The Effects of High Protein Diets and L-Phenylalanine on Energy and Glucose Homeostasis

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Abstract

Obesity is a major global health concern. Current anti-obesity therapies are either ineffective, or highly invasive and difficult to provide on a global scale. Therefore, there is a growing need for new treatments. High protein diets have been shown to be effective at promoting weight loss, improving glucose tolerance and supressing appetite, but compliance is low. Understanding the mechanisms underlying the beneficial effects of high protein diets could aid the development of new anti-obesity therapies.

Acute studies have shown protein loads to stimulate the secretion of anorectic hormones which can promote satiety and decrease food intake. However, the long-term effects of high protein intake on the regulation of gut hormones is less clear. This thesis presents a series of studies investigating the mechanisms which may mediate the effects of protein on energy and glucose homeostasis.

The long-term effects of high protein diets on the synthesis and secretion of gut hormones in mice was investigated, and a trend was observed for increased PYY expression in the jejunum, the site of protein digesta absorption.

Amino acids are products of protein digestion and have been suggested to regulate satiety and appetite in response to protein intake. Findings from our group have identified various appetite supressing amino acids including L-Cysteine, L-Arginine and L-Phenylalanine. The mechanism underlying L-Phenylalanine's anorectic effects were further investigated.

L-Phenylalanine decreased food intake, without apparent behavioural side effects, increased neuronal activation in the dorsal vagal complex (DVC), and stimulated the secretion of anorectic gut hormones and pancreatic hormones. The secretion of GLP-1 and PYY were blunted by a calcium sensing receptor (CaSR) antagonist *in vitro,* suggesting the CaSR may mediate the anorectic effects of L-Phenylalanine.

Vagal afferents transmit peripheral sensory information from the gut to the central nervous system via nuclei in the DVC. The activation of the DVC in response L-Phenylalanine suggested the vagus nerve might be involved in mediating these responses. The CaSR is one of the few nutrient sensors expressed in the vagus. Knockdown of the CaSR in the vagal afferents of mice inhibited protein induced glucagon secretion. This suggests the CaSR may play an important role in an entero-pancreatic vagal circuit that requires further investigation. The effect of CaSR knockdown on L-Phenylalanine's anorectic effects were inconclusive.

These studies examined the effects of L-Phenylalanine and high protein diets on appetite regulating hormones. They contribute to our understanding of the mechanism underlying the satiating effects of protein and identify vagally expressed CaSR as a potential nutrient sensor important in the regulation of pancreatic hormone secretion in response to protein intake. Further studies are required to determine the physiological importance of CaSR signalling in the vagus and the therapeutic potential of the CaSR system in the management of obesity.

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Declaration of Contributors

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

Calcium sensing receptor floxed mice were kindly donated by Wenhan Chang.

Chapter 3:

Studies in rats were performed in collaboration with Dr Amin Alamshah. C-Fos counting was performed by Miss Ye Cao in mice, Miss Sijing Cheng in rats and Mr Robert Hansford in the PBN of mice. All three individuals were blinded from treatment groups. The macro for automated c-Fos quantification was written by Mr Steven Rothery (Imperial College FILM facility). Miss Isabel Fernandes Freitas assisted with *in vivo* work.

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Dedicated to the memory of Dr Amin Alamshah; a wonderful mentor and colleague. You will be greatly missed.

Abbreviations

5-HT3	Serotonin 3 receptor
7TM	Seven transmembrane
AAV	Adeno-associated virus
AA	Amino acid
ACTH	Adrenocorticotropic hormone
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AgRP	Agouti-related peptide
AHA	Anterior hypothalamic area
APC	Anterior piriform cortex
AP	Area postrema
ARC	Arcuate nucleus
ATF4	Activating transcription factor 4
AUC	Area under the curve
BAT	Brown adipose tissue
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BH_4	Tetrahydrobiopterin
BMI	Body mass index
BSA	Bovine serum albumin
CART	Cocaine and amphetamine-regulated transcript
CaSR	Calcium sensing receptor
CCK	Cholecystokinin
CCK-A	Cholecystokinin receptor 1
CCK-B	Cholecystokinin receptor 2
CLAMS	Comprehensive laboratory animal monitoring system
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DAPI	4’,6-diamidino-2-phenylindole
DIO	Diet-induced obese
DIT	Dietary induced thermogenesis
DMEM	Dulbecco's Modified Eagle Medium

DMH	Dorsomedial nucleus of the hypothalamus
DMN	Dorsal motor nucleus of the vagus
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase 4
DVC	Dorsal vagal complex
EDTA	Ethylenediaminetetraacetic acid
EEC	Enteroendocrine cell
elf2α	Eukaryotic initiation factor 2 alpha
ENS	Enteric nervous system
ERK1/2	Extracellular signal-regulated kinases 1 and 2
GABA	γ-Aminobutyric acid
GCG	Preproglucagon
GCGR	Glucagon receptor
GCN2	General control non-repressed 2 protein
GEE	Generalised estimating equation
GHSR	Growth hormone secretagogue receptor
GI	gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GIP-R	Glucose-dependent insulinotropic peptide receptor
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
GPR142	G-protein-coupled receptor 142
HCD	High carbohydrate diet
HFD	High fat diet
HPD	High protein diet
HPLC	High pressure liquid chromatography
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IBMX	3-isobutyl-1-methylxanthine
ICV	Intracerebroventricular
IMP	Inosine monophosphate

