signal in DIO mice suggests that the reduction in food intake and the consequent weight loss are not driven by initial changes in ghrelin signalling. Our results may thus reflect the reduced

body weight of these animals, rather than a specific effect of L-Phe. Further studies could elucidate mechanisms involved in weight loss in DIO mice. For example, using c-fos studies to investigate changes in neuronal activation in regions involved in the control of appetite, and PCR to study changes in expression of specific appetite regulating molecules, such as NPY, AgRP and POMC, might give valuable clues as to the mechanisms mediating the effects of   chronic administration of L-Phe on energy homeostasis.

Further work is required to establish the role of gut hormones and CaSR. The work described in this chapter focused on the peripheral effects of L-Phe administration. I have assumed that the effects observed are mediated in the GI tract, which is in accord with the effects on *in vitro* L cell models and direct administration of low doses of L-Phe into the ileum. However, it is possible that the effects observed are mediated by central circuits. There is evidence that specific amino acids are sensed directly in CNS (Blouet et al., 2009, Blouet and Schwartz, 2012). Direct central administration in rodents as well as IP administration may reveal further information regarding the role of L-Phe in the CNS.

Further work is also required to examine whether the effects of L-Phe are enantiomer specific. I investigated the role of D-phenylalanine (D-Phe) on food intake and gut hormone release at considerably lower doses and concentrations than those used for L-Phe, and observed no effects (data not shown). However, due to poor solubility, I was unable to confirm the lack of effects at doses comparable to those of L-Phe used. Given that CaSR specifically responds to the L-form of amino acid, D-Phe is assumed not to effect food intake via the CaSR.

I also investigated the effect of L-Phe on energy expenditure in the CLAMS and found no significant difference in oxygen consumption, carbon dioxide production and RER in rats. In addition the administration of L-Phe had no significant effect on the animals' activity. However, the energy expenditure studies carried out in animals on fed background and

therefore should in future be repeated in fasted animals to investigate potential effects on energy expenditure.

I observed no visible signs of aversion or discomfort in either acute or chronic L-Phe studies in rats and mice. L-Phe reduced food intake at relatively low doses compared to other amino acids (section 2.3.2 and 2.3.4). However, detailed behavioural studies are required to confirm that the reduction in food intake is not due to any behavioural side effects.

The work described in this chapter suggests a role for L-Phe in appetite regulation. The data generated collectively suggest that the anorectic effect of L-Phe may be mediated via changes in gut hormone levels, and that these changes may be regulated via the CaSR.

Chapter IV:

The Effect of Encapsulated Amino Acids on Food Intake

4.1 Introduction

The mechanisms by which protein is sensed in order to modulate energy homeostasis are unclear, but it may be the amino acids products of protein digestion that are detected, rather than the protein itself (Veldhorst et al., 2008). The work described in the previous two chapters established L-arginine and L-phenylalanine as anorectic agents in rodents able to reduce appetite and alter gut hormone release.

The three nutrient-sensing G-protein coupled 7TM receptors that are broadly activated by amino acids, T1R1-T1R3, GPRC6A and CaSR, have been detected in the GI tract and are potentially involved in nutrient sensing and food intake via regulation of the release of signalling molecules from the gut (Wellendorph et al., 2009a). The expression of these receptors varies throughout the GI tract, but all are relatively highly expressed in the distal small intestine and the colon (Wellendorph et al., 2010, Reimann et al., 2012). In addition, there are high numbers of GLP-1 and PYY secreting L-cells in the distal small intestine and the colon (Symonds et al., 2015). Most amino acids generated from protein digestion are absorbed in the small intestine, with only low concentrations reaching the colon. The normal physiological sensing of these amino acids to regulate food intake is therefore likely to take place in the small intestine, rather than the colon, which may play a more important role in pathophysiology, when absorption is compromised (Freeman and Kim, 1978). However, higher concentrations of amino acids reaching the distal small intestine may signify the ingestion of a large protein meal that should be associated with longer satiety. Free amino acids are quickly absorbed in upper gastrointestinal tract following oral administration, and may not reach the distal small intestine. Therefore, in order to best exploit the putative appetite reducing effects of amino acids in the distal small intestine, it may be necessary to target their delivery. Microencapsulation offers a putative method by which to do this.

4.1.1 Microencapsulation in the food industry

Microencapsulation is a technique used within variety of industries including the pharmaceutical, food and agricultural industries. Microencapsulation is the process whereby different agents referred to as payload are stored within a microscopic coating matrix or shell which serves as a protective barrier to delay the degradation or release of the agents. The protective coating or shell is typically comprised of insoluble molecules that do not react with the core molecule. This coating can account for between 1 and 80% of the microencapsulate particle by weight, and depending on the intended utility, a diverse range of molecules can be used as the component of the shell or coating including many naturally occurring molecules such as sugars, proteins, lipids and waxes (Gaonkar et al., 2014).

The encapsulation process can protect the active payload against degradation as a result of exposure to environmental factors such as water, heat or light. In addition, encapsulation can be used to mask the undesirable taste, odour or colour of the payload which may interfere with the product performance. One important application of microencapsulation is to control the delivery of payload, a process known as controlled release or controlled delivery. Controlled delivery is achieved by utilizing various release mechanisms which include temperature, moisture, pH and enzymatic action. Controlled release can facilitate the delivery of payload to a specific desired location within the body, for example, specific regions of the GI tract (Gaonkar et al., 2014).

4.1.2 Microencapsulation materials

A wide range of substances have been used for encapsulation processes. However, only certain substances are considered to be safe to coat or encapsulate for use in food industry. The materials used for encapsulations are required to be food-grade and to have the ability to form an effective barrier between the payload and its surroundings. Possible materials include biomolecules such as proteins, lipids, fats and gums. Among such materials, polysaccharides are widely used in food encapsulation, with starches and cellulose and their

derivatives amongst the most common. Proteins such as caseins, gluten and gelatine are also used, and lipids such as fatty acids, fatty alcohols and waxes.

The choice of encapsulation matrix material greatly depends on the application. Natural waxes, for example, are suitable for aroma encapsulation due to their ease of handling, stability, inert chemical properties and safety. An important advantage of natural waxes such as beeswax, or plant waxes such as candelila and carnauba wax, is that they can be considered to be food constituents at the purity grade used in encapsulation processes (Nedovica et al., 2011).

Carnauba wax is extracted from the leaves of palm Copernica cerifera, native to northern Brazil. Carnauba wax is significantly less viscous and has a higher melting point, is more elastic and more resistant to deformation than other naturally occurring waxes. These properties make it a suitable agent in encapsulation. In particular, due to its high melting point, it has been widely used in controlled release and delivery systems (Milanovic et al., 2010).

4.1.3 Encapsulation techniques

There are several methods available for microencapsulation of food ingredients. Spay drying is one of the most widely used. It is an economically viable technique that produces good quality encapsulates of less than approximately 40 microns. There are, however, disadvantages in utilizing this method, including the complexity of the required equipment and the difficulty in controlling the size of particles. Alternative methods include spray-chilling, freeze-drying, melt extrusion and melt injections. Spray-chilling or spray-cooling techniques are often used in production of lipid-coated materials, with the melting point of the lipids used, determining which is preferred (Gaonkar et al., 2014).

Extrusion methods involve dropping droplets of an aqueous solution of polymer into a gelling bath. The technique is particularly common in the production of very small particles.

Emulsification is another common technique used in encapsulation of water soluble agents (Gaonkar et al., 2014).

4.1.4 Hypothesis and Aim

Hypothesis: Encapsulating specific amino acids will allow their targeted delivery to more distal regions of the small intestine, increasing their effect on the release of anorectic gut hormones, and thus on food intake.

Aim:

To investigate

- The effect of oral administration of encapsulated L-Arg.HCl and L-Phe on food intake and gut hormone release in rats.
- Whether encapsulation matrices alter the amino acid release profile in the GI tract compared to the un-encapuslated forms, resulting in an altered food intake time profile in rats.

4.2 Methods

4.2.1 Animals

Male C57BL/6 mice (Harlan, Bicester, Oxon, UK) weighing 18-20g (6-8 weeks old) were grouped housed (four per cage) under controlled temperature (21-23ºC) and light cycle (12 hours light, 12 hours dark) with *ad libitum* access to standard chow diet (RM1, Special Diet Services Ltd, Witham, Essex, UK) and water, unless otherwise stated.

Male Wistar rats (Charles River, Margate, Kent, UK) weighing 180-220g were individually housed under controlled temperature (21-23ºC) and light cycle (12 hours light, 12 hours dark) with *ad libitum* access to standard chow diet (RM1, Special Diet Services Ltd, Witham, Essex, UK) and water, unless otherwise stated.

All animals were randomised according to their body weight for all the studies. Animals were handled and acclimatised to the procedures prior to commencement of studies.

All animal procedures were approved and carried out according to the British Home Office Animals Scientific Procedures Act 1986 (project License: 70/8068).

4.2.2 The effect of proteinogenic amino acids on GLP-1 release in culture

The ability of individual proteinogenic amino acids to stimulate GLP-1 release was examined in a series of secretion experiments carried out in primary mice colonic L-cell culture. Amino acids were made up at 100mM concentration in secretion buffer (refer to Appendix 1). L-Tryptophan and L-tyrosine were not tested due to poor solubility. Mice colonic crypt isolation and secretion assays were performed as previously described in sections 2.2.7.1 and 2.2.7.2 respectively, with the cultures being incubated with the amino acids or control treatments for two hours. GLP-1 release was measured using a specific in-house RIA assay as described in section 2.2.7.3. GLP-1 release is presented as fold change from the baseline control.

4.2.3 Amino acid encapsulation

Microencapsulation was carried out by the project's industrial collaborator TasteTech Ltd. TasteTech is a specialist manufacturer of controlled release flavourings and ingredients (TasteTech). Microencapsulation was carried out using a variety of vegetable oils and naturally occurring waxes using matrix microencapsulation processes. Particle sizes were approximately 100 microns. The resulting microencapsulation products consist of particles containing typically 40-60% (weight:weight ratio) of amino acid, depending on the matrix used for the encapsulation process.

4.2.3.1 Encapsulated amino acids products

A number of different encapsulated forms of L-Arg.HCl and encapsulated L-Phe were developed by TasteTech. The products, payloads and encapsulation matrices are listed in table 4.2.1. The difference in the milling process of the amino acid payload in products #43773 and #43767 are highlighted. Cone and rotary ball milling were carried out in products #43773 and #43767 respectively. Rotary ball milling results in production of smaller payload particles compared to the cone milled particles.

Product ID	Encapsulation Matrix	Amino acid payload
43773	49.5% Candelilla Wax + 1% Silica	49.5% L-Arg.HCl (cone milled)*
43767	49.5% Candelilla Wax + 1% Silica	49.5% L-Arg.HCl (rotor milled)*
43770	47.5% Fully hydrogenated rapeseed oil + 2% Carnauba Wax + 1% Silica	49.5% L-Arg.HCl
43771	24.5% Fully hydrogenated rapeseed oil + 24.5% Carnauba Wax + 1% Silica	50% L-Arg.HCl
43772	49.5% Carnauba Wax + 1% Silica	49.5% L-Arg.HCl
43999	50% Synthetic Microcrystalline wax	50% L-Arg.HCl
44000	25% Synthetic Microcrystalline wax + 25% Carnauba Wax	50% L-Arg.HCl
44001	25% Glyceryl Behenate + 25% Carnauba wax	50% L-Arg.HCl
44002	25% Bees wax + 25% Carnauba wax	50% L-Arg.HCl
44003	25% Rice bran wax + 25% Carnauba wax	50% L-Arg.HCl
44004	25% Sunflower wax + 25%Carnauba wax	50% L-Arg.HCl
44007	50% Paraffin wax	50% L-Arg.HCl
44008	25% Paraffin wax + 25% Carnauba wax	50% L-Arg.HCl
44009	60% Carnauba wax	40% L-Arg.HCl
44010	55% Carnauba wax	45% L-Arg.HCl
200240	60% Carnauba wax	40% L-Phenylalanine
200262	59% Carnauba wax + 1% Silica	40% L-Phenylalanine

Table 4.2.1 Encapsulated amino acids.
Encapsulated amino acids with their encapsulation matrices and amino acid payloads. The compositions presented as percent weight: weight ratio. * Cone and rotary ball milling technique used to generate payload particles.

4.2.4 The effect of encapsulated amino acids on food intake in rats

A series of feeding studies were performed to examine the effect of encapsulated amino acids on food intake in rats.

For studies performed in light phase, rats were fasted 16 hours overnight and subsequently received an oral gavage of treatment. Animals were immediately returned to their cages and a pre-weighed amount of food was provided. Food intake was measured at 1, 2, 3, 4, 6, 8 and 24 hours post administration.

For feeding studies performed in the dark phase, rats had *ad-libitum* access to food prior to commencement of the study. Animals were orally gavaged at the onset of dark phase (7:00 PM) and were immediately returned to their cages with a pre-weighed amount of food provided. Food intake was measured at 1, 2, 3, 4, 6, 8 and 24 hours post administration. Encapsulated L-Arg.HCl products were made up in either water or vehicle (20% TWEEN20, water). Encapsulated L-Phe were made up in vehicle (10% TWEEN20, water). Encapsulation controls consisted of the corresponding encapsulation matrix for each product, without amino acid payload, and were prepared to contain the same weight of encapsulation matrix as the test agent in each study. Promising candidates were also compared against encapsulation control mixed with un-encapsulated payload. Table 4.2.2 summarises the feeding studies described in this chapter.

Study Number	Product ID	Encapsulated Amino Acid	Dose (mmol/kg)	Animal Feeding State	Study Phase
1	43773	Encapsulated L-Arg.HCl	16	Fasted	Light
1	43767	Encapsulated L-Arg.HCl	16	Fasted	Light
2	43770	Encapsulated L-Arg.HCl	20	Fasted	Light
3	43771	Encapsulated L-Arg.HCl	20	Fasted	Light
4	43772	Encapsulated L-Arg.HCl	20	Fasted	Light
5	43772	Encapsulated L-Arg.HCl	12	*Ad-libitum* fed	Dark
6	43772	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
7	43999	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
8	44000	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
9	44001	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
10	44002	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
11	44003	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
12	44004	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
13	44007	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
14	44008	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
15	44009	Encapsulated L-Arg.HCl	12	*Ad-libitum* fed	Dark
16	44009	Encapsulated L-Arg.HCl	12	*Ad-libitum* fed	Light
17	44009	Encapsulated L-Arg.HCl	16	*Ad-libitum* fed	Dark
18	44010	Encapsulated L-Arg.HCl	12	*Ad-libitum* fed	Dark
19	200240	Encapsulated L-Phenylalanine	6	Fasted	Light
20	200262	Encapsulated L-Phenylalanine	6	Fasted	Light
21	200240	Encapsulated L-Phenylalanine	6	*Ad-libitum* fed	Dark
22	200262	Encapsulated L-Phenylalanine	6	*Ad-libitum* fed	Dark

Table 4.2.2 Feeding studies: Investigating the effect of encapsulated amino acids on food intake in rats.
Feeding studies investigating the effect of encapsulated amino acids on food intake in rats. The table represents study conditions including tested dose, study phase and feeding state of rats.

4.2.5 The effect of encapsulated L-Arg.HCl #43772 on plasma L-arginine, GLP-1 and PYY levels in rats

Rats were fasted for 16 hours overnight and then received an oral dose of 16 mmol/kg un-encapsulated or encapsulated L-Arg.HCl #43772. Animals were culled by decapitation at 30, 60 or 90 minutes following administration and trunk blood collected as previously described in section 2.2.7.5.

L-arginine levels in plasma were measured using a specific ELISA kit for L-arginine (Cloud-Clone Corp, Houston, USA). Rat plasma samples were diluted 1:60 and 50µl was assayed in duplicate according to the manufacturer's instructions.

GLP-1 and PYY levels were determined using specific in-house RIA assays as previously described in sections 2.2.7.3 and 2.2.7.4

4.2.6 The effect of OG administration of encapsulated L-Phe on food intake in *ad libitum* fed rats in the early hours of dark phase

Rats were acclimatised to the plexiglass cages for 24 hours to generate stable reference data against which to test the effects of treatments. Rats had *ad libitum* access to food and were orally gavaged with vehicle (10% TWEEN20, water), 6 mmol/kg L-Phe (Sigma, Poole, UK) or 6 mmol/kg encapsulated L-Phe at 7.00 PM (onset of dark phase). Animals were then individually placed in a 24-chamber open-circuit Oxymax CLAMS (Columbus Instruments, OH, USA) cages. Animals continued to have *ad libitum* access to food following administration. Food intake recordings were taken both at every minute and every 12 minutes intervals following administration of the treatments for 12 hours.

4.2.7 Statistical analysis

All data are expressed as mean ± SEM. All feeding studies were analysed using one-way ANOVA with appropriate Tukey's or Bonferroni's *post hoc* tests. Gut hormone study and the study measuring L-arginine levels in rats were analysed using one-way ANOVA with Tukey's

post hoc test. Amino acid secretion *in vitro* data was expressed as fold change hormone release from the baseline control and were analysed using one-way ANOVA with Bonferroni's *post hoc* test. Feeding studies in CLAMS metabolic cages were analysed using two-way ANOVA with Bonferroni's *post hoc* test and the generalized estimating equation analysis (STATA9 software) for overall significance between treatment groups. $P < 0.05$ was considered statistically significant. All analysis was carried out using Graphpad Prism software (Prism 6.03, GraphPad Software Inc, CA, USA).

4.3 Results

4.3.1 The effect of proteinogenic amino acids on GLP-1 release from mice primary colonic culture

The effect of individual proteinogenic amino acids on GLP-1 release was examined following incubation of colonic culture with 100mM of each amino acid. Amongst the tested amino acids, L-Phe, L-Gln and L-Arg significantly stimulated GLP-1 release (control: 1.00 ± 0.19 fold change vs. L-Phe: 2.40 ± 0.43 fold change, $p<0.01$. vs. L-Gln: 2.84 ± 0.47 fold change, $p<0.001$. vs L-Arg: 3.58 ± 0.65 fold change, $p<0.001$, n=8 plates). There was no significant difference on GLP-1 release following incubation with the other amino acids tested. L-Tyr and L-Trp were not tested due to poor solubility.
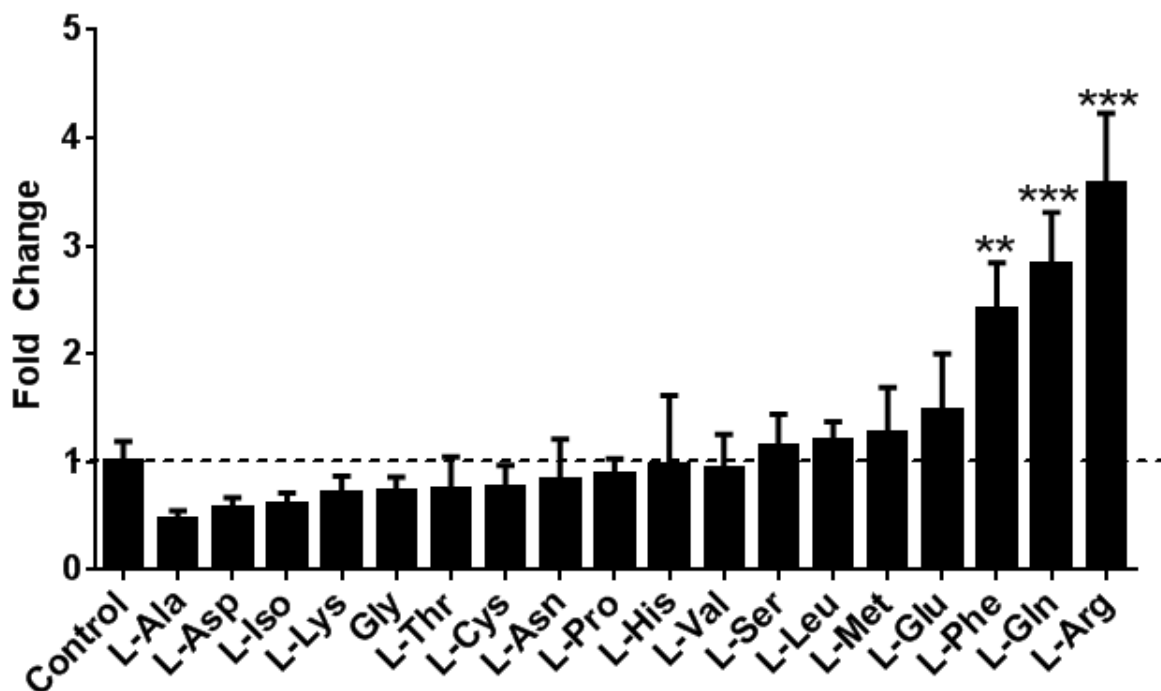
Figure 4.3.1 The effect of proteinogenic amino acids on GLP-1 release from primary mice colonic culture.
The effect of 100mM individual proteionogenic amino acids on GLP-1 release following 2 hours incubation. The data represents fold change from the baseline for every amino acid, apart from L-Tyr and L-Trp, which were not measured due to poor solubility. For L-Glu, L-Asp, L-Arg and L-Lys, neutral salts glutamate, aspartate, L-Arg.HCl and L-Lys.HCl were used respectively to avoid any pH dependent effects. Data presented as mean ± SEM. n = 8 plates from 8 mice. **P<0.01, ***p<0.001 vs control.