IP	Intraperitoneal
IP_3	Inositol trisphosphate
IR	Immunoreactive
LHA	Lateral hypothalamic area
L-Phe	L-Phenylalanine
MBH	Mediobasal hypothalamus
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MCH	Melanin concentrating hormone
MSH	Melanocyte stimulating hormone
mTORC1	Mammalian target of rapamycin complex 1
NEP24.11	Neutral endopeptidase 24.11
NG	Nodose/jugular ganglion complex
NMR	Nuclear magnetic resonance
NPD	Normal protein diet
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OG	Oral gavage
OXM	Oxyntomodulin
OX-R1	Orexin receptor 1
PAH	Phenylalanine hydroxylase
PBN	Parabrachial nucleus
PBS	Phosphate buffer saline
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
PEG	Poly ethylene glycol
PEPT-1	Peptide transporter 1
PFA	Paraformaldehyde
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
PYY	Peptide YY
RER	Respiratory exchange rate

RIA	Radioimmunoassay
RM1	Rat and mouse diet 1 (maintenance)
RM3	Rat and mouse diet 3 (breeding)
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SEM	Standard error of the mean
SGLT1	Sodium glucose co-transporter 1
SK6	p70 S6 kinase 1
SNAT2	Sodium-coupled neutral amino acid transporter 2
SON	Supraoptic nucleus
T1R1/T1R3	Taste receptor 1 member 1- taste receptor 1 member 3
TAE	Tris-acetate-EDTA buffer
TBS	Tris-buffered saline
TCA	Tricarboxylic acid cycle
TRH	Thyrotropin-releasing hormone
TTBS	Tris-buffered saline + 0.1% Triton X-100
VCO_2	Carbon dioxide production
VFT	Venus fly trap
VMH	Ventromedial nucleus of the hypothalamus
VO_2	Oxygen consumption
WHO	World Health Organization
WPRE	Woodchuck hepatitis post-transcriptional regulatory element
WT	Wild-type

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Chapter 1: General Introduction

1.1. Obesity

Throughout most of history, due to a lack of resources and tools, humans have faced food deprivation. Therefore, the ability to generate energy stores for times of scarcity was crucial for survival and subsequently favoured by evolution. However, in more recent years, this genetic predisposition, in the context of an excess of readily available highly calorific food and a sedentary lifestyle, have driven huge increases in obesity and other metabolic disorders.

Obesity is the result of a metabolic imbalance in which energy intake is greater than energy expenditure, leading to excessive adiposity that can be detrimental to one's health. The World Health Organisation (WHO) uses body mass index (BMI), a ratio between an individual's body weight and height squared (kg/m^2), to classify adults as obese (BMI ≥ 30 kg/m^2) or overweight (BMI ≥ 25 kg/m^2) (WHO 2018). However, this does not truly represent adiposity. Nonetheless, it is useful for determining obesity at a population level, when more detailed information is unavailable.

According to the WHO, globally in 2016, 39% of adults were overweight and 13% were obese. Obesity is highly prevalent in both developing and developed countries, and in both older and younger generations, with 124 million children and adolescents being categorised as obese in 2016 (WHO 2018). This poses a major health concern; in children, obesity is associated with increased hypertension and metabolic risk factors (Pulgarón 2013) and in adults with an increased risk of cancer, diabetes and stroke (Wang, McPherson et al. 2011). This results in an economic burden on the UK national health system that is predicted to increase by £1.9 billion by 2030. This does not include the indirect costs from increased disability and decreased productivity in the population (Wang, McPherson et al. 2011). Therefore, there is an immediate need for effective anti-obesity therapies which are currently lacking.

1.1.1. Current obesity therapies

Current anti-obesity therapies include lifestyle changes, as well as pharmacological and surgical interventions. Lifestyle interventions are the first line of treatment. These include increasing energy expenditure, through exercise, and decreasing energy intake, through dieting, to restore energy balance. However long-term adherence is low (Inelmen, Toffanello et al. 2004, Moroshko, Brennan et al. 2011).

When lifestyle interventions fail, NICE recommends the use of pharmacological agents such as Orlistat, a gastrointestinal and pancreatic lipase inhibitor which prevents fat absorption. Consequently it has limited efficacy in diets with a low fat intake (<20%) and gastrointestinal side effects, such as fatty stool and faecal urgency, are commonly reported (Kang and Park 2012). Weight

loss from Orlistat is modest, with patients losing 2.9kg more than placebo treated patients after a year or more of treatment (Padwal, Rucker et al. 2003).

Liraglutide (Saxenda) is a glucagon-like peptide 1 (GLP-1) analogue. It was approved for the treatment of obesity in the UK in 2017 but is not NICE recommended or prescribed by the NHS. However, at a lower dose, it has been prescribed as an anti-diabetic agent since 2009. According to a double-blinded, randomised control study, a greater weight loss was observed in obese non-diabetic patients taking Liraglutide, compared to Orlistat and placebo controls, after two years of treatment (Astrup, Carraro et al. 2011). However, withdrawal due to adverse effects was greater in patients taking liraglutide, with gastrointestinal discomfort, particularly nausea, being highly common. Interestingly, liraglutide is also able to promote weight loss in patients with melanocortin 4 receptor (MC4R) mutations, a common cause of monogenic obesity (Iepsen, Zhang et al. 2018).

Concerns regarding the safety of liraglutides were raised following several case reports of patients on liraglutide developing pancreatitis (Franks, Lee et al. 2012, Jensen, Saha et al. 2015); a concern that has been raised with other GLP-1 based therapies (Denker and Dimarco 2006). Rate of acute pancreatitis as well as amylase and lipase levels were examined in the randomised controlled trials LEADER and SCALE, which examined cardiovascular safety and weight management, respectively, in response to liraglutide. In both studies there was a reversible, dose-dependent increase in amylin/lipase levels which was not associated with the rate of acute pancreatitis (Steinberg, Buse et al. 2017, Steinberg, Rosenstock et al. 2017). In the SCALE study, the risk of pancreatitis in the liraglutide group was greater than the placebo control, but the difference was small (0.4% Vs. 0.1%) (Steinberg, Rosenstock et al. 2017). On the other hand, in the LEADER study, there were fewer cases of pancreatitis in the liraglutide treated group (Steinberg, Buse et al. 2017).

The most effective weight loss therapy is bariatric surgery. This is normally recommended for individuals that either have a BMI >40 kg/m^2 or a BMI >35kg/m^2 with another comorbid disease that would benefit from weight loss (NICE 2014). In addition to sustained weight loss, bariatric surgery can result in improvement and remission of metabolic disorders such as type II diabetes. With current laproscopic techniques, amongst other factors, serious morbidity and mortality have decreased to 4% and 0.1-0.3% respectively, though this varies depending on the type of bariatric surgery (Nguyen and Varela 2016). However, bariatric surgery is still invasive, carries surgical risks and is impractical considering the extent of the population that is obese.

1.2. Energy homeostasis

Energy homeostasis is the balanced regulation of energy intake and expenditure; it is fundamental for survival. As a result, there are multiple systems, both central and peripheral, in place to regulate energy input and output.

1.2.1. Central energy homeostasis

The central nervous system receives both hormonal and neuronal inputs from across the periphery, including the gastrointestinal (GI) tract and adipose tissue; it integrates this information and coordinates an appropriate response to regulate energy homeostasis. The hypothalamus and caudal brain stem are key regions in the homeostatic regulation of food intake. Other central regions involved in appetite regulation, for example the mesocorticolimbic system, are thought to play a role in the hedonic regulation of food intake, but can also respond to hormonal signals from the gut, such as ghrelin and PYY_{3-36} (Skibicka, Hansson et al. 2011, Stadlbauer, Weber et al. 2014). Recent work establishing the circuitry of appetite control has also identified roles for specific neuronal populations in the amygdala and the thalamus in food intake (Campos, Bowen et al. 2016, Ong, Liu et al. 2017). However, although all of these regions are also susceptible to modulation by peripheral gut hormones, whether directly or indirectly, they will not be discussed in this thesis.

1.2.1.1. Hypothalamus

The hypothalamus is located at the base of the brain, below the thalamus and above the brain stem. It is comprised of multiple nuclei that are involved in regulating different aspects of homeostasis such as thermoregulation, fluid balance, glucose and energy homeostasis.

The role of the hypothalamus in energy homeostasis has been investigated for over 70 years. Initial experiments involving lesions to the hypothalamus in animals led to the 'dual centre' hypothesis (Hetherington and Ranson 1940, Anand and Brobeck 1951), which suggested that the ventromedial nucleus is a satiety centre and the lateral hypothalamus a feeding centre. However, it is now clear that the regulation of energy homeostasis involves a more complex interplay between nuclei of the hypothalamus and the rest of the brain, and that specific neuronal circuits within a single nucleus can have disparate roles in energy homeostasis.

The arcuate nucleus (ARC), ventromedial nucleus (VMH) dorsomedial nucleus (DMH), paraventricular nucleus (PVN) and lateral hypothalamus (LHA) are nuclei in the hypothalamus associated with the regulation of energy homeostasis, with the ARC being one of the most intensely studied. The organisation of these nuclei is shown in figure 1.2.1.1.