4.3.2 The effect of encapsulated L-Arg.HCl on food intake in rats: Part 1

The encapsulation of L-Arg.HCl was carried out using natural vegetable fat and waxes. The initial batch of encapsulated L-Arg.HCl was produced using carnauba and candellila wax coatings (Table 4.3.1). Carnuaba wax and candellila wax are two well-characterised waxes routinely used for microencapsulation in the food industry (Gaonkar et al., 2014).

Product ID	Encapsulation Matrix	L-Arg.HCl composition
43773	49.5% Candelilla Wax + 1% Silica	49.5% L-Arg.HCl (cone milled)
43767	49.5% Candelilla Wax + 1% Silica	49.5% L-Arg.HCl (rotor milled)
43770	47.5% Fully hydrogenated rapeseed oil + 2% Carnauba Wax + 1% Silica	49.5% L-Arg.HCl
43771	24.5% Fully hydrogenated rapeseed oil + 24.5% Carnauba Wax + 1% Silica	50% L-Arg.HCl
43772	49.5% Carnauba Wax + 1% Silica	49.5% L-Arg.HCl

Table 4.3.1 First generation encapsulated L-Arg.HCl products.
The encapsulation matrix and L-Arg.HCl composition for each product is presented. The compositions are presented as percent weight: weight ratio.

4.3.2.1 The effect of OG administration of encapsulated L-Arg.HCl #43767 and #43773 on food intake in fasted rats in the early light phase

Encapsulated L-Arg.HCl #43767 (49.5% Candelila wax + 1% Silica + 49.5% L-Arg.HCl, rotor milled) and encapsulated L-Arg.HCl #43773 (49.5% Candelila wax + 1% Silica + 49.5% L-Arg.HCl, cone milled) were administered at an estimated dose of 16 mmol L-Arg.HCl /kg to fasted rats. Encapsulated product #43767 significantly reduced food intake compared to the encapsulation control during the 0-1 hour post administration. (Encapsulation control: 6.31 ± 0.47g vs. #43767: 4.71 ± 0.24g, $p < 0.05$, n=5-11). Encapsulated #43773 and un-encapsulated L-Arg.HCl reduced food intake at 0-1 hours at a similar magnitude to #43767, but these effects did not reach statistical significance. There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.2).

Figure 4.3.2 The effect of encapsulated L-Arg.HCl #43767 and #43773 on food intake in fasted rats in the early light phase.
The effect of OG of water (control), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, encapsulated L-Arg.HCl #43767 or encapsulated L-Arg.HCl #43773 on food intake in overnight fasted rats injected in early hours of the light phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6 and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 5-11. *p<0.05 vs encapsulation control.

4.3.2.2 The effect of OG administration of encapsulated L-Arg.HCl #43770 on food intake in fasted rats in the early light phase

Oral gavage of 20 mmol/kg encapsulated L-Arg.HCl #43770 (47.5% fully hydrogenated rapeseed oil + 2% carnauba wax + 1% silica + 49.5% L-Arg.HCl) significantly reduced food intake compared to vehicle control and encapsulation control at 0-1 hour post administration (vehicle: 6.34 ± 0.31g vs Encapsulated L-Arg.HCl #43770: 4.60 ± 0.42g, p<0.05, n=8-12; encapsulation control: 6.50 ± 0.45g vs. Encapsulated L-Arg.HCl #43770: 4.60 ± 0.42g, p<0.05, n=8-12). Similarly, un-encapsulated L-Arg.HCl significantly reduced food intake compared to the vehicle control group at 0-1 hour post administration (vehicle: 6.34 ± 0.31g vs un-encapsulated L-Arg.HCl: 4.71 ± 0.52g, p<0.05, n=8-12). Food intake remained lower in both encapsulated and un-encapsulated L-Arg.HCl at 1-2 and 2-3 hours interval following administration; however this was not statistically significant (Fig. 4.3.3). There were no significant differences in food intake between groups at any other time point at which it was measured.
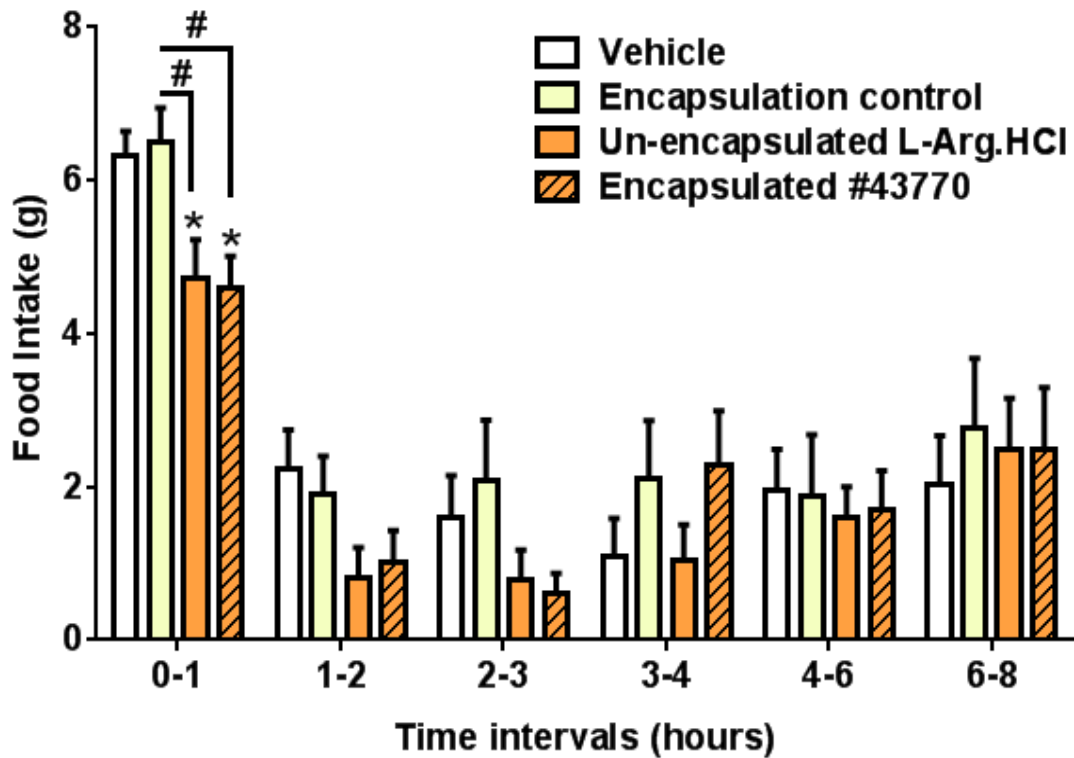
Figure 4.3.3 The effect of encapsulated L-Arg.HCl #43770 on food intake in fasted rats in the early light phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 20 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #43770 on food intake in overnight fasted rats injected in early hours of the light phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6 and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 8-12. *p<0.05 vs vehicle; #p<0.05 vs encapsulation control.

4.3.2.3 The effect of OG administration of encapsulated L-Arg.HCl #43771 on food intake in fasted rats in the early light phase

Oral gavage of 20 mmol/kg encapsulated L-Arg.HCl #43771 (24.5% fully hydrogenated rapeseed oil + 24.5% carnauba wax + 1% silica + 49.5% L-Arg.HCl) reduced food intake at 0-1 hour interval to a similar magnitude to un-encapsulated L-Arg.HCl (vehicle:.6.02 ± 0.41g vs. encapsulated L-Arg.HCl #43771: 4.39 ± 0.47g, p>0.05, n=8-12). In addition, un-encapsulated L-Arg.HCl effect on food intake achieved statistical significance compared to vehicle control and encapsulation control (vehicle: 6.02 ± 0.41g vs. un-encapsulated L-Arg.HCl: 3.81 ± 0.41g, p<0.01; encapsulation control: 5.86 ± 0.50g vs. un-encapsulated L-Arg.HCl: 3.81 ± 0.41g, p<0.01, n=8-12). Food intake was significantly lower in the un-encapsulated L-Arg.HCl group at 1-2 hour compared to controls; however, there was no difference in food intake in encapsulated #43771 group compared to the controls. There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.4).
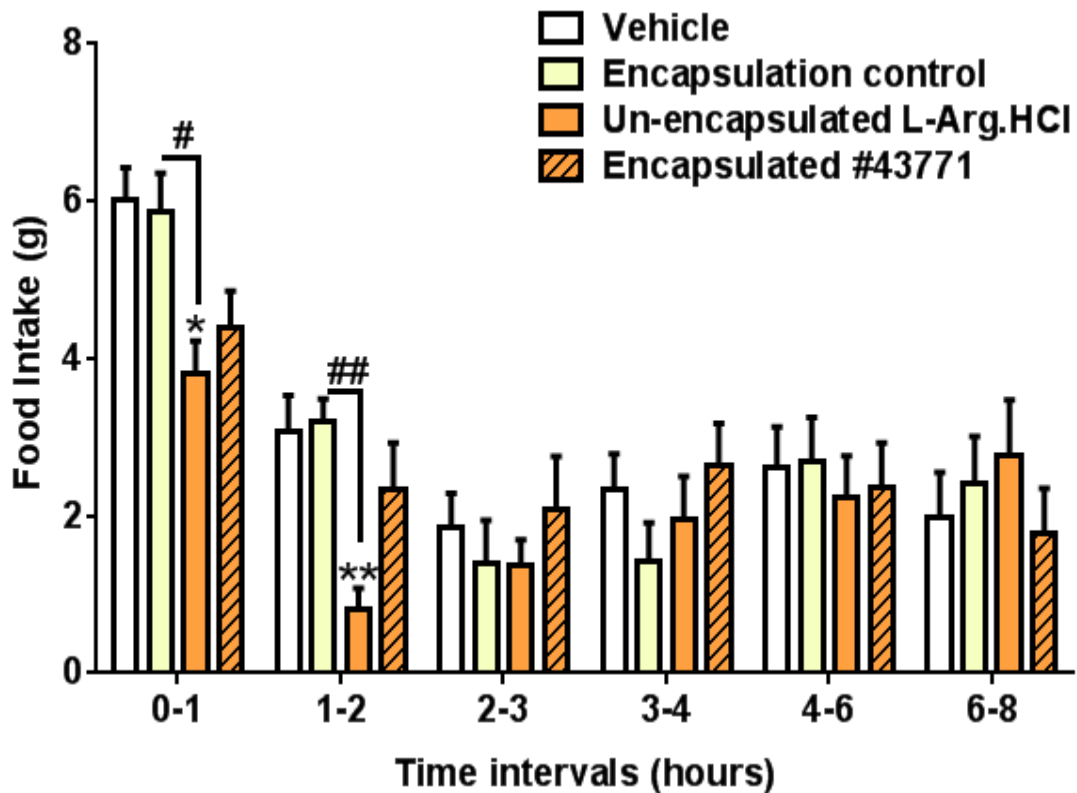
Figure 4.3.4 The effect of encapsulated L-Arg.HCl #43771 on food intake in fasted rats in the early light phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 20 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #43771 on food intake in overnight fasted rats injected in early hours of the light phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6 and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 8-12. *p<0.05, **p<0.01vs vehicle; #p<0.05, ##p<0.01 vs encapsulation control.

4.3.2.4 The effect of OG administration of encapsulated L-Arg.HCl #43772 on food intake in fasted rats in the early light phase

Oral gavage of 20 mmol/kg encapsulated L-Arg.HCl #43772 (49.5% carnauba wax + 1% silica + 49.5% L-Arg.HCl) significantly reduced food intake at 0-1 hour post administration compared to water and encapsulation control (water: 6.58 ± 0.41g vs. encapsulated L-Arg.HCl #43772: 4.49 ± 0.37g, p<0.05; encapsulation control: 6.42 ± 0.45g vs. Encapsulated L-Arg.HCl #43772: 4.49 ± 0.37g, p<0.05, n=8-12). Similarly, un-encapsulated L-Arg.HCl at an identical dose of 20 mmol/kg significantly reduced food intake at 0-1 hour compared to water and encapsulation control (water: 6.58 ± 0.41g vs. un-encapsulated L-Arg.HCl: 3.98 ± 0.72g, p<0.01; encapsulation control: 6.42 ± 0.45g vs. Encapsulated L-Arg.HCl: 3.98 ± 0.72g, p<0.01, n=8-12). Encapsulated L-Arg.HCl #43772 significantly reduced good intake compared to the water control at 2-6 hours (water: 7.87 ± 0.45g vs. encapsulated L-Arg.HCl #43772: 5.26 ± 0.54g, p<0.01, n=8-12). There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.5).

Figure 4.3.5 The effect of encapsulated L-Arg.HCl #43772 on food intake in fasted rats in the early light phase.
The effect of OG of water (control), encapsulation control, 20 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #43772 on food intake in overnight fasted rats injected in early hours of the light phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, 2-6 and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 8-12. *p<0.05, **p<0.01 vs water control. #p<0.05, ##p<0.01 vs encapsulation control.

4.3.2.4.1 The effect of OG administration of encapsulated L-Arg.HCl #43772 on food intake in *ad libitum* fed rats during the early dark phase

Initial screening of the encapsulated products suggested that product #43772, which used a higher percentage carnauba wax coating, was a more suitable candidate for further validation. Therefore the effect of oral administration of #43772 was investigated during the dark phase in rats.

Oral gavage of #43772 was examined at doses of 12 and 16 mmol/kg in two independent feeding studies. Oral gavage of 12 mmol/kg encapsulated L-Arg.HCl #43772 significantly reduced food intake during 0-1 hour following administration (Water: 2.13 ± 0.27g vs. encapsulated L-Arg.HCl #43772: 0.39 ± 0.12g, $p < 0.01$, n=8-12). The anorectic effect appeared greater than that of un-encapsulated L-Arg.HCl.

Similarly, 16 mmol/kg encapsulated L-Arg.HCl #43772 significantly reduced food intake at 0-1 hour in rats following oral gavage (Water: 2.76 ± 0.39g vs. encapsulated L-Arg.HCl #43772: 0.69 ± 0.17g, $p < 0.001$, n=8-11). Although un-encapsulated L-Arg.HCl alone, and in a mixture with the encapsulation control significantly reduced food intake compared to water control (Water: 2.13 ± 0.27g vs. encapsulation control and L-Arg.HCl mixture: 1.34 ± 0.30g, $p < 0.05$; Water: 2.13 ± 0.27g vs vs. un-encapsulated L-Arg.HCl: 1.43 ± 0.37g, $p < 0.05$, n=8-11), the anorectic effect appeared greater in encapsulated #43772. There were no significant differences in food intake between groups at any other time point at which it was measured (Fig 4.3.6).

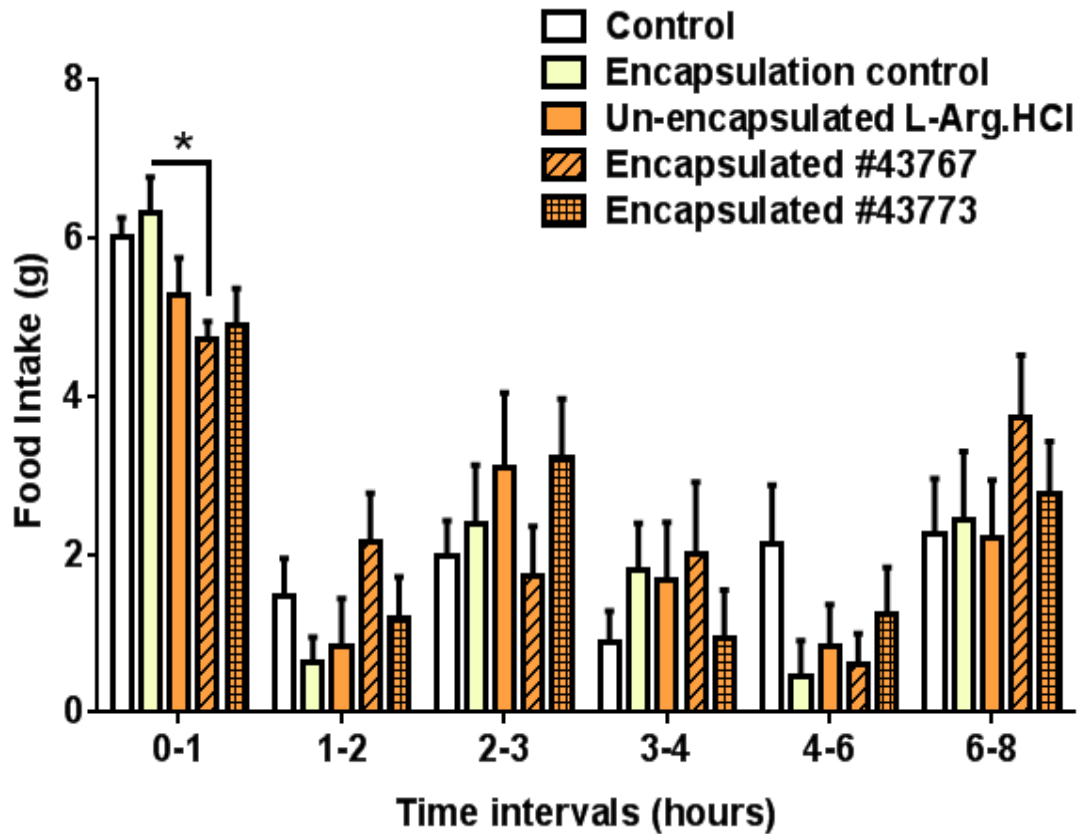**Figure 4.3.6 The effect of OG of encapsulated L-Arg.HCl #43772 on food intake in *ad libitum* rats during the early dark phase.**

The effect of OG of water (control), encapsulation control, 12 mmol/kg encapsulated L-Arg.HCl #43772 or un-encapsulated L-Arg.HCl on food intake at 0-1 hour in *ad libitum* fed rats during the dark phase (A), Data is presented as mean ± SEM. n = 8-12. **p<0.01. The effect of OG of water, encapsulation control and en-encapsulated L-Arg.HCl, 16 mmol/kg encapsulated L-Arg.HCl #43772 or un-encapsulated L-Arg.HCl on food intake at 0-1 hour in ad-libitum fed rats during dark phase (B), Data is presented as mean ± SEM. n = 8-11. *p<0.05, ***p<0.001 vs water control.

4.3.2.4.2 The effect of OG of encapsulated L-Arg.HCl #43772 on circulating L-arginine levels in fasted rats in the early light phase

In order to investigate the effect of encapsulation on L-Arg.HCl release, L-arginine levels were measured in plasma following oral administration of L-Arg.HCl #43772 and un-encapsulated L-Arg.HCl.