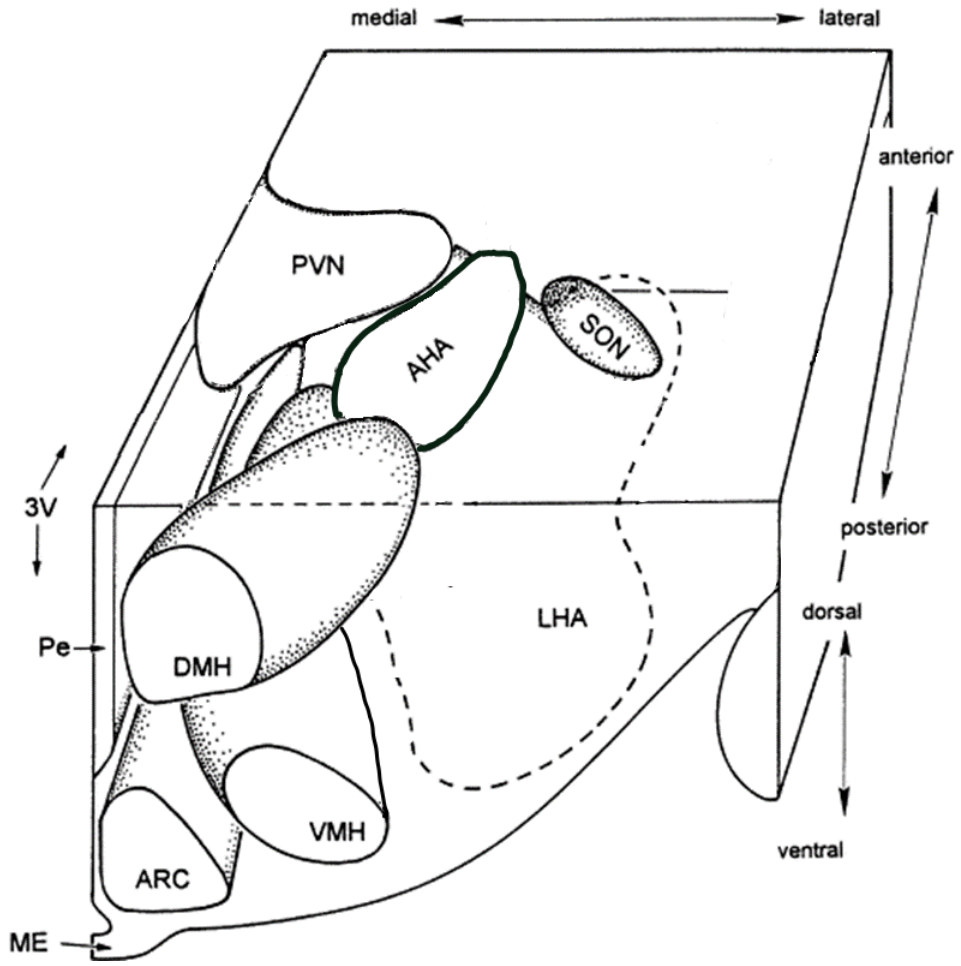


Figure 1.2.1.1 Schematic diagram of the structure and organisation of the rat hypothalamus.

ARC, arcuate nucleus; VMH, ventromedial nucleus; DMH, dorsomedial nucleus; Pe, periventricular nucleus; PVN, paraventricular nucleus; AHA, anterior hypothalamic area; SON, supraoptic nucleus; LHA, lateral hypothalamic area. Adapted from (Berthoud 2002)

1.2.1.2. Arcuate nucleus

The ARC is situated at the base of the third ventricle. In humans it is known as the infundibular nucleus. Within it there are two key neuronal populations important in the regulation of energy homeostasis: the orexigenic agouti related protein (AgRP)/neuropeptide Y (NPY) neurons, and the anorectic proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons, shown in figure 1.2.1.2. Both these neuronal populations express a variety of sensors, making the arcuate nucleus an important region in the detection and integration of peripheral signals, which are then communicated to the rest of the central nervous system through extensive projections to hypothalamic and extra-hypothalamic regions of the brain to regulate energy homeostasis.

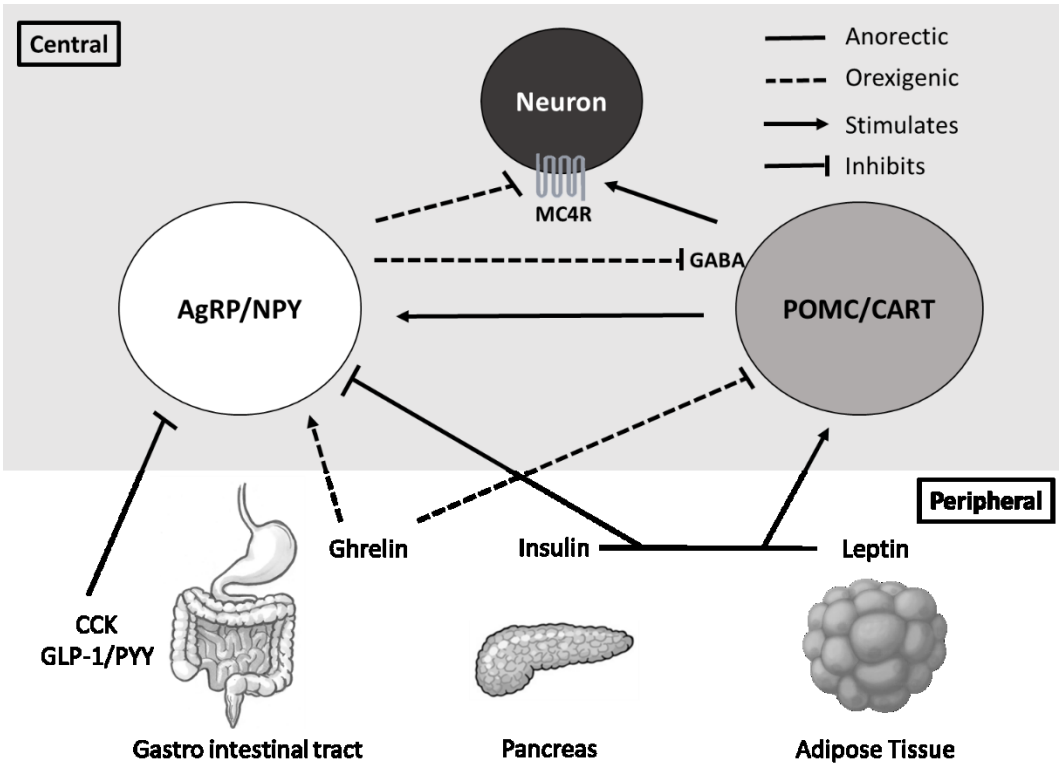


Figure 1.2.1.2 Diagram illustrating peripheral signal integration in arcuate nucleus AgRP/NPY and POMC/CART neurons projecting to other neuronal populations

CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; PYY, peptide YY; AgRP, agouti related peptide; NPY, neuropeptide Y; MC4R, melanocortin 4 receptor; GABA, Gamma-Aminobutyric Acid; POMC, proopiomelanocortin; Cart, cocaine amphetamine regulated transcript.

1.2.1.2.1. AgRP

In rodents, central AgRP expression is restricted to the ARC and median eminence (Shutter, Graham et al. 1997). In humans it is expressed in the hypothalamus and subthalamic nucleus. Outside of the brain AgRP is expressed in the adrenal medulla and at low levels in the testis (Shutter, Graham et al. 1997). AgRP acts on the melanocortin system, antagonising melanocortin 3 and 4 receptors (MC3R and MC4R) (Ollmann, Wilson et al. 1997, Haskell-Luevano and Monck 2001). It also acts as an inverse agonist at the melanocortin 4 receptor (Haskell-Luevano and Monck 2001, Nijenhuis, Oosterom et al. 2001). However, ablation of AgRP neurons leads to starvation independently of the melanocortin system, most likely via a γ-Aminobutyric acid (GABA) mediated mechanism; these neurons appear to inhibit a neuronal population in the parabrachial nucleus that powerfully suppresses food intake when released from this inhibition (Wu, Howell et al. 2008, Wu, Boyle et al. 2009).

The role of AgRP neurons in energy homeostasis is highlighted by optogenetic and chemogenetic studies that acutely activate AgRP neurons resulting in increases in food intake, energy expenditure

and motivation to work for food in mice (Aponte, Atasoy et al. 2011, Krashes, Koda et al. 2011). Chronic activation of these neurons in mice drives weight gain and increased fat mass; chemogenetic inactivation suppresses food intake (Krashes, Koda et al. 2011). Interestingly optogenetic stimulation of AgRP neurons can increase food intake following lithium chloride, which causes gastric discomfort, but not lipopolysaccharide, a component of bacterial cell walls (Essner, Smith et al. 2017). This would suggest that the orexigenic signal can be overridden. It is worth noting that (Aponte, Atasoy et al. 2011, Essner, Smith et al. 2017) and (Krashes, Koda et al. 2011) utilised mouse lines that express Cre recombinase in AgRP expressing neurons, the expression of which has been shown to overlap to some degree with POMC during development and in adult mice (Lam, Cimino et al. 2017).

Additionally, in rats, central AgRP injections increase food intake (Rossi, Kim et al. 1998) and fasting increases AgRP expression (Bi, Robinson et al. 2003). In mice, AgRP knock-out from birth has no effect on food intake and body composition (Qian, Chen et al. 2002), however when AgRP neurons are ablated in adult mice using diphtheria toxin, food intake is reduced (Gropp, Shanabrough et al. 2005); further supporting the importance of these neurons in feeding. This also highlights some of the restrictions of germline knockout models, in which rodents can compensate for the genetic ablation, and emphasises the benefits of using technologies such as inducible cre lines and optogenetics which allow a more time-specific manipulation of the DNA.