Oral gavage of 16 mmol/kg of un-encapsulated and encapsulated L-Arg.HCl #43772 elevated L-arginine levels in circulation at 30, 60 and 90 minutes following administration. The effect reached statistical significance at 30 minutes in encapsulated L-Arg.HCl compared to the basal levels (Basal L-arginine: 18.32 ± 4.09 µg/ml vs. Encapsulated L-Arg.HCl at 30 minutes: 142.2 ± 37.62 µg/ml, $p<0.05$, $n=4-6$). There was no significant difference between the L-arginine levels following oral administration of encapsulated and un-encapsulated L-Arg.HCl at the other time point (Fig.4.3.7).
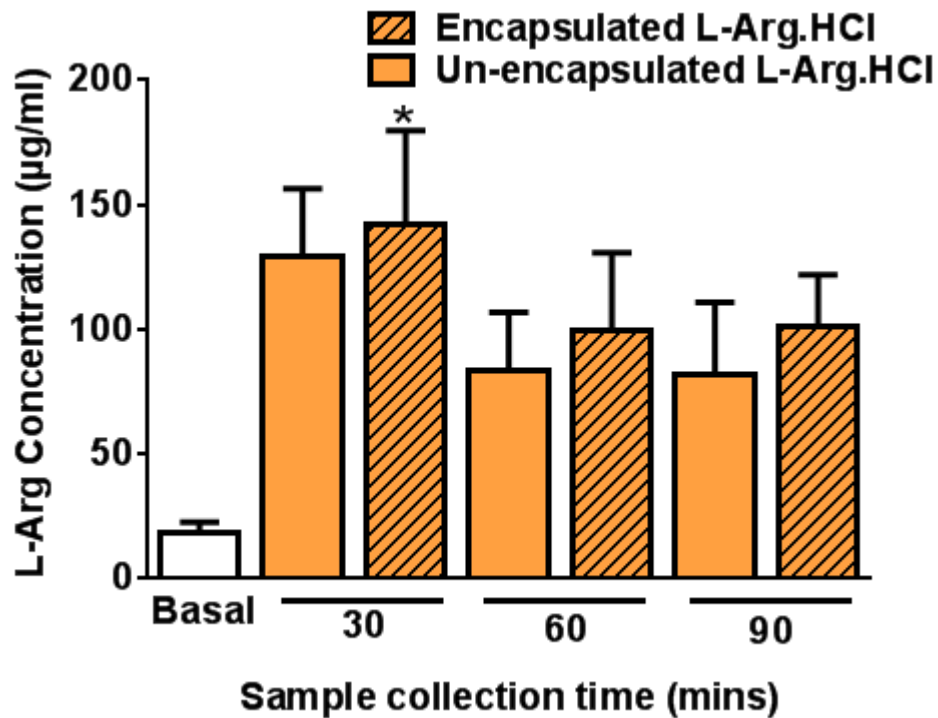
Figure 4.3.7 The effect of OG of encapsulated L-Arg.HCl on circulating L-arginine levels in fasted rats in the early light phase.
The effect of OG of 16 mmol/kg un-encapsulated and encapsulated L-Arg.HCl #43772 on L-arginine levels in circulation in overnight fasted rats measured at 30, 60 and 90 minutes post administration. Basal arginine was measured from untreated rats. Data is presented as mean ± SEM. n = 4-6, *p<0.05 vs basal L-arginine

4.3.2.4.3 The effect of encapsulated L-Arg.HCl #43772 on GLP-1 and PYY release in fasted rats in the early light phase

Oral gavage of 16 mmol/kg both un-encapsulated and encapsulated L-Arg.HCl #43772 significantly elevated basal GLP-1 and PYY levels in rats at 30, 60 and 90 minutes post administration (Basal GLP-1: 26.49 ± 6.94 pmol/L vs. un-encapsulated L-Arg.HCl at 60 minutes: 60.97 ± 5.94 pmol/L, $p<0.05$; vs. encapsulated L-Arg.HCl at 60 minutes: 59.97 ± 3.30 pmol/L, $p<0.05$; vs. encapsulated L-Arg.HCl at 90 minutes: 72.09 ± 8.41 pmol/L, $p<0.001$, n=4-6).

At 90 minutes, there was a significant difference in GLP-1 levels between rats treated with un-encapsulated and those treated with encapsulated L-Arg.HCl (Un-encapsulated L-Arg.HCl: 72.09 ± 8.41pmol/L vs. encapsulated L-Arg.HCl #43772: 36.39 ± 5.65pmol/L, $p<0.01$, n=4-6) (Fig 4.3.8 A).

Similarly, PYY levels were elevated at 30, 60 and 90 minutes post administration. At 30 minutes post administration, PYY levels were significantly higher compared to the basal PYY levels (Basal PYY: 29.47 ± 21.31 pmol/L vs. encapsulated L-Arg.HCl at 30 minutes: 106.6 ± 25.62 pmol/L, $p<0.05$, n=4-6). However there were no significant difference in PYY levels between encapsulated and un-encapsulated L-Arg.HCl at each time point (Fig. 4.3.8 B).
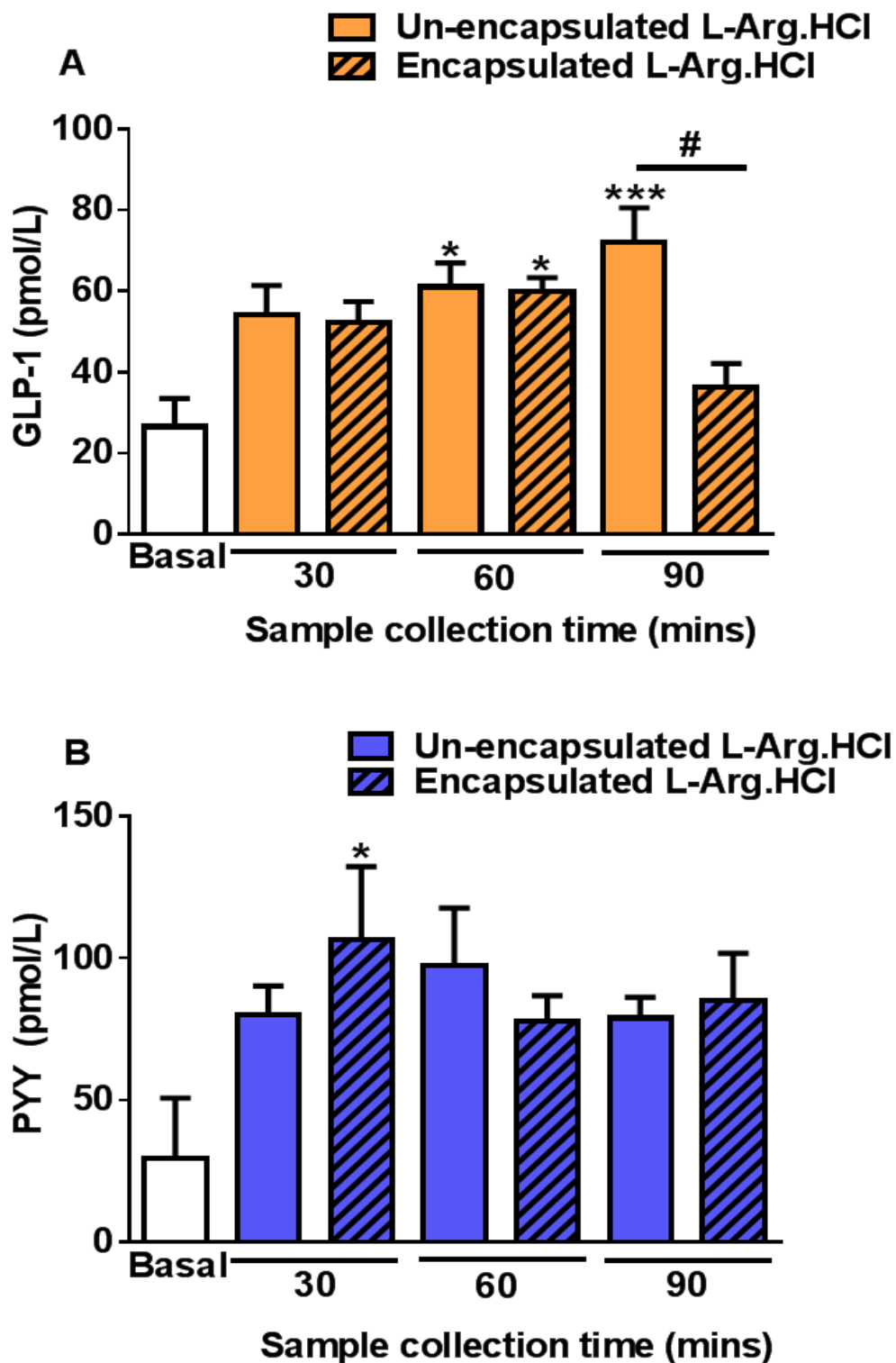
Figure 4.3.8 The effect of OG of encapsulated L-Arg.HCl #43772 on circulating GLP-1 and PYY levels in fasted rats in the early light phase.
The effect OG of 16 mmol/kg un-encapsulated L-Arg.HCl and encapsulated L-Arg.HCl #43772 on plasma GLP-1 (A) and PYY (B) levels in overnight fasted rats. Basal levels were measured from untreated rats. Data is presented as mean ± SEM. n = 4-6, *$p<0.05$ ***$p<0.001$ vs basal levels; #$p<0.05$ vs un-encapsulated L-Arg.HCl at 90 minutes.

4.3.3 The effect of encapsulated L-Arg.HCl on food intake in rats: Part 2

Following the testing of the initial encapsulated products, it appeared that a higher percentage carnauba wax resulted in a more efficient coating matrix than others tested as it appeared to alter the magnitude of the anorectic response and to delay the anorectic effect in rats. Therefore, a series of encapsulated L-Arg.HCl products were developed using a combination of carnauba wax and other available natural wax products such as bees wax and paraffin wax. The details of the encapsulated products are summarised in Table 4.3.2. The effect of oral administration of these products on food intake was investigated in *ad libitum* fed rats in the early dark phase, as it was thought that they might be more sensitive to the effects of the products than fasted rats.

Product ID	Encapsulation Matrix	L-Arg.HCl composition
43999	50% Synthetic Microcrystalline wax	50% L-Arg.HCl
44000	25% Synthetic Microcrystalline wax + 25% Carnauba Wax	50% L-Arg.HCl
44001	25% Glyceryl Behenate + 25% Carnauba wax	50% L-Arg.HCl
44002	25% Bees wax + 25% Carnauba wax	50% L-Arg.HCl
44003	25% Rice bran wax + 25% Carnauba wax	50% L-Arg.HCl
44004	25% Sunflower wax + 25%Carnauba wax	50% L-Arg.HCl
44007	50% Paraffin wax	50% L-Arg.HCl
44008	25% Paraffin wax + 25% Carnauba wax	50% L-Arg.HCl
44009	60% Carnauba wax	40% L-Arg.HCl
44010	55% Carnauba wax	45% L-Arg.HCl

Table 4.3.2 Second generation encapsulated L-Arg.HCl products.
The encapsulation matrix and L-Arg.HCl composition for each product is presented. The compositions are presented as percent weight: weight ratio.

4.3.3.1 The effect of OG administration of encapsulated L-Arg.HCl #43999 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 16 mmol/kg un-encapsulated L-Arg.HCl significantly reduced food intake at 0-1 hour following administration compared to the vehicle control (vehicle:2.50 ± 0.023g vs. un-encapsulated L-Arg.HCl: 1.70 ± 0.31g, $p < 0.05$, n=6-7). Oral administration of encapsulated L-Arg.HCl #43999 (50% synthetic microcrystalline wax + 50% L-Arg.HCl) had no significant effect on food intake at 0-1 hour. Food intake was lower to similar magnitude as un-encapsulated L-Arg.HCl during 1-2 hour time interval; however these observations did not reach statistical significance. There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.9).

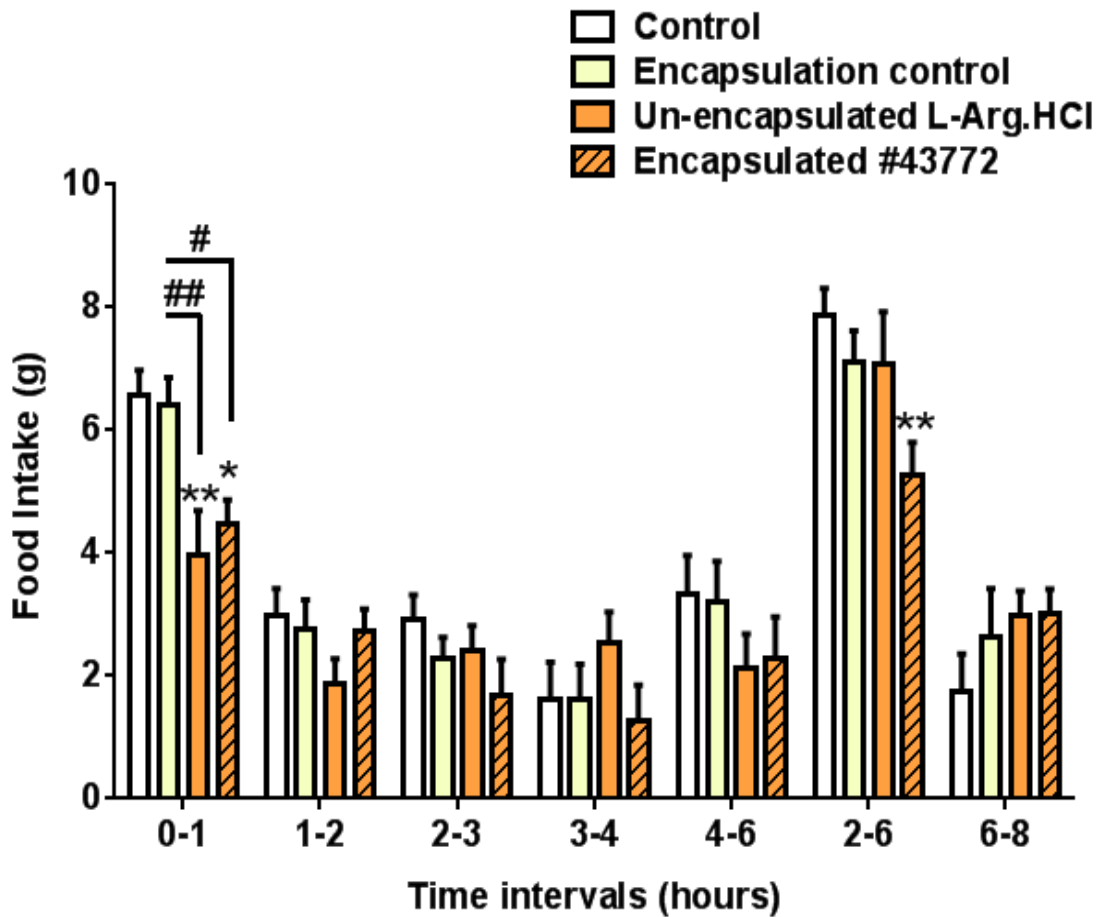**Figure 4.3.9 The effect of encapsulated L-Arg.HCl #43999 on food intake in *ad libitum* fed rats in the early dark phase**

The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #43999 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7. *p<0.05 vs vehicle control.

4.3.3.2 The effect of OG administration of encapsulated L-Arg.HCl #44000 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 16 mmol/kg encapsulated L-Arg.HCl #44000 (25% synthetic microcrystalline wax + 25% carnauba wax + 50% L-Arg.HCl) had no significant effect on food intake in rats. Oral gavage of 16 mmol/kg un-encapsulated L-Arg.HCl significantly reduced food intake at 0-1 hour following administration compared to the vehicle control (vehicle:2.50 ± 0.023g vs. un-encapsulated L-Arg.HCl: 1.70 ± 0.31g, $p < 0.05$, n=6-7). There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.10).
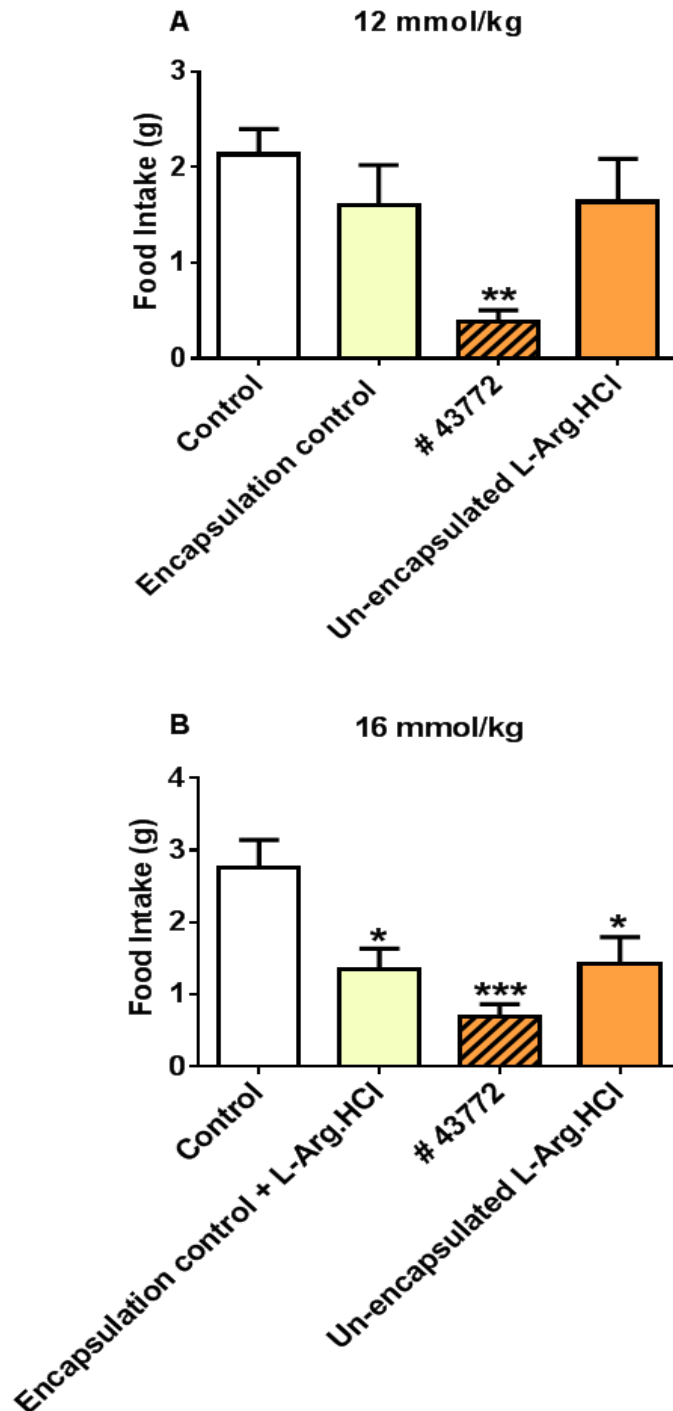
Figure 4.3.10 The effect of encapsulated L-Arg.HCl #44000 on food intake in *ad libitum* fed rats in the early dark phase.

The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44000 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7. *p<0.05 vs vehicle control.

4.3.3.3 The effect of OG administration of encapsulated L-Arg.HCl #44001 on food intake in *ad libitum* fed rats in the early dark phase

Oral administration of encapsulated L-Arg.HCl #44001 (25% glyceryl behenate + 25% carnauba wax + 50% L-Arg.HCl) at a dose of 16 mmol/kg had no significant effect on food intake in rats. In comparison, un-encapsulated L-Arg.HCl reduced food intake in the early hours following administration. The effect was statistically different compared to encapsulated L-Arg.HCl during 2-3 hour time interval (un-encapsulated L-Arg.HCl: 1.10 ± 0.64g vs. encapsulated L-Arg.HCl #44001: 3.03 ± 0.32g, $p < 0.05$, n=6-7). There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.11).

Figure 4.3.11 The effect of encapsulated L-Arg.HCl #44001 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44001 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7. *p<0.05 vs encapsulated L-Arg.HCl.

4.3.3.4 The effect of OG administration of encapsulated L-Arg.HCl #44002 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 16 mmol/kg encapsulated L-Arg.HCl #44002 (25% bees wax + 25% carnauba wax + 50% L-Arg.HCl) reduced food intake to a similar magnitude to un-encapsulated L-Arg.HCl at 0-1 hour post administration. Food intake was similarly lower in both encapsulated and un-encapsulated forms of L-Arg.HCl during 2-3 hour time interval. However, these effects did not reach statistical significance, and there were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.12).

Figure 4.3.12 The effect of encapsulated L-Arg.HCl #44002 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44002 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7.

4.3.3.5 The effect of OG administration of encapsulated L-Arg.HCl #44003 on food intake in *ad libitum* fed rats in the early dark phase

Oral administration of 16 mmol/kg encapsulated L-Arg.HCl #44003 (25% rice bran wax + 25% carnauba wax + 50% L-Arg.HCl) reduced food intake at 0-1 hour post administration when compared to vehicle and encapsulation control. Un-encapsulated L-Arg.HCl significantly reduced food intake compared to the encapsulation control during 0-1 hour time point (encapsulation control: 3.08 ± 0.26g vs. un-encapsulated L-Arg.HCl: 1.35 ± 0.34g, $p < 0.05$, n=6-7). There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.13).

Figure 4.3.13 The effect of encapsulated L-Arg.HCl #44003 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44003 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7. *$p<0.05$ vs encapsulation control.

4.3.3.6 The effect of OG administration of encapsulated L-Arg.HCl #44004 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 16 mmol/kg un-encapsulated L-Arg.HCl significantly reduced food intake during 0-1 hour time interval post administration (vehicle: 2.70 ± 0.42g vs. Un-encapsulated L-Arg.HCl: 1.35 ± 0.34g, $p < 0.05$, n=6-7). However, oral administration of encapsulated L-Arg.HCl #44004 (25% sunflower wax + 25% carnauba wax + 50% L-Arg.HCl) had no significant effect on food intake in rats. There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.14).

Figure 4.3.14 The effect of encapsulated L-Arg.HCl #44004 on food intake in *ad libitum* fed rats in the early dark phase.

The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44004 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7. *p<0.05 vs vehicle control.

4.3.3.7 The effect of OG administration of encapsulated L-Arg.HCl #44007 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 16 mmol/kg encapsulated L-Arg.HCl #44007 (50% paraffin + 50% L-Arg.HCl) had no significant effect on food intake in rats There were no significant differences in food intake between groups at any time point at which it was measured (Fig. 4.3.15).

Figure 4.3.15 The effect of encapsulated L-Arg.HCl #44007 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44007 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7.

4.3.3.8 The effect of OG administration of encapsulated L-Arg.HCl #44008 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 16 mmol/kg encapsulated L-Arg.HCl #44008 (25% paraffin wax + 25% carnauba wax + 50% L-Arg.HCl) reduced food intake at 0-1 hour time interval following administration compared to vehicle and to similar magnitude to un-encapsulated L-Arg.HCl. However, this effect did not reach statistical significance, and were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.16).
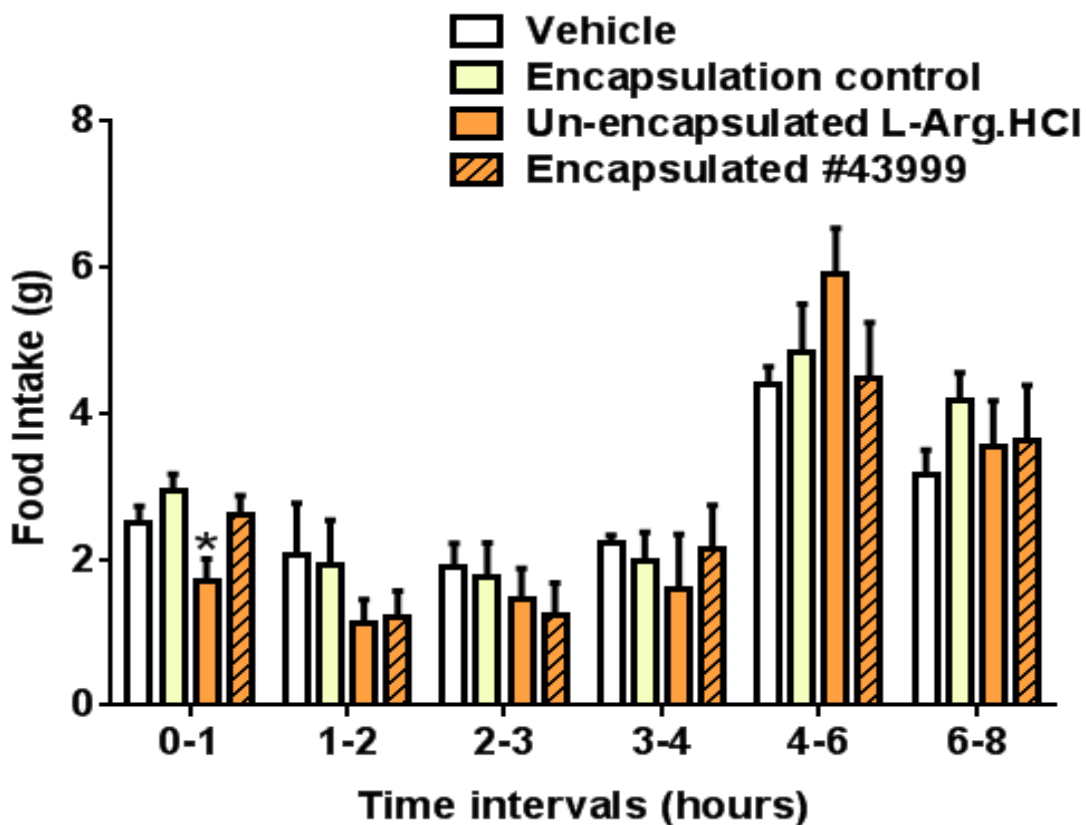
Figure 4.3.16 The effect of encapsulated L-Arg.HCl #44008 on food intake in *ad libitum* fed rats in the early dark phase.

The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44008 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 6-7.

4.3.3.9 The effect of OG administration of encapsulated L-Arg.HCl #44009 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 12 mmol/kg encapsulated L-Arg.HCl #44009 (60% carnauba wax + 40% L-Arg.HCl) had no significant effect on food intake during 0-1 hour following administration. Due to initial solubility issues, a lower dose of 12 mmol/kg was used. Un-encapsulated L-Arg.HCl significantly reduced food intake compared to the vehicle control group (vehicle: 2.50 ± 0.27g vs. un-encapsulated L-Arg.HCl: 0.78 ± 0.21g, $p < 0.001$, n=9-11) and encapsulated L-Arg.HCl #44009 (encapsulated L-Arg.HCl #44009: 1.72 ± 0.23g vs. un-encapsulated L-Arg.HCl: 0.78 ± 0.21g, $p < 0.05$, n=9-11) at 0-1 hour time point. There were no significant differences in food intake between groups at any other time point at which it was measured (Fig. 4.3.17).
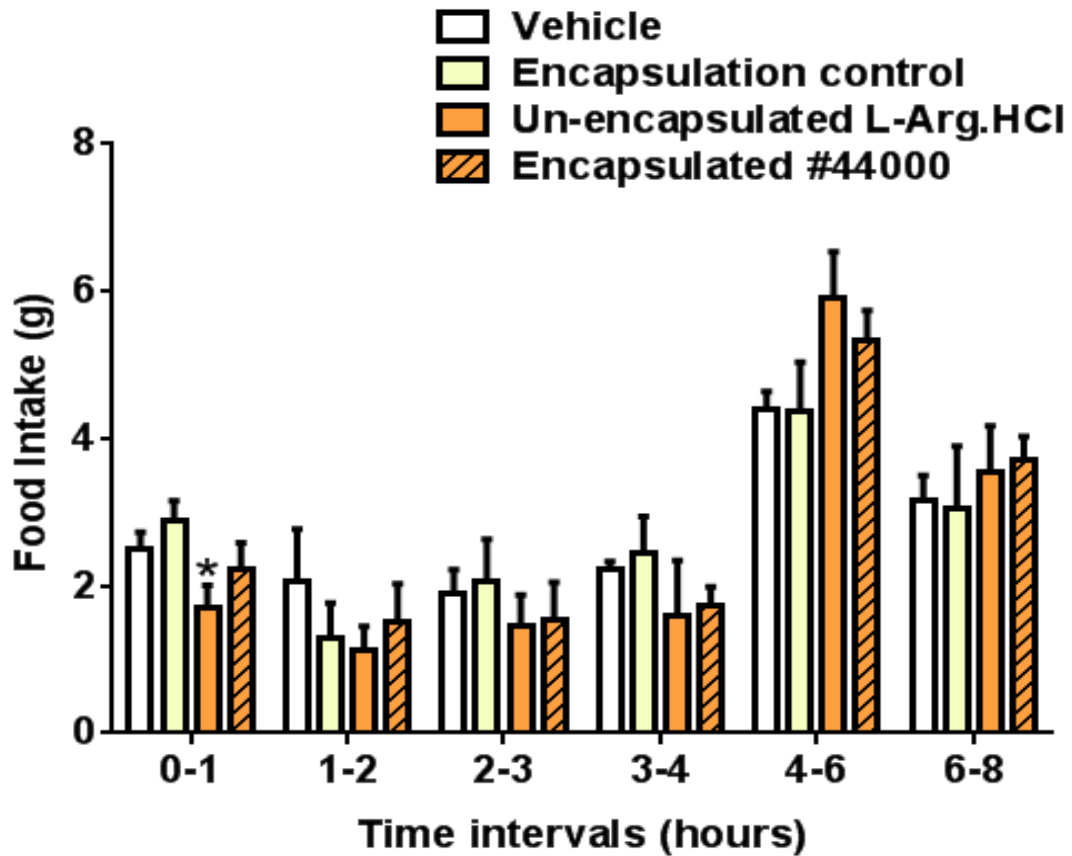
Figure 4.3.17 The effect of encapsulated L-Arg.HCl #44009 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 12 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44009 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 9-11.*p<0.05 vs encapsulated L-Arg.HCl #44009, ***p<0.001 vs vehicle control.

4.3.3.9.1 The effect of OG of encapsulated L-Arg.HCl #44009 on food intake in *ad libitum* fed rats during the light and dark phase

The effect of oral administration of encapsulated L-Arg.HCl #44009 was further examined using 12 and 16 mmol/kg in *ad libitum* fed rats administered in light phase and dark phase respectively.

Oral gavage of 12 mmol/kg encapsulated L-Arg.HCl #44009 reduced food intake at 0-1 hour post administration in *ad libitum* fed rats during early hours of light phase (Encapsulation control: 2.46 ± 0.49g vs. encapsulated L-Arg.HCl #44009: 1.46 ± 0.34g, $p>0.05$, n=9-11). Similarly un-encapsulated L-Arg.HCl significantly reduced food intake at 0-1 hour time point (Encapsulation control: 2.46 ± 0.49g vs. un-encapsulated L-Arg.HCl: 1.01 ± 0.44g, $p<0.05$, n=9-11) (Fig. 4.3.18 A). Furthermore, the cumulative food intake between 1-4 hours post administration was lower in encapsulated L-Arg.HCl #44009 compared to the encapsulation control and un-encapsulated L-Arg.HCl, though this effect did not quite achieve statistical significance (Encapsulation control: 2.84 ± 0.60g vs. encapsulated L-Arg.HCl #44009: 1.33 ± 0.44g, $p=0.056$, n=9-11) (Fig. 4.3.18 B).

Oral gavage of 16 mmol/kg encapsulated L-Arg.HCl #44009 significantly reduced food intake in *ad libitum* fed rats during 0-1 hour post administration in dark phase (Encapsulation control: 2.43 ± 0.20g vs. encapsulated L-Arg.HCl #44009: 1.72 ± 0.23g, $p<0.05$, n=9-11). Encapsulated L-Arg.HCl had also reduced food intake significantly and to a greater magnitude compared to the encapsulated form (Encapsulation control: 2.43 ± 0.20g vs. un-encapsulated L-Arg.HCl: 0.78 ± 0.21g, $p<0.001$, n=9-11) (Fig. 4.3.18 C). Cumulative food intake at 1-4 hours post administration showed a significant reduction in food intake with both encapsulated and un-encapsulated form of L-Arg.HCl (Fig. 4.3.18 D). There were no significant differences in food intake between groups at any other time point at which it was measured.
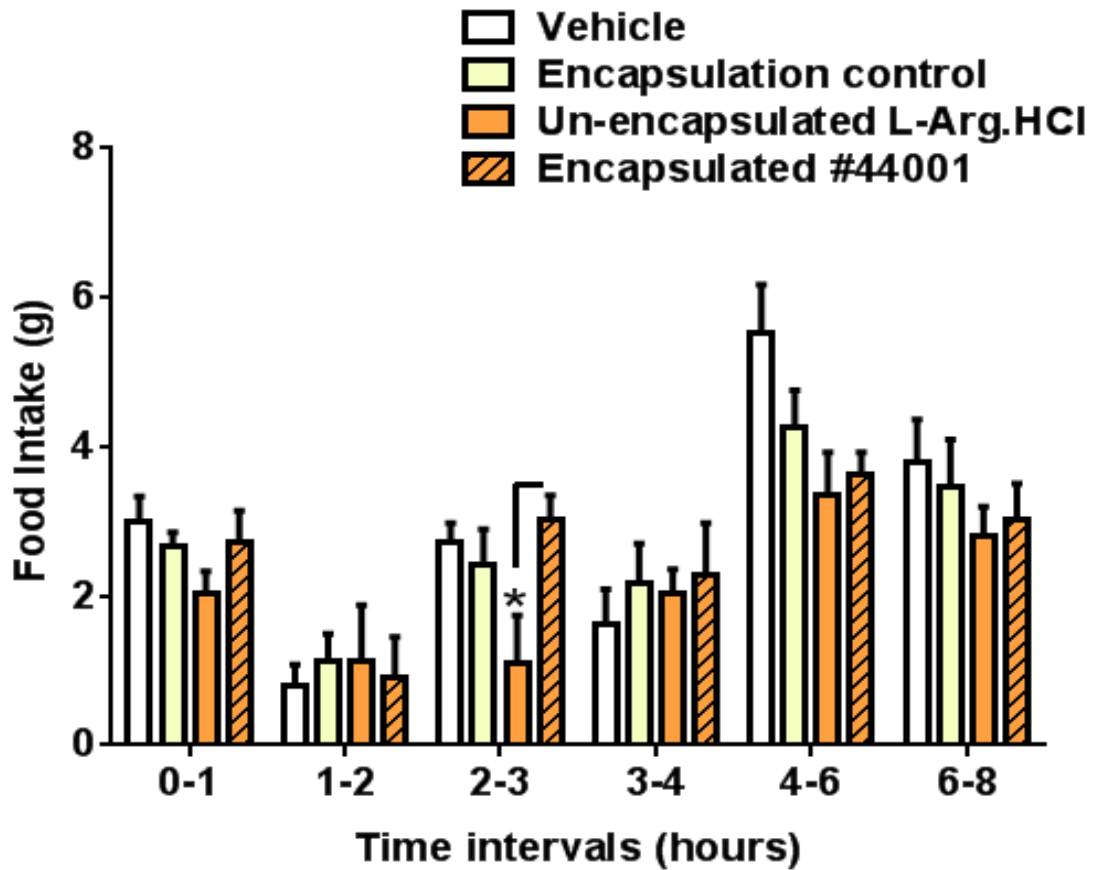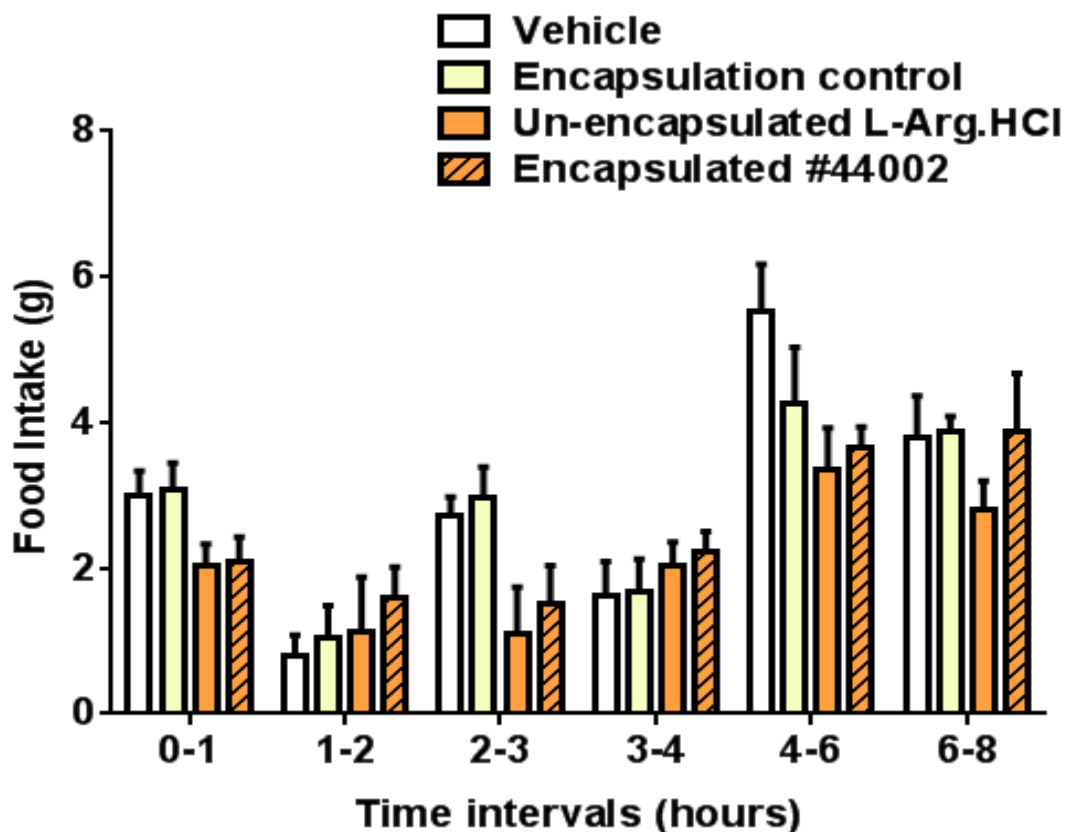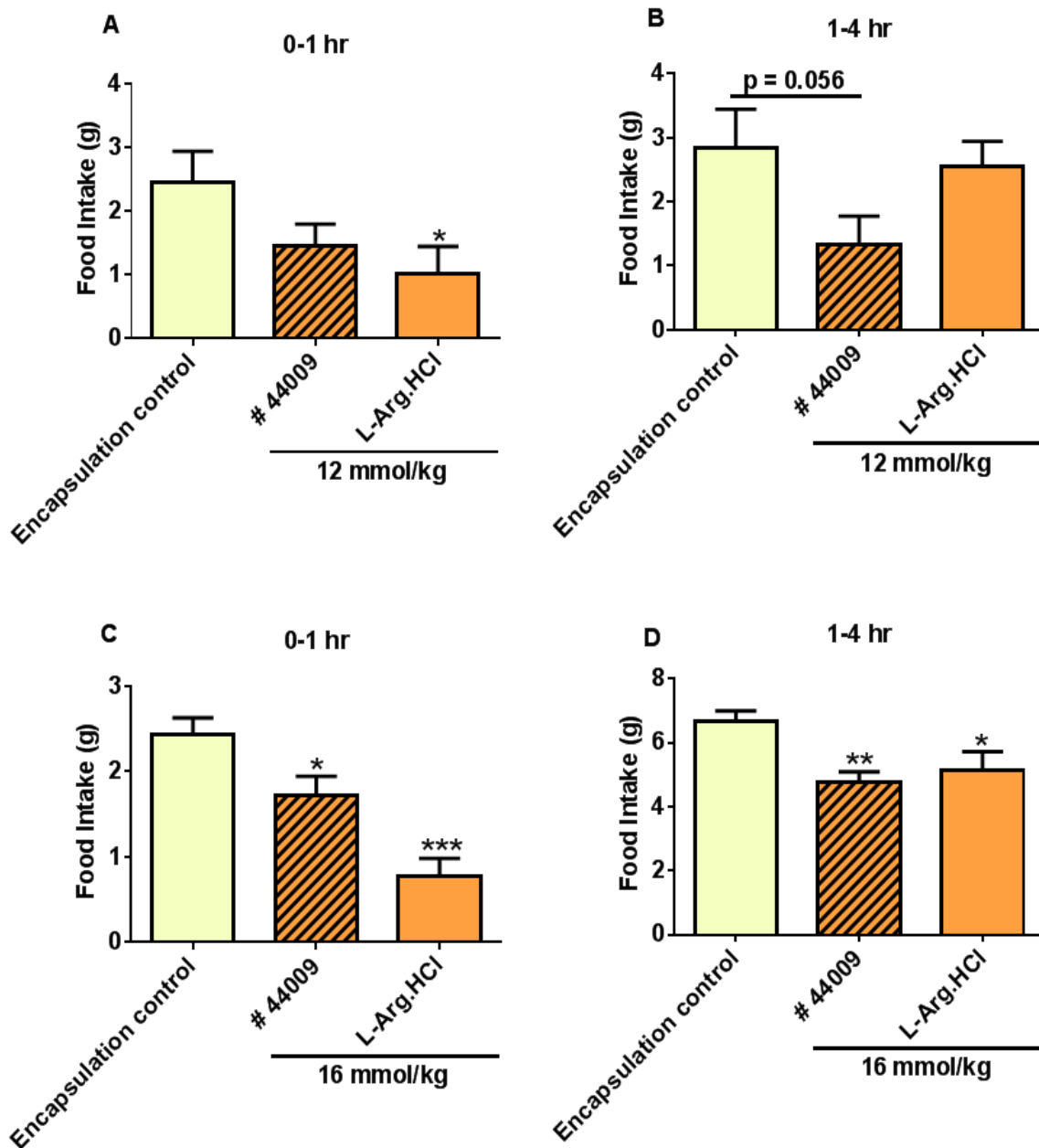
Figure 4.3.18 The effect of encapsulated L-Arg.HCl #44009 on food intake in *ad libitum* fed rats.
The effect of OG of encapsulation control, 12 mmol/kg encapsulated L-Arg.HCl #43772 or un-encapsulated L-Arg.HCl on food intake at 0-1 (A) and 1-4 hour (B) during light phase; and 16 mmol/kg encapsulated L-Arg.HCl #43772 or un-encapsulated L-Arg.HCl on food intake at 0-1 (C) and 1-4 (D) hour during dark phase in *ad libitum* fed rats. Data is presented as mean ± SEM. n = 9-11. *p<0.05, ***p<0.01 vs encapsulation control.