1.2.1.2.2. NPY

NPY is a potent orexigenic neuropeptide that is highly expressed in the ARC and DMH (Bi, Robinson et al. 2003). Its role in energy homeostasis seems to differ across nuclei. For more information on the role of DMH NPY on energy homeostasis see section 1.2.1.5.

The activity of ARC NPY neurons correlates with the feeding status of mice (Takahashi and Cone 2005). During fasting, NPY expression and firing is increased in the ARC, suggesting a physiological role for NPY in appetite regulation (Bi, Robinson et al. 2003, Takahashi and Cone 2005). NPY acts as an endogenous agonist at the Y_1 and Y_5 receptors that are expressed in multiple nuclei including the ARC and PVN (Durkin, Walker et al. 2000, Kopp, Xu et al. 2002). The PVN is highly innervated by NPY neurons. Administration of NPY into the PVN stimulates food intake in satiated rats (Stanley and Leibowitz 1985). Moreover, when administered chronically, NPY administration into the cerebral ventricles or the PVN of rodents results in obesity (Glenn Stanley, Kyrkouli et al. 1986, Zarjevski, Cusin et al. 1993).

Overexpression of NPY in the LHA or PVN in rats results in hyperphagia characterised by increased meal frequency in rats overexpressing NPY in the PVN, and increased meal size in rats

overexpressing NPY in the LHA (Tiesjema, Adan et al. 2007). On the other hand, NPY knockdown in rats using anti-sense sera results in hypophagia and weight loss (Gardiner, Kong et al. 2005). This effect is not seen in germline knockout mice, presumably due to developmental compensation (Erickson, Clegg et al. 1996). However, germline NPY knockout did attenuate the obese phenotype observed in leptin deficient mice, hinting at the importance of NPY neurons in leptin signalling (Erickson, Hollopeter et al. 1996).

ARC NPY neurons express the leptin receptor (Draper, Kirigiti et al. 2010). Leptin down regulates fasting induced NPY expression and neuronal firing in rats and mice, respectively (Korner, Savontaus et al. 2001) (Takahashi and Cone 2005). It plays an important role in regulating the intrinsic excitability of NPY neurons through, in part, the modulation of voltage gated potassium channels (Baver, Hope et al. 2014). NPY neurons are also susceptible to regulation by other peripheral signals including peptide YY (PYY), as shown in figure 1.2.2.1.

1.2.1.2.3. POMC

POMC expression is dynamic throughout development. However, central POMC expression is limited to the ARC and the nucleus tractus solitarius (NTS) (Padilla, Reef et al. 2012, Wang, He et al. 2015). The POMC precursor molecule can be post-translationally processed in a tissue-specific manner into a series of peptides including melanocyte stimulating hormones (α-, β- and y-MSH), adrenocorticotrophic hormone (ACTH) and β-endorphins.

Transciptomic studies have shown that POMC neurons are highly heterogeneous and express various receptors for peripheral signals such as leptin (25% of neurons) and insulin (57% of neurons)(Lam, Cimino et al. 2017). Following fasting, POMC expression in the hypothalamus decreases, (Mizuno, Kleopoulos et al. 1998). Additionally, in humans, mutations in POMC signalling lead to early-onset obesity, red hair pigmentation and adrenal insufficiency (Krude, Biebermann et al. 1998). This suggests a physiological role for the peptide in the regulation of energy homeostasis.

When administered centrally, α-MSH, an endogenous agonist of MC3R and MC4R G-protein coupled receptors, inhibits food intake in fasted rats (Tsujii and Bray 1989). Although MC3R is highly concentrated in the hypothalamus (Roselli-Rehfuss, Mountjoy et al. 1993), it has been suggested that hypothalamic MC3R plays a limited role in food intake, as central administration of MC3R selective agonists failed to supress food intake (Abbott, Rossi et al. 2000). Furthermore, MC3R deficient mice are hypophagic despite their increased adiposity (Chen, Marsh et al. 2000). Consequently, MC4R is thought to mediate the food intake effects of α-MSH.

In humans and rodents, function compromising mutations in the MC4R result in an obese and hyperphagic phenotype (Huszar, Lynch et al. 1997, Yeo, Farooqi et al. 1998), indicating its importance in providing an inhibitory tone on feeding. Central administration of AgRP, an endogenous inverse agonist of the MC4R, increases food intake in rats and inhibits the anorectic effects of α-MSH (Rossi, Kim et al. 1998). While AgRP also antagonises the MC3R, central administration of a MC4R specific antagonist also increases food intake in rodents (Kask, Mutulis et al. 1998, Kask, Rago et al. 1998, Vergoni, Bertolini et al. 1998, Skuladottir, Jonsson et al. 1999), suggesting that the MC4R may be sufficient to mediate the effects of AgRP on appetite.