4.3.3.10 The effect of OG administration of encapsulated L-Arg.HCl #44010 on food intake in *ad libitum* fed rats in the early dark phase

Oral gavage of 12 mmol/kg encapsulated L-Arg.HCl #44010 (55% carnauba wax + 45% L-Arg.HCl) had no significant effect on food intake in rats, and there were no significant differences in food intake between groups at any time point at which it was measured (Fig. 4.3.19).
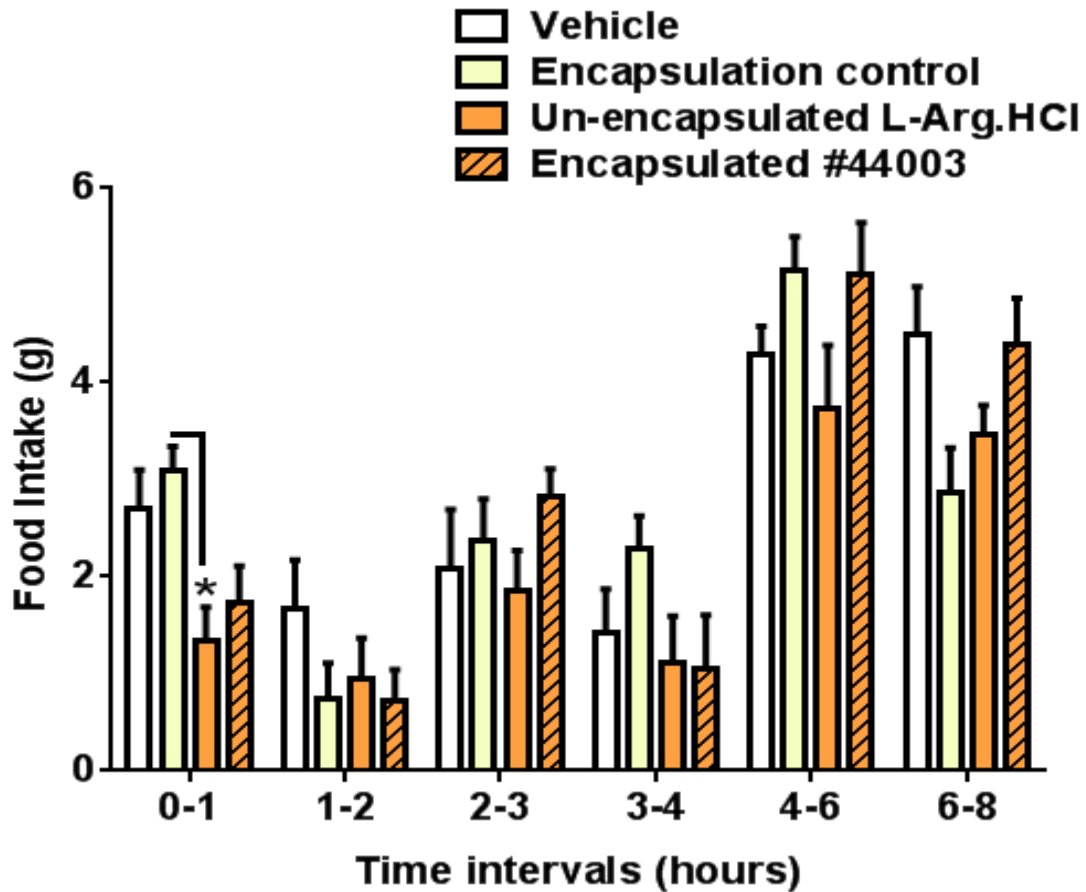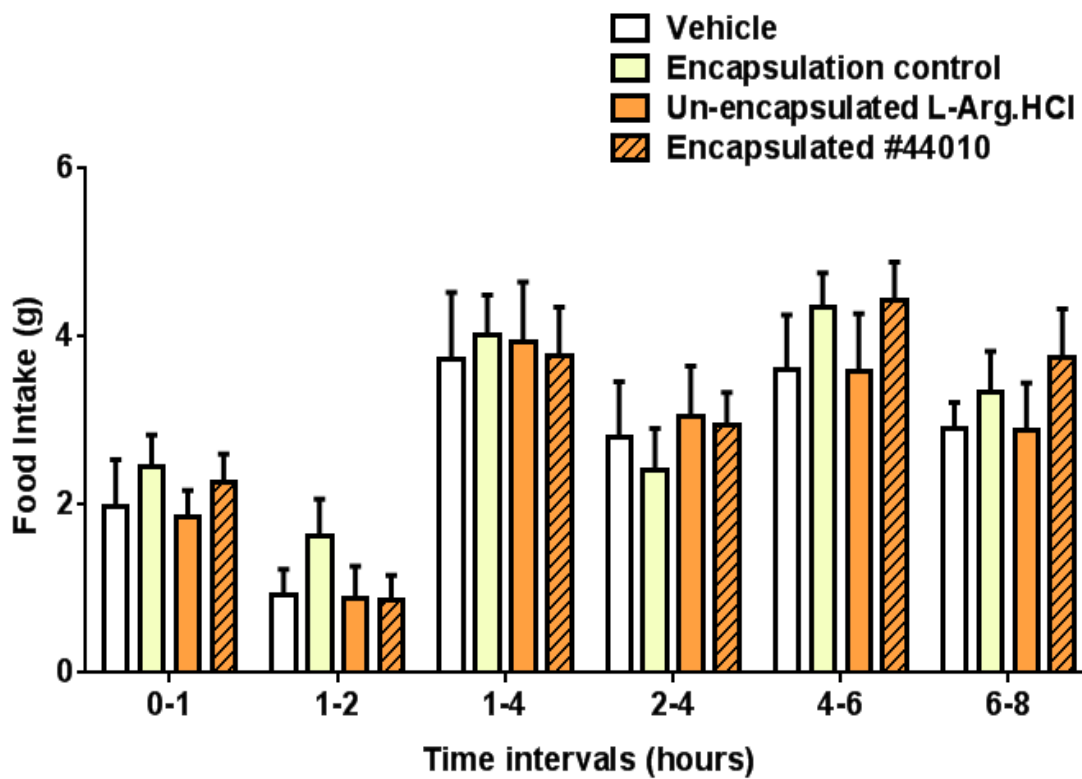

Figure 4.3.19 The effect of encapsulated L-Arg.HCl #44010 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (20% TWEEN20, water), encapsulation control, 16 mmol/kg un-encapsulated L-Arg.HCl, or encapsulated L-Arg.HCl #44010 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 1-4, 2-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 4-7.

4.3.4 The effect of encapsulated L-Phe on food intake in rats

As previously demonstrated, L-Phe inhibited food intake and modulated gut hormone release in rodents following oral administration (Sections 3.3.1, 3.3.2 and 3.3.6), and stimulated GLP-1 release from mice primary L-cell cultures (section 4.3.1). L-Phe therefore appeared a suitable candidate for encapsulation. In collaboration with TasteTech, two forms of encapsulated L-Phe were developed (Table 4.3.3) and subsequently tested in rats.

Product ID	Encapsulation Matrix	L-Phe composition
200240	60% Carnauba wax	40% L-Phenylalanine
200262	59% Carnauba wax + 1% Silica	40% L-Phenylalanine

Table 4.3.3 Encapsulated L-Phe products.
The encapsulated L-Phe and their matrices composition. The compositions are presented as percent weight: weight ratio.

4.3.4.1 The effect of OG administration of L-Phe on food intake in rats

In order and prior to investigating the anorectic effect of encapsulated L-Phe products, the effect of L-Phe on food intake was established in rats both in the fasted state in the early light phase, and in the fed state in the early dark phase.

Oral gavage of 3 and 6 mmol/kg L-Phe reduced food intake at 0-1 hour time point in fasted rats administered during early hours of light phase. The effect reached statistical significance with the higher dose of 6 mmol/kg compared to the vehicle control (vehicle: 7.44 ± 0.41g vs. 6 mmol/kg L-Phe: 5.83 ± 0.45g, $p<0.05$, n=10) (Fig. 4.3.20 A).

Likewise, oral gavage of 3 and 6 mmol/kg L-Phe reduced food intake at 0-1 hour time point in *ad libitum* fed rats administered at the onset of dark phase. The effect was statistically significant at the higher dose compared to the vehicle control (vehicle: 3.60 ± 0.50g vs. 6 mmol/kg L-Phe: 1.73 ± 0.43g, $p<0.01$, n=10). (Fig. 4.3.20 B). There were no significant differences in food intake between groups at any other time point at which it was measured.
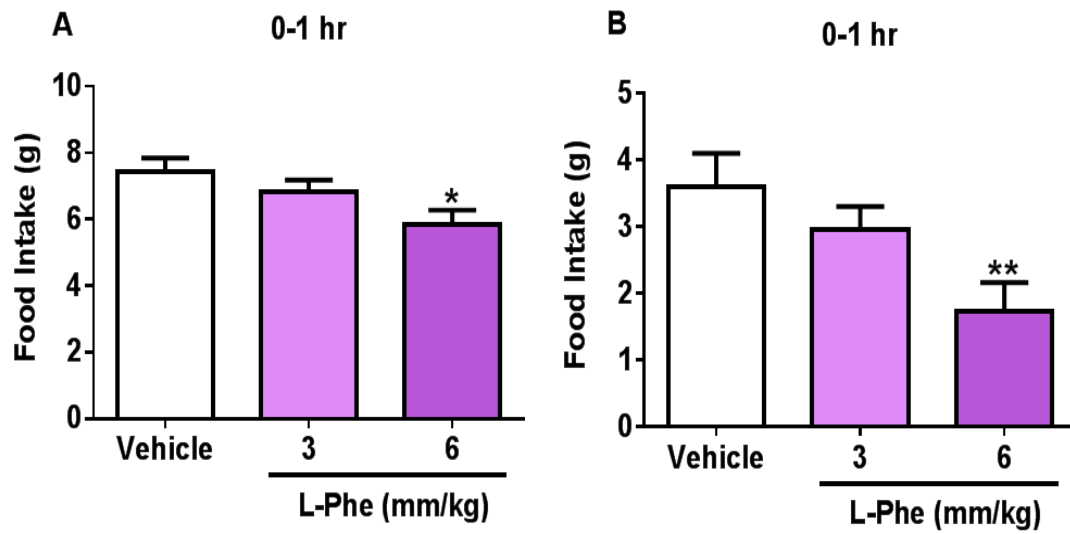
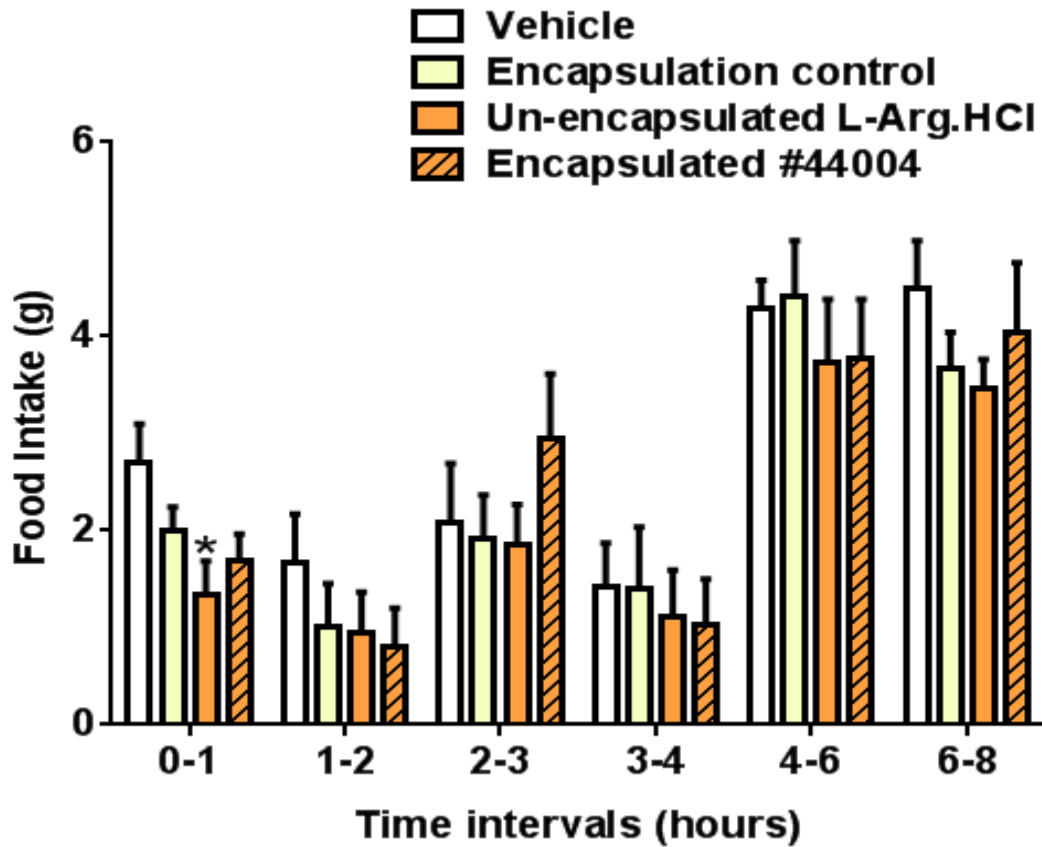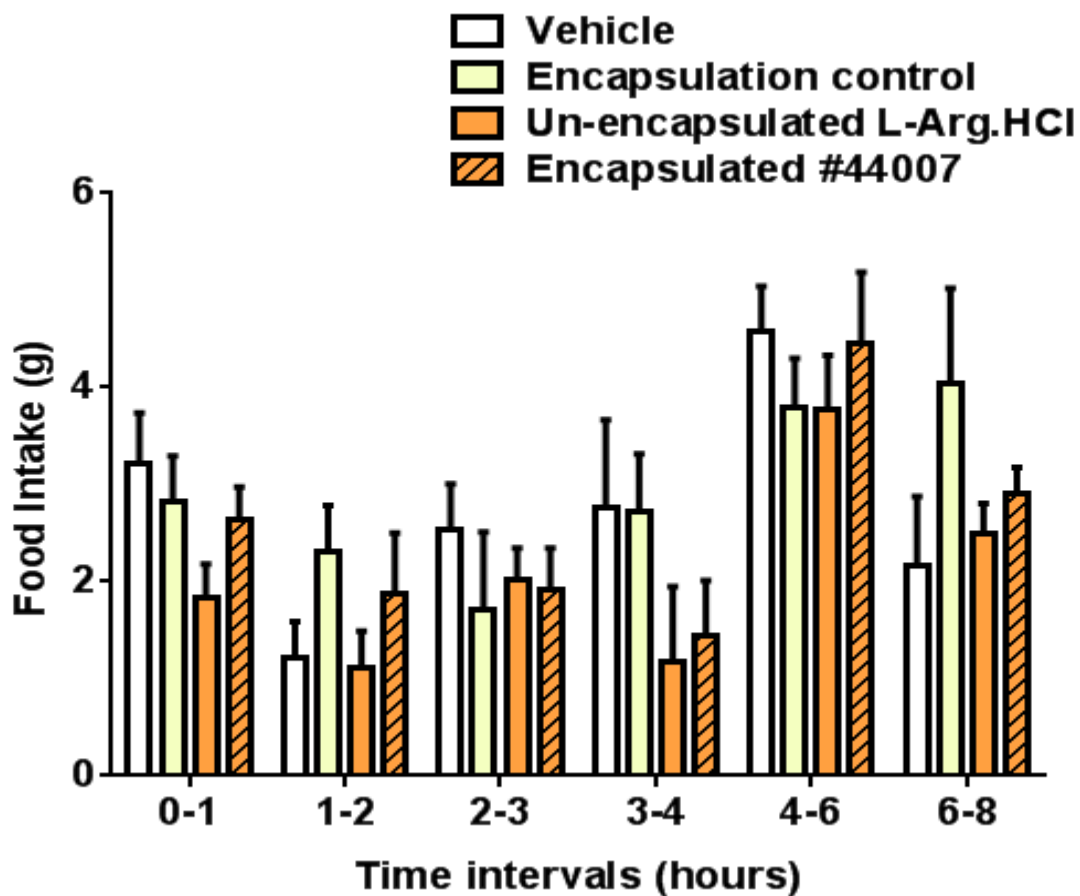**Figure 4.3.20 The effect of OG of L-Phe on food intake in fasted and fed rats.**
The effect of OG of vehicle (10% TWEEN20, water) 3, and 6 mmol/kg L-Phe in overnight fasted rats at 0-1 hour during early hours of light phase (A) and *ad-libitum* fed rats during early hours of dark phase (B). Data is presented as mean ± SEM. n = 10 per group. *p<0.05, **p<0.01 vs vehicle control.

4.3.4.2 The effect of OG administration of encapsulated L-Phe #200240 on food intake in fasted rats during the light phase

Oral gavage of 6 mmol/kg encapsulated L-Phe #200240 had no significant effect on food intake in rats compared to vehicle control or un-encapsulated L-Phe (Fig. 4.3.21).
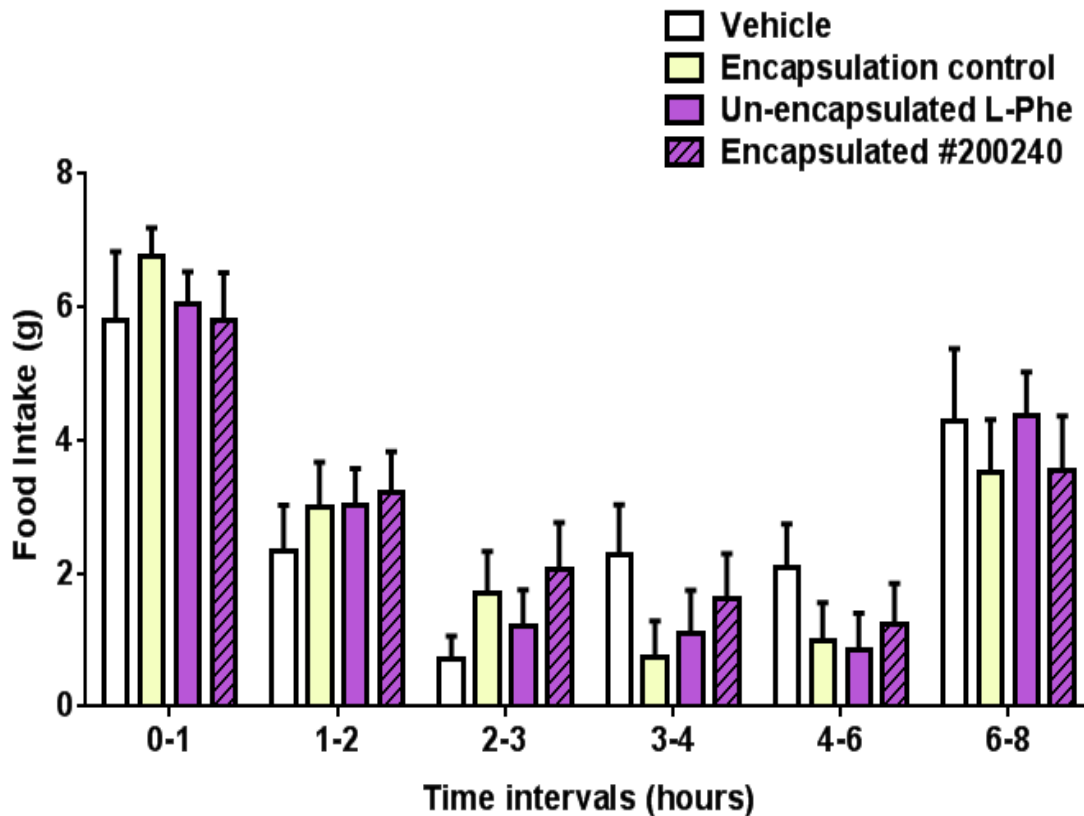
Figure 4.3.21 The effect of encapsulated L-Phe #200240 on food intake in fasted rats in the early light phase.
The effect of OG of vehicle (10% TWEEN20, water), encapsulation control, 6 mmol/kg un-encapsulated L-Phe, or encapsulated L-Phe #200240 on food intake in overnight fasted rats injected at early hours of the light phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 7-8.

4.3.4.3 The effect of OG administration of encapsulated L-Phe #200262 on food intake in fasted rats during the light phase

Oral gavage of 6 mmol/kg un-encapsulated L-Phe significantly reduced food intake at 0-1 hour post administration compared to the encapsulation group (encapsulation control: 7.40 ± 0.51 vs. un-encapsulated L-Phe: 5.10 ± 0.34g, p<0.05, n=7-8) . However, the encapsulated L-Phe #200262, at same dose, had no significant effect on food intake in rats compared vehicle control or un-encapsulated L-Phe. The food intake in encapsulated treated group was lower at 1-2 and 3-4 intervals compared to the un-encapsulated L-Phe, however these effects were not statistically significant. There were no significant differences in food intake between groups at any other time point at which it was measured.
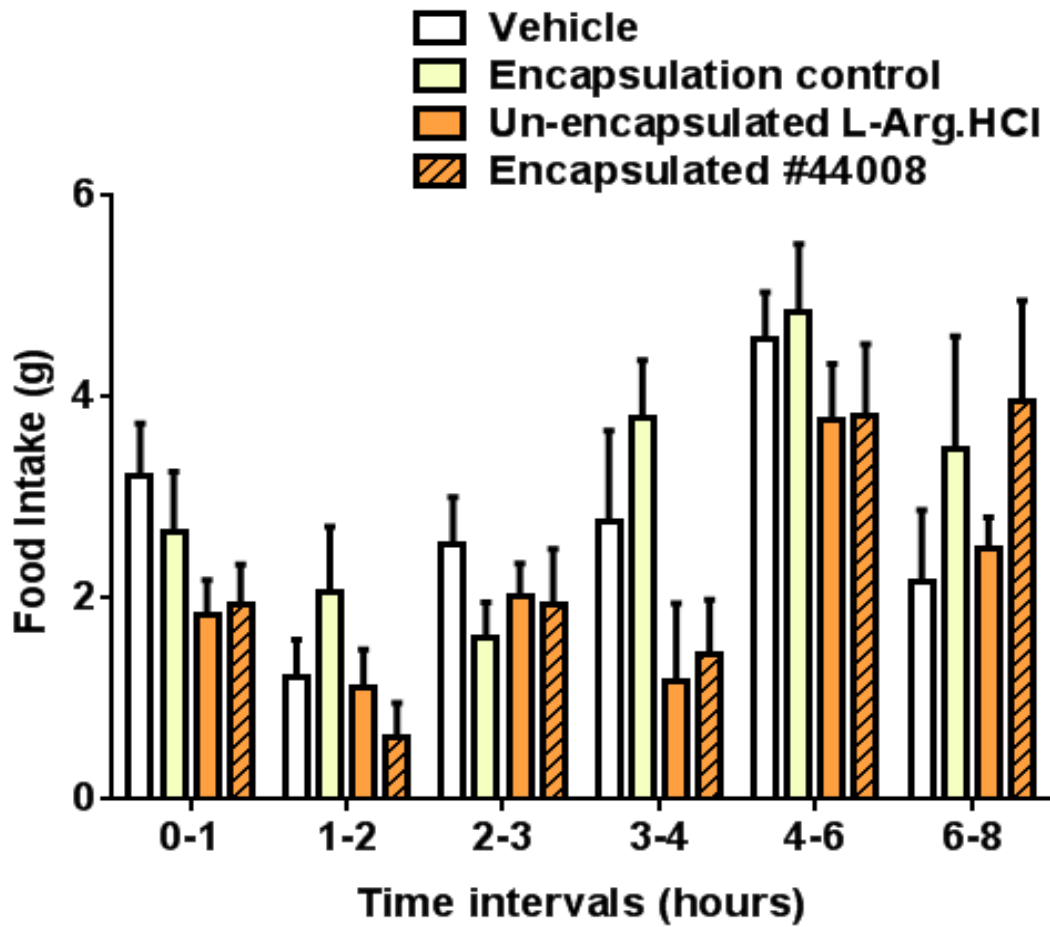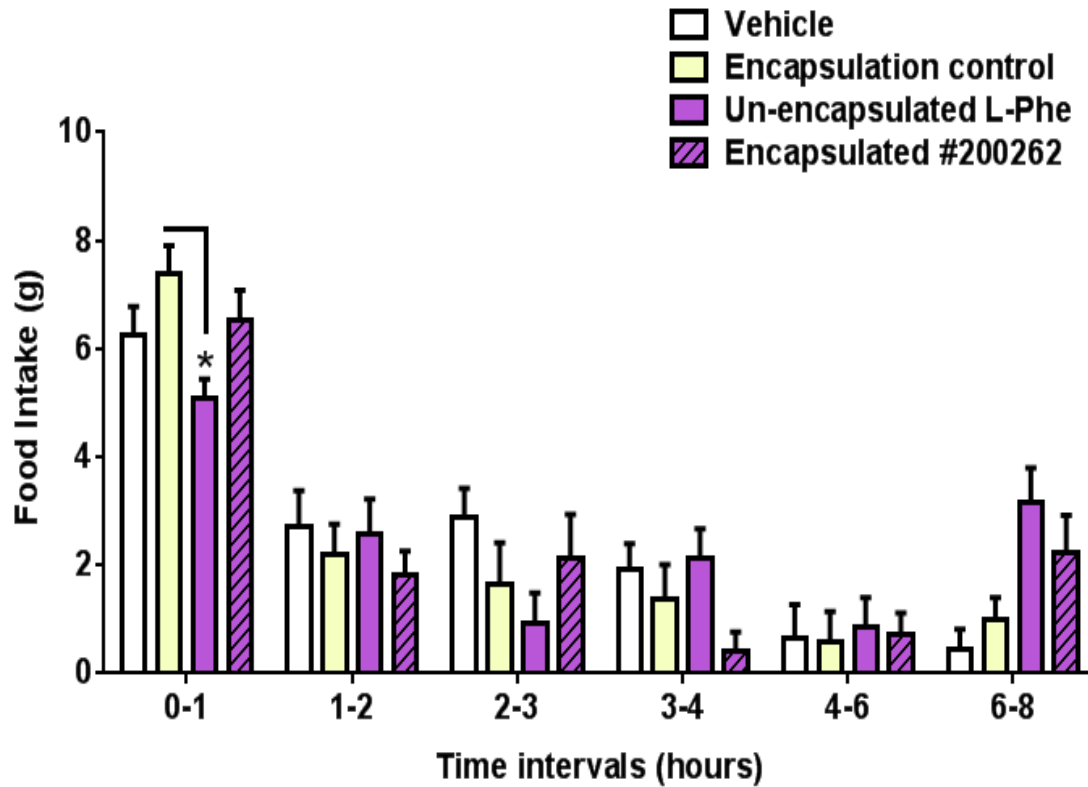
Figure 4.3.22 The effect of encapsulated L-Phe #200262 on food intake in fasted rats in the early light phase.
The effect of OG of vehicle (10% TWEEN20, water), encapsulation control, 6 mmol/kg un-encapsulated L-Phe, or encapsulated L-Phe #200262 on food intake in overnight fasted rats injected at early hours of the light phase. The graph shows the food intake at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 hours intervals following oral administration. Data is presented as mean ± SEM. n = 7-8. *p<0.05 vs encapsulation control.

4.3.4.4 The effect of OG administration of encapsulated L-Phe #200240 on food intake in *ad libitum* fed rats during the early dark phase

Oral gavage of 6 mmol/kg encapsulated L-Phe #200240 during the dark phase had no significant effect on food intake in rats compared to vehicle control or un-encapsulated L-Phe. The encapsulated L-Phe treated group food intake was lower during 1-3 hour interval compared to other groups; however this effect was not statistically significant (Fig. 4.3.23).
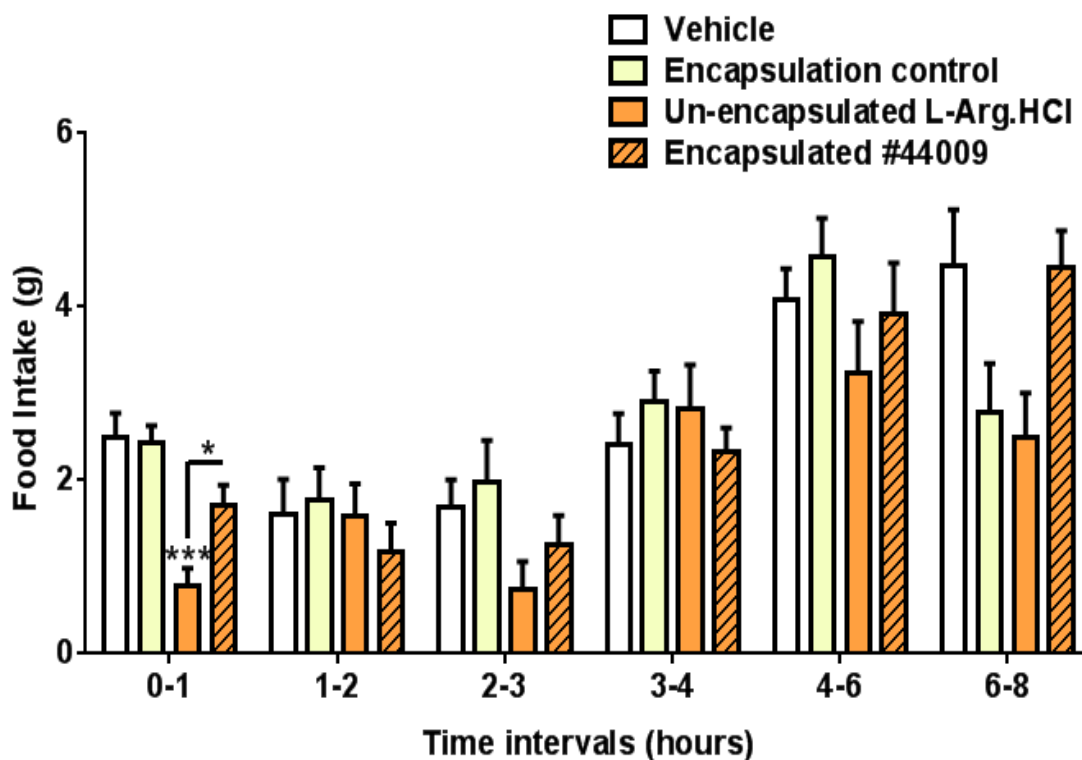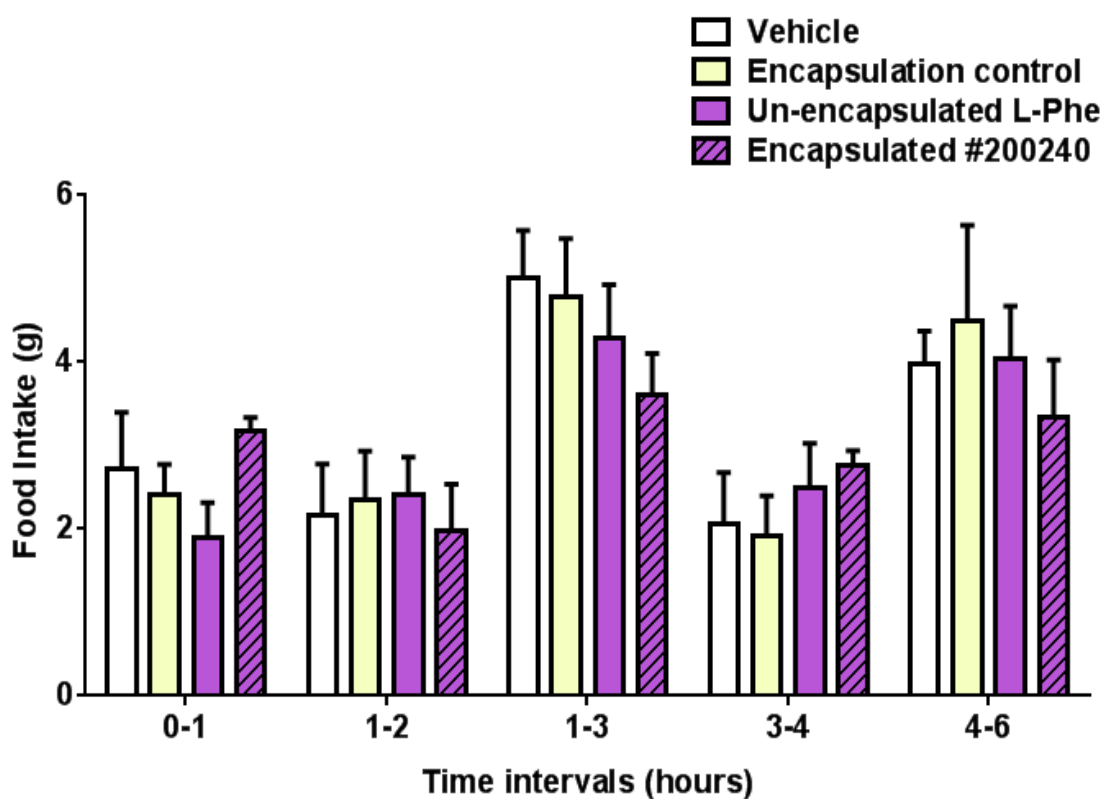

Figure 4.3.23 The effect of encapsulated L-Phe #200240 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (10% TWEEN20, water), encapsulation control, 6 mmol/kg un-encapsulated L-Phe, or encapsulated L-Phe #200240 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 1-3, 3-4, and 4-6 hours intervals following oral administration. Data is presented as mean ± SEM. n = 7-8.

4.3.4.5 The effect of OG administration of encapsulated L-Phe #200262 on food intake in *ad libitum* fed rats during the early dark phase

Oral gavage of 6 mmol/kg un-encapsulated L-Phe significantly reduced food intake at 0-1 hour post administration compared to the vehicle control group (vehicle: 3.40 ± 0.55 vs. un-encapsulated L-Phe: 1.40 ± 0.42g, p<0.05) . The encapsulated L-Phe #200262, at same dose reduced food intake; however the effect was not statistically significant. The food intake was significantly lower in un-encapsulated L-Phe group compared to the encapsulation group (un-encapsulated L-Phe: 0.94 ± 0.41 vs. encapsulation control: 2.96 ± 0.59g, p<0.05, n=7-8) and encapsulated L-Phe #200262 (un-encapsulated L-Phe: 0.94 ± 0.41 vs. encapsulated L-Phe #200262: 3.36 ± 37g, p<0.01, n=7-8) (Fig. 4.3.24). There were no significant differences in food intake between groups at any other time point at which it was measured.
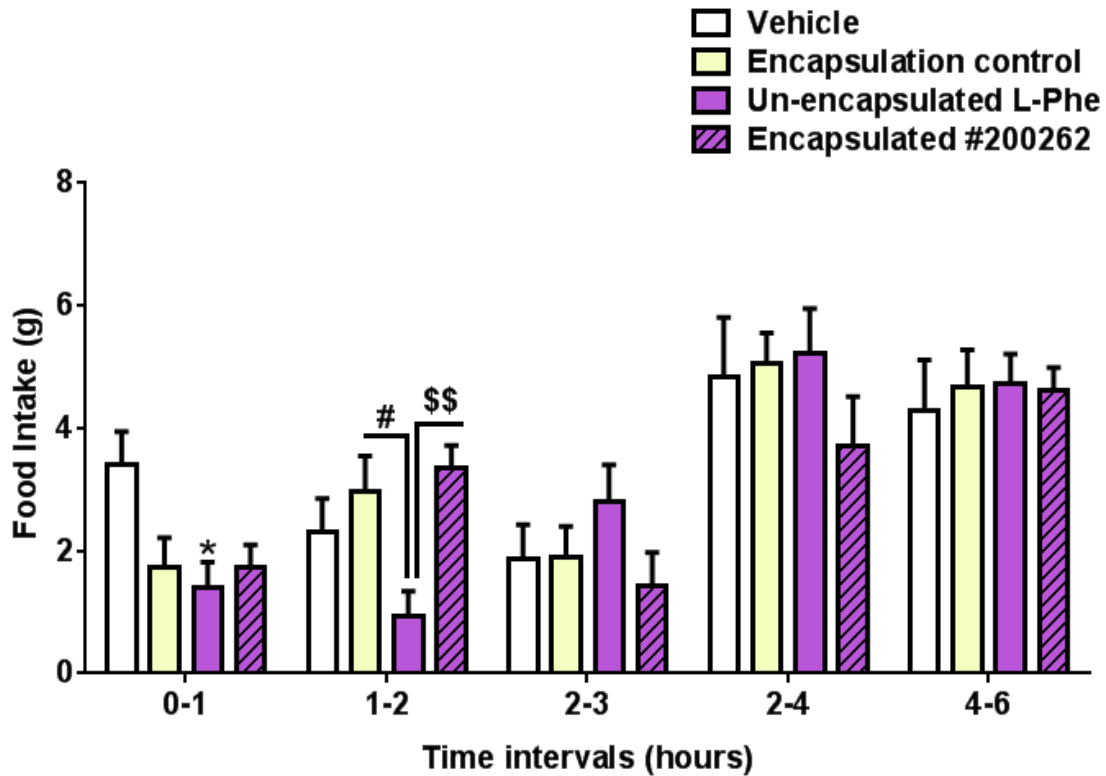
Figure 4.3.24 The effect of encapsulated L-Phe #200262 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (10% TWEEN20, water), encapsulation control, 6 mmol/kg un-encapsulated L-Phe, or encapsulated L-Phe #200262 on food intake in *ad libitum* fed rats injected at onset of the dark phase. The graph shows the food intake at 0-1, 1-2, 2-3, 2-4, and 4-6 hours intervals following oral administration. Data is presented as mean ± SEM. n = 7-8. *p<0.05 vs. vehicle control; #p<0.05 vs. encapsulation control; $$p<0.01 vs. encapsulated L-Phe #200262.