MC4R agonists have been considered for the treatment of obesity. Setmelanotide, a second generation MC4R agonist, promotes weight loss and ameliorates hunger in patients with POMC (Kühnen, Clément et al. 2016), MC4R (Collet, Dubern et al. 2017) and leptin receptor (Clément, Biebermann et al. 2018) deficiencies, without any apparent cardiovascular adverse events. It has also been found to acutely increase resting energy expenditure (Chen, Muniyappa et al. 2015) and is currently undergoing phase III clinical trials. Other MC4R agonists such as melanotan II have been discontinued due to their side effects (Fani, Bak et al. 2013).

1.2.1.3. Paraventricular nucleus

The PVN is located adjacent to the third ventricle and sends projections to other hypothalamic nuclei, for example the ARC, and extra hypothalamic nuclei, such as the NTS in the brain stem and the mesocorticolimbic system. The PVN also sends projections to spinal centres important in the autonomic nervous system (Saper, Loewy et al. 1976, Swanson 1977, Geerling, Shin et al. 2010).

The PVN and ARC are interconnected, with many ARC POMC and AgRP neurons projecting to the PVN. Within the PVN, the MC4R, a receptor endogenously modulated by α-MSH and AgRP, is highly expressed (Jacqueline, Unga et al. 2013). Intra-PVN administration of AgRP-like peptides, that antagonise the MC4R, increases feeding in rats, while administration of MC4R agonists supresses food intake (Giraudo, Billington et al. 1998). NPY and CART also increase and decrease food intake, respectively, when administered directly into the PVN (Stanley and Leibowitz 1984, Stanley, Chin et al. 1985, Stanley, Small et al. 2001).

Within the PVN there are various neuronal cell types including vasopressin- and oxytocin-expressing magnocellular neurons important in homeostasis and social behaviours (Stoop 2012), and parvocellular neurons. Parvocellular neurons project to many regions associated to appetite regulation, including within the PVN itself, other regions of the hypothalamus, the brainstem and the median eminence. These neurons are heterogenous and express a variety of neuropeptides and hormones, including corticotropin-releasing hormone (CRH), important in the hypothalamic-

pituitary-adrenal stress axis, and thyrotropin-releasing hormone (TRH), important in in the hypothalamic-pituitary-thyroid axis. TRH neurons are heavily innervated by AgRP/NPY and POMC/CART neurons, and the expression of TRH decreases during fasting (Fekete, Légrádi et al. 2000). Central administration of TRH and CRH decreases food intake, promotes weight loss and increases energy expenditure (Vijayan and McCann 1977, Suzuki, Kohno et al. 1982, Arase, York et al. 1988).

The PVN is sensitive to the effects of orexigenic and anorectic peptides. Central intra-ventricular of anorectic GLP-1 (Turton, O'Shea et al. 1996) and orexigenic NPY (Xu, Li et al. 1995) stimulates c-Fos expression the PVN of rats, as does peripheral administration of anorectic leptin (Elmquist, Ahima et al. 1997). When administered directly into the PVN, leptin (Shih, Au et al. 2003) and GLP-1 (McMahon and Wellman 1998) decreased the food intake of fasted rodents. On the other hand, administration of NPY, galanin, orexin, AgRP, or noraderenaline into the PVN stimulated feeding (Leibowitz, Roossin et al. 1984, Stanley and Leibowitz 1984, Kyrkouli, Stanley et al. 1990, Edwards, Abusnana et al. 1999). Moreover, PVN lesions in rats result in weight gain and hyperphagia, though to a lesser extent than VMH lesions (Weingarten, Chang et al. 1985, Fukushima, Tokunaga et al. 1987).

1.2.1.4. Ventromedial nucleus

The VMH sends and receives projections to and from other appetite regulating centres of the brain including the ARC, brainstem and amygdala (Saper, Swanson et al. 1976, Koylu, Couceyro et al. 1997, Sternson, Shepherd et al. 2005). Bilateral lesions to the VMH result in hyperphagia and weight gain (Shimizu, Oomura et al. 1987).

Brain-derived neurotrophic factor (BDNF), a gene locus associated with obesity in human genome-wide association studies, is highly expressed in the VMH (Speliotes, Willer et al. 2010). It's expression in the VMH is suppressed post-fasting (Xu, Goulding et al. 2003) and increased following glucose and leptin administration (Komori, Morikawa et al. 2006, Unger, Calderon et al. 2007). Acute administration of BDNF into the VMH results in increased energy expenditure, hypophagia and weight loss (Wang, Bomberg et al. 2007, Wang, Bomberg et al. 2010). Chronic administration is protective against diet-induced obesity (Godar, Dai et al. 2011). Moreover, BDNF knock down in developed mice results in hyperphagia and obesity (Yang, An et al. 2016). This suggests VMH BDNF plays an important role in appetite regulation.