4.3.4.6 The effect of OG administration of encapsulated L-Phe #200240 on food intake in *ad libitum* fed rats in the early hours of dark phase

In order to further investigate the effect of oral gavage of encapsulated L-Phe on food intake in rats, CLAMS metabolic cages were used. Two independent feeding studies were performed in which the effect of OG of either 6 mmol/kg un-encapsulated L-Phe or 6 mmol/kg encapsulated L-Phe #200240 were investigated against the vehicle.

Oral gavage of both un-encapsulated and encapsulated L-Phe reduced cumulative food intake in rats during 0-4 hours post administration (Fig. 4.3.25 A,C) compared to the vehicle control (Cumulative food intake at 240 minutes: Vehicle: 6.16 ± 0.43g vs. un-encapsulated L-Phe: 7.11 ± 1.15g; Vehicle: 8.04 ± 1.12g vs encapsulated L-Phe #200240: 6.38 ± 0.68g). However, OG administration of encapsulated L-Phe resulted in a sustained reduction in cumulative food intake over 12 hours period compared to the un-encapsulated L-Phe (Cumulative food intake at 12 hours: Vehicle: 19.44 ± 0.83g vs. un-encapsulated L-Phe: 19.47 ± 0.79g; Vehicle: 20.03 ± 1.23g vs encapsulated L-Phe #200240: 17.13 ± 0.76g, $p < 0.05$, n=5-6 per group) (Fig. 4.3.25 B,D).

0-4 hr

A

0-12 hr

B

219
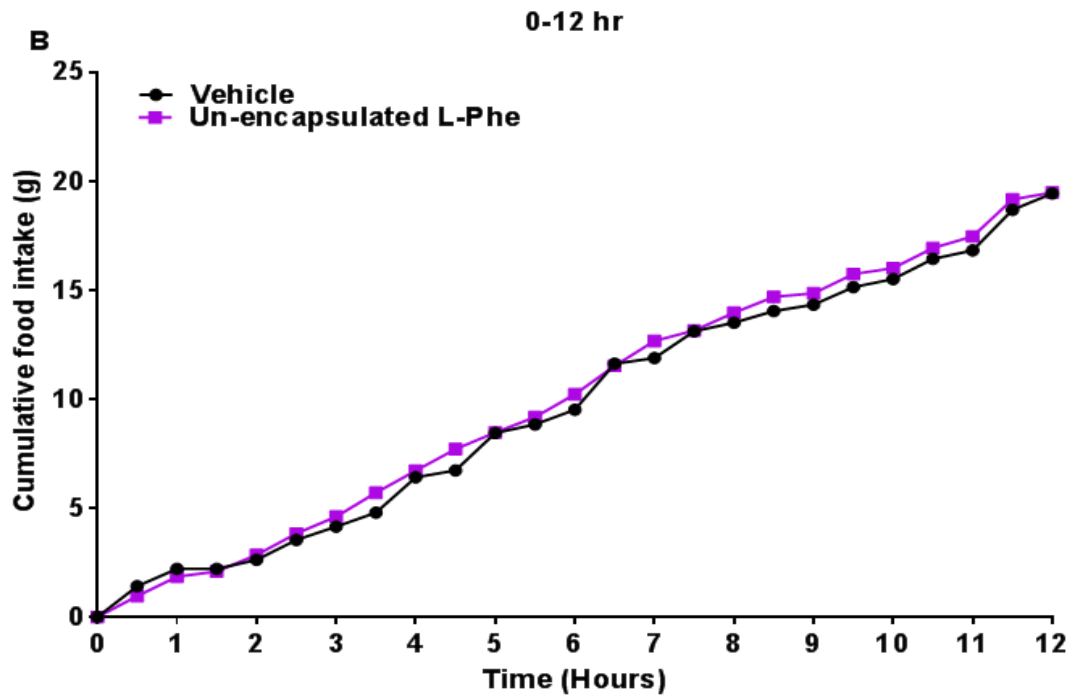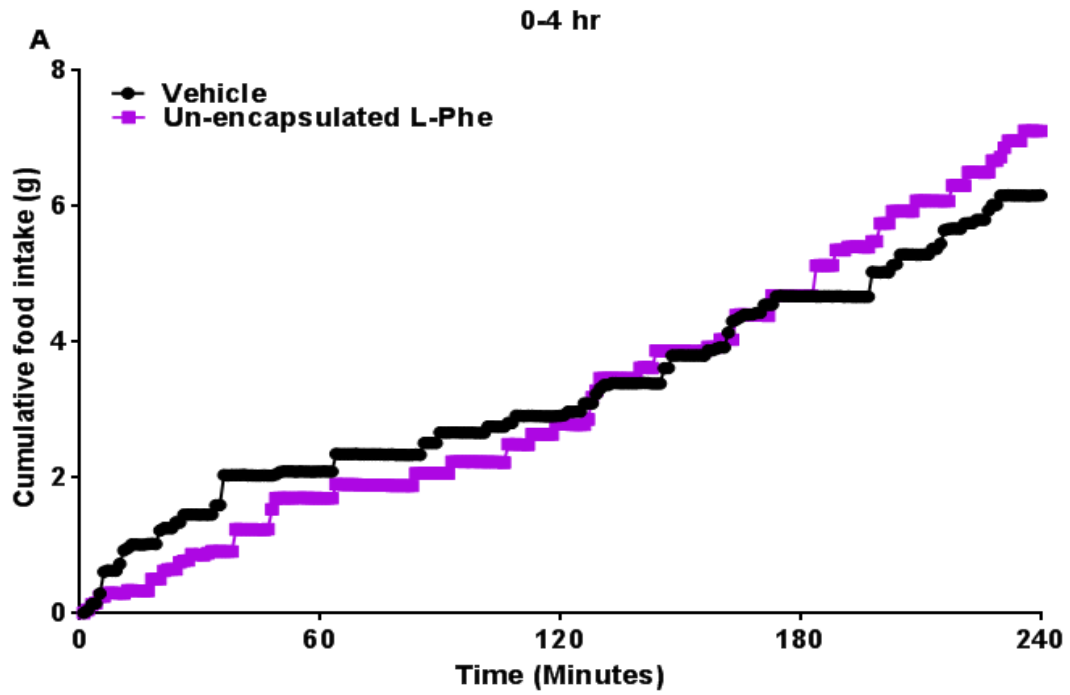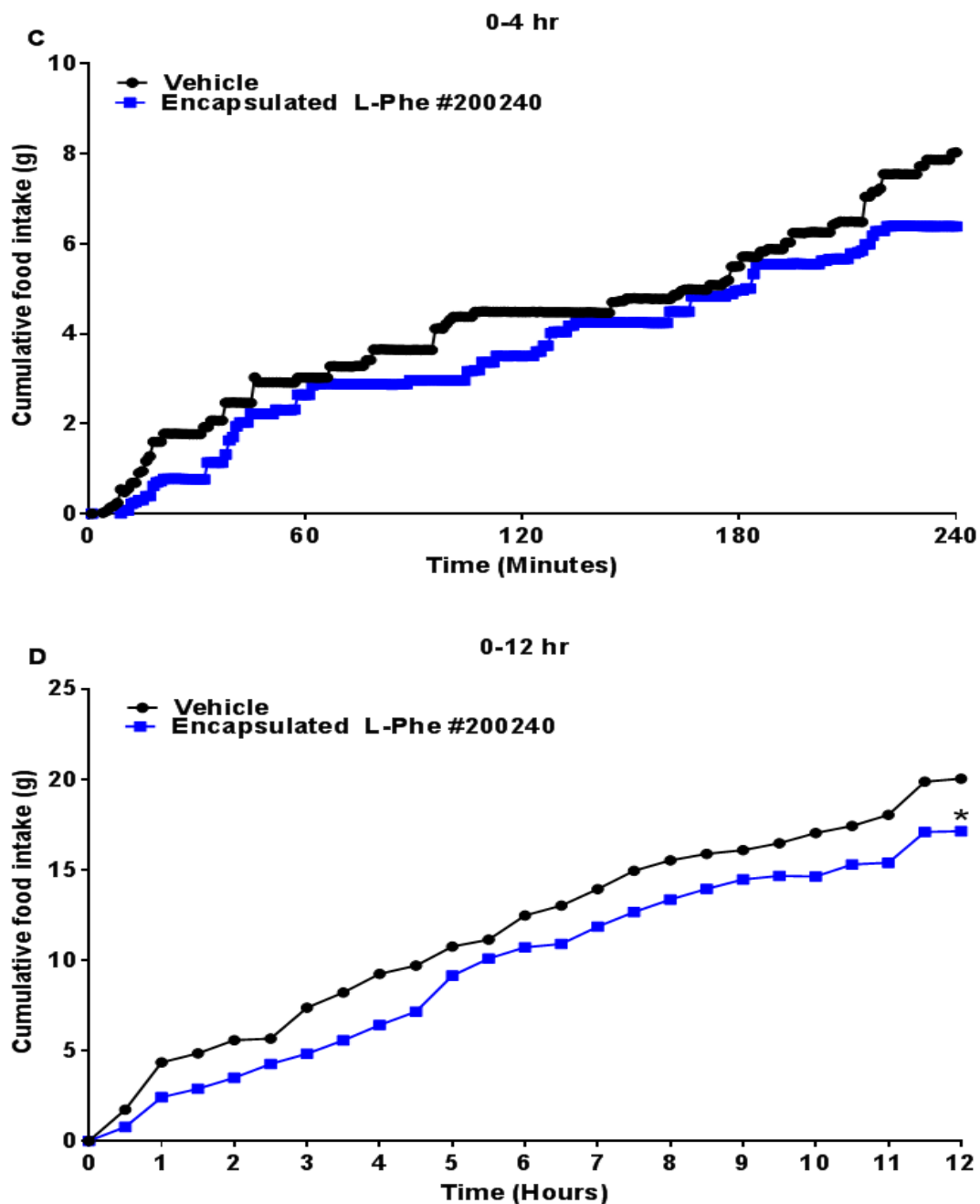
Figure 4.3 25 The effect of un-encapsulated and encapsulated L-Phe #200240 on food intake in *ad libitum* fed rats in the early dark phase.
The effect of OG of vehicle (10%TWEEN20, water) and 6 mmol/kg encapsulated L-Phe on 0-4 (A) and 0-12 (B) hour cumulative food intake in *ad libitum* fed rats injected at onset of the dark phase. N=6 per group.
The effect of OG of vehicle (10%TWEEN20, water) and 6 mmol/kg encapsulated L-Phe #200240 on 0-4 (C) and 0-12 (D) hour cumulative food intake in *ad libitum* fed rats injected at onset of the dark phase. N=6 per group. Data is presented as mean ± SEM. $p<0.05$ vs Vehicle at 12 hours time point.

4.4 Discussion

The aim of this project was to produce, optimise and validate encapsulated amino acids as novel food ingredients to promote satiation. Preliminary screenings of proteinogenic amino acids *in vitro*, together with the *in vivo* anorectic observations suggested L-Arg.HCl and L-Phe as suitable candidates for encapsulation. In collaboration with the project industrial partner, TasteTech Ltd, a series of encapsulated amino acids products were generated using various food-grade vegetable fat and natural waxes as the coating matrix. The work in this chapter examined the anorectic effect of encapsulated L-arginine and L-phenylalanine in rats. More than ten distinct encapsulated L-arginine products (Table 4.2.1) and two initial encapsulated L-Phe products were tested (Table 4.2.2). In addition, the work examined the effect of encapsulation on circulating L-arginine and gut hormone levels in rats. The encapsulation process delayed the anorectic effects in rats. However, no significant differences in gut hormone or circulating amino acid levels were observed following oral administration of the lead encapsulated products compared to an un-encapsulated control.

First generation encapsulated L-Arg.HCl products utilised candellila and carnauba wax as coating agents. Silicon dioxide (silica) was also incorporated within the matrix. Silica acts as a wetting agent, altering the behaviour of water molecules surrounding the encapsulation coating. This aids the solubility of the encapsulated product, but can ultimately result in rupture of the particles and release of the payload (Table 4.3.1). In the products studied, the application of silica appeared to have resulted in earlier release of the payload. Initial feeding studies demonstrated that carnauba wax was a more efficient coating matrix as it appeared to delay the anorectic effects of L-Arg.HCl in rats and alter the magnitude of response compared to the un-encapsulated form. Candellila wax-coated encapsulated products (#43773 and #43767) produced similar food intake profile to the un-encapsulated L-Arg.HCl, suggesting relatively early payload release following administration. In addition, products milled using two different techniques, rotary ball and cone milling, were tested. Rotary ball milling results in the generation of smaller payload particles than cone milling. Larger

particles may protrude through the coating matrix, but smaller particles may be more susceptible to penetration by water due to their larger total surface area. However, there was no significant difference between the two products tested.

Amongst the tested products, encapsulated L-Arg.HCl #43772 delayed the anorectic effects of L-Arg.HCl. Therefore, its effect on food intake, gut hormone release and L-arginine levels in circulation was further investigated. Increasing the percentage of carnauba wax delayed the anorectic effect compared to the un-encapsulated L-Arg.HCl. Oral administration of encapsulated L-Arg.HCl #43772 resulted in a significantly lower cumulative food intake during 2-6 hours interval and appeared to drive a stronger anorectic response in rats compared to the un-encapsulated L-Arg.HCl, an effect which was hypothesised to be due to its release more distally in the small intestine. These observations suggested that the encapsulation matrix was able to delay the absorption and degradation of L-Arg.HCl as the product travelled down the GI tract, releasing its payload more distally in the small intestine.

In order to investigate whether the anorectic profile of encapsulated #43772 was due to an improved effect on anorectic gut hormone release, rats were orally gavaged with encapsulated L-Arg.HCl #43772 or un-encapsulated L-Arg.HCl and gut hormone levels measured at 30, 60 and 90 minutes following administration. Oral gavage of encapsulated L-Arg.HCl #43772 elevated plasma GLP-1 and PYY levels. However, there was no significant difference in GLP-1 levels at 30 and 60 minutes compared to the un-encapsulated L-Arg.HCl. Interestingly, 90 minutes post administration, GLP-1 levels were significantly lower in encapsulated L-Arg.HCl compared to the un-encapsulated L-Arg.HCl. It is possible that this effect reflects a type I error (a false positive due to experimental error), or that the encapsulation process resulted in an unexpected change in L-Arg.HCl release in the gut. In the same study, no significant differences were observed in PYY levels between encapsulated and un-encapsulated L-Arg.HCl. At 30 minutes post administration, PYY levels appeared to be higher compared to the un-encapsulated L-Arg, but this effect did not achieve statistical significance.

In the same study, L-arginine plasma levels were measured as a surrogate of L-Arg.HCl release in the gut, and to investigate whether L-arginine profile in circulation corresponded with the food intake profile. Oral gavage of encapsulated L-Arg.HCl elevated L-arginine levels in circulation. The levels were highest at 30 minutes post administration. However there was no significant difference between encapsulated and un-encapsulated L-Arg.HCl. These data suggest that the encapsulation process is not influencing the anorectic effects of L-Arg.HCl by modulating its release or its effects on GLP-1 and PYY. However, further work is required to establish a detailed timeline of payload release. It would be interesting to carry out continuous blood sampling to gain a detailed profile of plasma L-arginine following administration, particularly using a longer timeline, and to measure the levels of L-arginine released in the gut, perhaps by tagging the payload using a small tracer molecule.

Second generation encapsulated L-Arg.HCl products (Table 4.3.2) were coated using higher percentage of carnauba wax and various other natural waxes. It was hypothesised that application of a mixture of other naturally occurring waxes with carnauba wax may improve the coating resistance and hence result in a more digestion resistant product. Results from feeding studies suggested that products coated using a combination of waxes were not effective encapsulation matrices as the majority led to effects on food intake less effective than, or indistinguishable from, un-encapsulated L-Arg.HCl. Initial screenings suggested two products with a higher percentage of carnauba wax (55% and 60%) were more efficient in protecting the payload and delaying the anorectic effects. Further studies suggested that encapsulated L-Arg.HCl #44009 (60% Carnauba wax) significantly reduced cumulative food intake at 1-4 hours following administration, whereas the un-encapsulated L-Arg.HCl control did not. However, this effect was not large, and did not appear as significant in *ad libitum* fed animals, and it was decided that further matrix development was required to produce an effective product.

Subsequently, the effect of encapsulating L-Phe on food intake was investigated using two encapsulated L-Phe products. Following earlier observations, a higher percentage (60%) of

carnauba wax was used in presence and absence of 1% silica in the coating. Initial findings suggested that administration of encapsulated L-Phe products resulted in similar effects on food intake to the un-encapsulated L-Phe, though further studies are required to confirm these observations. Application of silica in the coating (encapsulated L-Phe # 200262) may have resulted in an earlier release of the L-Phe particles compared to the encapsulated L-Phe #200240.

In order to further examine the anorectic effect of encapsulated L-Phe on food intake CLAMS metabolic cages were used. CLAMS cages enabled detailed analysis of food intake in rats without interfering with the normal feeding behaviour. The effect of OG administration of encapsulated L-Phe #200240 and un-encapsulated L-Phe were examined in two separate feeding studies. Oral gavage of 6 mmol/kg un-encapsulated L-Phe resulted in an early (0-1 hour) reduction in food intake in rats. However, compared to un-encapsulated L-Phe, oral gavage of encapsulated L-Phe #200240 had a lesser effect on food intake during the first 2 hours. The reduction in the cumulative food intake is lost at approximately 2 hours following administration in un-encapsulated L-Phe. However, encapsulating L-Phe appeared to have extended its anorectic effects, compared to un-encapsulated controls, as the reduction in cumulative food intake was observed during the later 2-4 hour period. In addition, encapsulated L-Phe resulted in a greater sustained reduction in food intake over 12 hours period post administration compared to the un-encapsulated L-Phe. This is promising and suggests that not only the microencapsulation delays the anorectic effect; it also leads to a sustained reduction in food intake. Further work is required to establish whether chronic administration of encapsulated product can affect body weight.

One major limitation of such feeding experiments was the vehicle system for the delivery of the encapsulated solution to rats. Encapsulation products were largely insoluble in water due to their hydrophobic encapsulation matrices. Therefore, for the majority of the feeding studies a vehicle control was used. The challenge was to use a delivery system that neither affected the encapsulation matrices nor the food intake. A series of safe and commercially

available organic solvents were tested to determine the most suitable vehicle for the delivery of encapsulated products. Following these pilot studies, TWEEN20, a polysorbate surfactant, was used as a vehicle throughout the feeding studies. Due to its relatively non-toxic properties, TWEEN20 is commonly used in food industry as a wetting agent. Our initial studies suggested that TWEEN20 at the levels used had no effect on food intake in rodents and therefore was a suitable delivery system. However, it remains a concern that the use of TWEEN20 may perhaps influence particle integrity negatively, leading to earlier release.

In addition, the study phase, the n numbers and also the use of TWEEN20 as a vehicle delivery system have led to variations observed between feeding studies performed in this chapter. Depending of the encapsulation matrix of a particular product, water or TWEEN20 was used as a vehicle. In addition the volume of gavage may have been changed to facilitate the delivery of the product in rats. Furthermore, using a rodent model in feeding studies where subtle changes occur proved to be challenging, as it ican be difficult to detect small changes in feeding behaviour in rodents. However, such differences in study protocols were carefully noted and further experiments were carried out to investigate any significant discrepancies within the experiments. The results presented in this chapter are the outcome of an initial screening process in rats model performed over a period of three years.

In summary, the present finding suggests that encapsulation may be a viable approach to facilitate the targeted delivery of the amino acids in the gut. However, further matrix optimization is required to improve the controlled release of the payload.

Chapter V:
General Discussion

Obesity is a major health concern and the fifth leading cause of deaths worldwide. It is estimated that 2.8 million adults die every year as a consequence of being overweight or obese (WHO, 2015). Despite this, there is no effective and safe treatment for obesity. Bariatric surgery is the most effective treatment option. However, it is impractical for all patients due to its costs, invasive nature and associated risks (Maggard et al., 2005). Diets and life style modifications remain the first choice of treatment. High protein diets are associated with effective weight loss and improved weight management compared to other macronutrient diets (Halton and Hu, 2004). However, diets are often only effective short term and are difficult to adhere to over longer period of times. Therefore, understanding the molecular mechanisms by which high protein diets exert their beneficial effects may offer an alternative option for treatment of obesity. A number of hypotheses have been proposed to contribute to the satiating effects of high protein diets. Evidence suggests that specific amino acids released following protein digestion in the GI tract may play an important role. Different proteins produce different levels of satiety which may be due to amino acid constitutes of different proteins (Veldhorst et al., 2008). Furthermore, the satiety effects of proteins have been attributed to concentrations of certain specific amino acids in circulation (Veldhorst et al., 2009e).

The work previously carried out in out laboratory demonstrated that individual amino acids such as L-cysteine have potent anorectic properties (McGavigan et al., 2015). The work carried out in this thesis investigated the anorectic properties of L-arginine and L-phenylalanine in rodents and the mechanisms by which such effects are mediated. Furthermore, the thesis examined the utility of targeted delivery of L-arginine and L-phenylalanine in the GI tract.

Oral gavage of L-Arg.HCl significantly reduced food intake in mice and rats. L-arginine is a basic amino acids, hence in order to avoid any pH dependent effects, the neutral salt of the amino acid was used in experiments. The anorectic effect of L-Arg.HCl was independent of any secondary behavioural side effects. A recent publication has examined the effect of oral

gavage of all 20 proteinogenic amino acids in rats and reported L-arginine, L-lysine and L-glutamic acid as the most anorectic amino acids (Jordi et al., 2013). The authors tested amino acids at a dose of 6.7 mmol/kg. Our studies demonstrate the anorectic effect of L-Arg.HCl at considerably higher doses than the one tested by the authors. L-arginine and L-lysine are basic amino acids, and L-glutamic acid is acidic in solution, and therefore the potential effects of pH should be considered carefully. Previous pilot studies performed within our group suggested that L-arginine does not reduce food intake at doses equal to or lower than 8 mmol/kg when the solution to be administered is neutralized. Thus, although primary behavioural studies suggested no secondary side effects, further detailed conditioned taste aversion studies are required to confirm that the anorectic effects reported are not secondary to side effects caused by the high pH. Jordi et al reported that both L-arginine and L-lysine caused visceral discomfort in rats when orally administrated (Jordi et al., 2013), an effect that may be explained by the pH of solutions.

The discovery of promiscuous L-amino acids receptors and their high expression in the GI tract suggests a role for these receptors in amino acid sensing in the gut (Wellendorph et al., 2009a, Wellendorph et al., 2010). Amino acids have shown to have different affinities for these receptors. Basic amino acids are potent activators of GPRC6A (Wellendorph et al., 2005). I hypothesised that the effect of L-Arg.HCl may be mediated via activation of GPRC6A. However, oral administration of L-Arg.HCl significantly reduced food intake in both WT and GPRC6a-KO mice, suggesting that GPRC6A is not required for the effect of L-Arg.HCl on food intake. However further studies are required to characterise the possible role of other L-amino acid receptors, including T1R1-T1R3 and CaSR. L-arginine has been reported to stimulate the release of GLP-1 and PYY from isolated rat small intestine via a CaSR-dependent mechanism (Mace et al., 2012). In addition, T1R1-T1R3 responds to L-arginine, an effect that is enhanced in presence of IMP (Nelson et al., 2002).

L-arginine has previously has shown to stimulate the release of insulin and growth hormone from pancreatic beta cells (Floyd et al., 1966, Adeghate et al., 2001) and the pituitary

(Villalobos et al., 1997), respectively. Therefore, I hypothesised that L-Arg.HCl may reduce food intake by stimulating the release of the anorectic gut hormones, GLP-1 and PYY. L-Arg.HCl stimulated the release of GLP-1 and PYY from a murine primary colonic L-cell culture. In accords with this observation, oral gavage of L-Arg.HCl significantly elevated GLP-1 and PYY levels in rats. However, blocking GLP-1R and Y2R failed to abolish the anorectic effect of L-Arg.HCl in mice, suggesting the anorectic effect is not dependent on the effects of GLP-1 and PYY_{3-36}. Further detailed studies are required to investigate the mechanisms by which L-Arg.HCl reduce food intake. Amino acid transporter systems play a crucial role in amino acids transport across the cellular membranes. Recent evidence suggests that in addition to their transport activity, transporter systems have receptor-like activities. For example, the SNAT2 transporter has been implicated in GLP-1 release induced by L-glutamine from murine primary intestinal cultures (Tolhurst et al., 2011). In addition, L-arginine-induced insulin release from the pancreatic beta-cells has shown to be mediated by the electrogenic transport of L-arginine into the beta-cells resulting in membrane depolarization and consequent insulin release (Smith et al., 1997).

There is evidence to suggest that amino acids can regulate appetite via centrally mediated mechanisms. The branched chain amino acid L-leucine reduces food intake by centrally activating the mTORC1 pathway (Cota et al., 2006). Further studies have demonstrated that MBH administration of L-leucine activates a specific neural energy regulatory circuit by activating POMC neuronal populations within the MBH, oxytoxin neurones within PVN, and neurones within the NTS, leading to an acute reduction in food intake (Blouet et al., 2009). However, oral supplementation of L-leucine has shown not to effectively alter food intake both in rodents and human (Pedroso et al., 2015). The IP administration of L-Arg.HCl significantly reduced food intake in rats, suggesting systemic L-Arg.HCl can be sensed, perhaps by central mechanisms. The recent study performed by Jordi and colleagues suggested that the anorectic effects of L-arginine are mediated by neuronal activity in AP and NTS (Jordi et al., 2013). In addition, previously published work from our laboratory

demonstrates that oral administration of L-cysteine increased the number of c-foc positive cells within AP (McGavigan et al., 2015); suggesting that the anorectic properties of L-cysteine may be mediated via direct sensing of this amino acid within the brainstem. This is perhaps unsurprising; the AP is a circumventricular organ with an incomplete blood-brain barrier exposed to circulatory stimuli including amino acids, and, indeed, is known to act as a sensor of circulating toxins. Our studies suggest that L-arginine does not cause aversive effects, but further studies are required to establish whether L-arginine acts directly on central pathways to regulate appetite.

Similar to L-arginine, oral gavage of L-Phe potently reduced food intake in both mice and rats. Compared to L-arginine, the effect was achieved at much a lower dose of 3 and 12 mmol/kg in rats and mice, respectively. The data suggests a potent effect of L-Phe on food intake compared to a recent study which found no significant effect on food intake following OG of 6.7 mmol/kg of L-Phe in rats (Jordi et al., 2013). It is possible that solubility difficulties led to these findings.

The work carried out therefore suggests L-arginine and L-phenylalanine may be useful as agents to suppress appetite. I have shown that oral administration of L-Phe reduces food intake in DIO mice as well as lean animals, and that the chronic administration of L-Phe reduces food intake and body weight in DIO mice. Similarly, previous work in our laboratory has found that L-arginine reduces food intake and body weight chronically in DIO mice in a similar fashion to L-Phe (data not shown). The doses of L-arginine and L-phenylalanine orally administered in our feeding studies are pharmacological. The amount of L-Arg.HCl administered acutely roughly equals the amount of L-arginine that a rodent would consume daily if placed on 45% high protein diet. In case of L-Phe, the amount administered was similar to their daily normal intake of L-Phe on normal chow diet. These findings suggest that our results may represent pharmacological activation of a physiological nutrient sensing system, and that both L-arginine and L-Phe may be pharmacologically useful in the suppression of appetite and the prevention or treatment of obesity.

Aromatic amino acids are potent agonists to CaSR (Wellendorph et al., 2009a). Several studies have shown that L-Phe stimulates the release of CCK via a mechanism that requires the activity of CaSR (Hira et al., 2008). I hypothesised that the anorectic effects of L-Phe were mediated by the activity of CaSR in the gut. L-Phe stimulated the release of GLP-1 from STC-1 cell-line, an effect that was partially blocked in presence of CaSR antagonist in culture. In addition, direct ileal administration of L-Phe reduced food intake in rats, and co-administration of a CaSR antagonist attenuated this anorectic effect. In addition, OG of a CasR agonist alone reduced food intake in rats. These observations collectively suggest a role for CaSR in mediating the anorectic effects of L-Phe. I also investigated the effect OG of L-Phe on plasma gut hormone levels. OG of 3mmol/kg L-Phe elevated GLP-1 levels and reduced ghrelin levels following administration. However, PYY levels remained unchanged. The data suggests that the anorectic effect of L-Phe may be mediated via the modulation of gut hormone release. However extensive studies are required to confirm these observations. It is possible that release of L-Phe further down the GI tract might result in an increase in PYY release. L-Phe stimulated the release of GLP-1 and PYY from murine primary intestinal L-cells, and Mace and colleagues demonstrated L-Phe induced GLP-1 release from isolated rat small intestine via a CaSR-dependent mechanism (Mace et al., 2012).

The work described in this thesis investigated the effect of targeted delivery of specific amino acids in food intake in rats. Three nutrient sensing L-amino acids receptors are expressed in the GI tract. The expression of these receptors is higher in the distal small intestine and colon. Furthermore, there are higher numbers of cells in distal gut that secrete both GLP-1 and PYY in response to a nutrient load. Therefore I hypothesised that targeted delivery of amino acids to more distal regions of the gut may enhance their anorectic effects. Much ingested protein is broken down to amino acids which are then absorbed in the upper small intestine, with only lower concentrations reaching the distal gut. Orally administered amino acids might be expected to be absorbed even quicker. Therefore, in collaboration with the project's industrial partner, a series of microencapsulated amino acids were produced and

their ability to reduce food intake in rats investigated. The encapsulation matrices used were composed of mixtures of natural vegetable oils and waxes; these substances are considered as foods, rather than medicines, which would ease their use as dietary supplements to treat or prevent obesity.

A series of feeding studies in rats investigated the effect of encapsulated L-Arg.HCl and L-Phe on food intake. Earlier observations presented similar food intake profiles compared to the un-encapsulated amino acids. Feeding study data suggested that compared to candellila wax, carnauba wax might delay payload release in the gut. However, the encapsulation matrix required further optimisation to improve the controlled release. Application of higher percentage of carnauba wax alone, seemed to further delay the release of the amino acid payload in the gut, as demonstrated by a delayed anorectic effect compared to the un-encapsulated L-Arg.HCl. However, although encapsulated L-Arg.HCl stimulated GLP-1 and PYY release, no significant differences were seen compared to the effects of the un-encapsulated form. Even if the effect of oral administration of L-Arg.HCl on appetite does not require GLP-1 and PYY_{3-36} signalling, increasing its ability to stimulate anorectic gut hormone release may well result in a significant pharmacological effect. Further work is thus required to improve the encapsulation matrix and ultimately the release profile.

The focus of this thesis was to establish the effect of L-arginine and L-phenylalanine on appetite in rodents, and therefore the majority of the work focused on the role of peripherally administered amino acids on food intake in rodents. The current evidence suggests that there may be overlapping mechanisms involved in mediating the effects of amino acids on food intake (Potier et al., 2009). The work carried out in this thesis investigated the role of gut hormones GLP-1 and PYY in mediating the effects of L-arginine and L-Phe on food intake. Although both GLP-1 and PYY levels were elevated following oral administration of L-Arg.HCl, our data suggests that the anorectic effect of L-arginine is not mediated via GLP-1 and PYY. Oral administration of L-Phe elevated GLP-1 and reduced ghrelin signal in rodents, and further studies performed here demonstrated that the anorectic effects may be

due to changes in gut hormone profile following administration. Although the majority of work here concentrated on GLP-1 and PYY, the role of other gut hormones cannot be ruled out. CCK release has been classically shown to be stimulated by L-Phe. Studies have demonstrated L-Phe induced CCK release from isolated I-cells (Wang et al., 2011a), STC-1 cell lines (Hira et al., 2008) and humans (Ballinger and Clark, 1994). In our studies, we observed no significant change in CCK levels following L-Phe administration in rats and mice (data not shown). In addition, though oral administration of L-Arg.HCl to rats did not change circulating acyl ghrelin levels (data not shown), oral L-Phe did significantly reduce acylated ghrelin levels. However, the data from the chronic L-Phe administration study suggestsed that the effects observed on energy homeostasis were not driven by changes in ghrelin.

Branched chain amino acids have shown to reduce food intake in rodents via centrally mediated signals (Cota et al., 2006). This effect has so far only been observed following MBH administration of L-Leucine (Blouet et al., 2009). However, a recent study suggested that a member of the soluble carrier family of proteins, SLC38A9, may play an important role in central L-arginine sensing via mTORC1 dependent mechanisms (Rebsamen et al., 2015, Wang et al., 2015). SLC38A9 has shown to have transceptor activity which can initiate mTORC1 signalling and activation through the Rag-Ragulator protein complex (Abraham, 2015). Interestingly, in our studies, IP administration of L-Arg.HCl significantly reduced food intake in rats suggesting a potential central mechanism for L-arginine induced satiety.

Furthermore, L-Phe is the precursor molecule for the biosynthesis of neurotransmitter dopamine in the CNS (Kapatos and Zigmond, 1977). Dopamine is implicated in regulation of food intake (Volkow et al., 2011). Therefore, changes in the concentration of centrally available L-Phe may indirectly influence food intake via dopamine dependent mechanisms. However, previous studies have concluded that dopamine synthesis and release is not significantly influenced by L-Phe availability in the brain, and extremely high doses of amino acids are needed to pharmacologically influence neurotransmitter availability in the CNS (Fernstrom, 1977, Lou, 1994, Meeusen and Watson, 2007).

The aim of this thesis was to investigate the role of amino acids in appetite regulation and protein-induced satiety. The work presented in this thesis investigated the effect of specific amino acids on appetite and gut hormone release. The findings reported here demonstrate that different amino acids have different satiating effects. This work suggests that the incorporation of amino acids into food stuffs might be useful in the treatment of obesity. However, further work is required to determine whether chronic administration of these agents can reduce long term food intake and body weight in humans before such agents can be introduced to the market. In addition, these data demonstrate the pharmacological effects of specific amino acids, and further work is required to determine whether protein-induced satiety occurs as a result of the sensing of multiple amino acids, perhaps via different mechanisms.

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Appendix 1: List of solutions

- **Phosphate buffer:** 48g $Na_2HPO_4.2H_2O$, 4.13g $KHPO_4$, 18.61g $C_{10}H_{14}H_2ONa_2.2H_2O$ (EDTA) and 2.5g NaN_3 (sodium azide) dissolved in 5 litres of distilled water that has been boiled and allowed to cool. pH adjusted to 7.4 with diluted HCl and stored at 4°C.

- **Phosphate buffer with gelatine:** 12.5g gelatine added to the boiled water as above prior to cooling, and before addition of the other reagents

- **Dextran-coated charcoal**: 2.4g charcoal and 0.24g dextran added to 100ml phosphate buffer with gelatine and stirred for 20 minutes.

- **Versene:** 16g NaCl, 0.4g KCl, 2.88g $Na_2HPO4.2H_2O$ (di-sodium hydrogen orthophosphate), 1.2g $C_{10}H_{14}N_2O_8Na_2.2H_2O$ (EDTA), 0.4g KH_2PO_4 (potassium dihydrogen orthophosphate) dissolved in 1 litre of distilled water. 3ml phenol red (Sigma, Poole, UK) added and made up to 2 litres with distilled water. Solution autoclaved prior to use.

- **Cell lysis buffer:** 0.25g $C_{24}H_{39}NaO_4.H_2O$ (sodium deoxycholate monohydrate), 0.88g NaCl, 0.5ml Igepal (Sigma, Poole, UK) and 2.5ml Tris HCl (1M, pH 8) added to 40ml of distilled water, 1 tablet complete EDTA-free protease cocktail inhibitor (Roche, Basel, Switzerland) was added to the buffer. Stored at 4°C.

- **Secretion buffer:** 0.335g KCl, 8.065g NaCl, 0.353g $NaHCO_3$, 0.187g $NaH_2PO_4.2H_2O$, 2.6ml 1M $CaCl_2$, 1.2ml 1M $MgCl_2$ and 2.383g HEPES dissolved in 1L of distilled water. pH adjusted to 7.4 using dilute NaOH. Stored at 4°C.

Appendix 2: Principles of radioimmunoassay

Radioimmunoassay (RIA) is a sensitive *in vitro* technique used to determine the concentrations of antigens using specific antibodies. The technique was used in this thesis to measure concentrations of gut hormones GLP-1 and PYY from cells, tissue and plasma samples. RIA is a competition-based binding assay between a known concentration of radio-labelled antigen and unlabelled antigen for binding to a known limiting concentration of antibody. The RIA reaction is incubated and allowed to reach equilibrium according to the following equation:

*Ag + Ab + Ag ↔*AgAb + AgAb
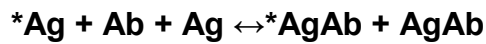
Ag = unlabelled antigen

*Ag = radiolabelled antigen

Ab = antibody

The amount of labelled antigen and antibody complexes formed depend on the concentration of the unlabelled antigen in the sample. Increasing the concentration of unlabelled antigen in the sample reduces the number of available binding sites for the labelled antigen to bind. Therefore, the amount of labelled antigen and antibody complexes is inversely proportional to the concentration of unlabelled antigen in the sample. The concentration of unknown antigen is interpolated from a standard curve devised using a range of known concentrations of the antigen.

Antibody-bound antigen is separated from the free unbound antigen using specific separation methods. GLP-1 assays were separated using charcoal adsorption technique, whereas PYY assays used a specific secondary antibody complex method of separation. Charcoal separation was carried out by addition of a charcoal suspension containing dextran to the reaction tube. Dextran covers large pores in the charcoal and therefore prevents adsorption of the bound complex. The addition of charcoal suspension followed by centrifugation of samples separates unbound antigen. The bound and unbound fractions are

then separated by aspirating the supernatant containing the bound fraction from the unbound charcoal pellet.

Secondary antibody separation was carried out for PYY assays using an anti-rabbit IgG antibody (Pharmacia Diagnostics, Uppsala, Sweden). Samples were incubated with the secondary antibody for a minimum of one hour prior to the addition of 0.01% Triton-X and 10% PEG as previously described in method sections 2.2.7.3 and 2.2.7.4. The samples were centrifuged and the bound and free fractions were separated by aspiration.

For both assays, free and bound fractions were measured using gamma scintillation counter (LB2111 Multi Crystal Gamma Counter, Berthold Technologies, Bad Wildbad, Germany) and hormones concentrations were determined using a non-linear plot (Prism version 6.03, GraphPad Software Inc, CA, USA).

All assays were performed in duplicates for plasma samples. Following tubes were added in duplicates at the beginning of each assay:

- Non-specific binding (NSB) tubes: labelled antigen without antibody added.
- Half and twice the required volume of labelled antigen added to check the quality and sensitivity of the labelled antigen used in assays.
- Zero tubes: tubes with no samples added throughout the assay to control for baseline drift within the assay.
- Quality control (QC): tubes containing a low, medium and high known concentration of measuring hormone to check for overall assays' performance.
- Excess antibody tubes: Antibody is added at 6X the amount normally required for each tube to assess the immunological integrity of labelled antigens.

Both GLP-1 and PYY peptides used in this thesis were iodinated by Professor Mohammad Ghatei (Section of Investigative Medicine, Imperial College London) using the Iodogen method (Wood et al., 1981). Iodinated peptides were purified by HPLC (Gilson 321-H1)

using a C18 column (Waters, Milford, USA). Fractions were tested and used at 900-1200 counts per minute per tube.