The VMH expresses an array of receptors that detect appetite regulating peptides, including leptin (Elmquist, Bjorbaek et al. 1998), NPY (Weinberg, Sirinathsinghji et al. 1996), CCK (Day, Hall et al. 1986), α-MSH and AgRP (Huang, Han et al. 2003). Direct administration of NPY into the VMH

increases food intake (Bouali, Fournier et al. 1995), whereas leptin microinjections decrease food intake (Jacob, Dziura et al. 1997). This further supports the involvement of the VMH in energy homeostasis, though much is still to be learned.

1.2.1.5. Dorsomedial nucleus

The DMH projects to hypothalamic nuclei (LHA, VMH, PVN, ARC), extra hypothalamic areas (brainstem) and circumventricular organs (subfornical organ) (Horst and Luiten 1986). Many of these projections are reciprocated.

The DMH plays an important role in mediating the thermoregulatory effects of leptin. Exposure to the cold or to peripheral leptin activates neurons in the DMH, measured using c-Fos as a surrogate marker (Zhang, Kerman et al. 2011, Rezai-Zadeh, Yu et al. 2014). Intra-DMH leptin administration stimulates sympathetic brown adipose tissue (BAT) tone (Enriori, Sinnayah et al. 2011) and optogenetic activation of leptin receptor positive DMH neurons results in increased energy expenditure and weight loss (Rezai-Zadeh, Yu et al. 2014). Moreover, leptin receptor knockdown in the DMH results in weight gain and decreased energy expenditure (Dodd, Worth et al. 2014, Rezai-Zadeh, Yu et al. 2014). Similarly, GLP-1 signalling in the DMH seems to be important in BAT thermogenesis and adiposity (Lee, Sanchez-Watts et al. 2018), and appears to modulate NPY mRNA expression in the DMH (Lee, Sanchez-Watts et al. 2018).

Unlike ARC NPY neurons, DMH NPY neurons do not co-localise with the leptin receptor (Draper, Kirigiti et al. 2010). NPY expression in the DMH is increased in rats chronically deprived from food (calorie restricted) (Bi, Robinson et al. 2003), and in various rodent models of obesity, including diet induced obese (DIO) mice (Guan, Yu et al. 1998) and MC4R knockout mice (Kesterson, Huszar et al. 1997). NPY overexpression in the DMH results in hyperphagia and weight gain in rats, whereas knockdown enhances thermogenesis, improves adiposity and protects against DIO (Chao, Yang et al. 2011) (Yang, Scott et al. 2009). Moreover, NPY neurons in the DMH project to the brainstem and modulate the satiating effects of CCK (Yang, Scott et al. 2009, de La Serre, Kim et al. 2016). Together, this data highlights the importance of NPY DMH neurons in energy homeostasis.

1.2.1.6. Lateral hypothalamus

The LHA was originally designated the “feeding centre” as LHA lesions resulted in hypophagia and weight loss (Anand and Brobeck 1951). In accordance, LHA electrical stimulation increased feeding (Delgado and Anand 1953).

The LHA neuronal population is heterogeneous, expressing various peptides involved in appetite regulation, including melanin-concentrating hormone (MCH), glutamate, neurotensin, galanin and

orexin/hypocretin (Qu, Ludwig et al. 1996, de Lecea, Kilduff et al. 1998, Leinninger, Jo et al. 2009). MCH and orexin/hypocretin neurons do not express the leptin receptor and form distinct neuronal populations (Leinninger, Jo et al. 2009). The expression of MCH and orexin in the LHA is increased during fasting, and their central administration stimulates food intake (Qu, Ludwig et al. 1996, Sakurai, Amemiya et al. 1998). Animal models overexpressing MCH are obese and hyperphagic (Ludwig, Tritos et al. 2001), whereas MCH knockout mice are hypophagic and lose weight, despite low levels of leptin (Shimada, Tritos et al. 1998).

On the other hand, neurotensin-expressing neurons of the LHA do express the leptin receptor. Neurotensin is an appetite regulating hormone found centrally and peripherally. However, the LHA is the only place where neurotensin expressing neurons colocalise with the leptin receptor. Leptin receptor knockout in neurotensin neurons of the LHA results in hyperphagic obese mice that have reduced energy expenditure (Leinninger, Opland et al. 2011).

Within the LHA there are also GABAergic and glutamatergic neurons that, based on optogenetic studies, have opposing effects on appetite regulation. Optogenetic stimulation of LHA GABAergic neurons results in voracious feeding (Jennings, Ung et al. 2015). Contrastingly, photoactivation of LHA glutamatergic neurons suppressed food intake in calorie restricted mice (Jennings, Rizzi et al. 2013).

The LHA also plays an important role in arousal that has not been discussed here but is well described in the literature (Chemelli, Willie et al. 1999, Harris and Aston-Jones 2006, Adamantidis and De Lecea 2009).

1.2.1.7. Supraoptic nucleus

The SON contains oxytocin and vasopressin secreting magnocellular neurons which co-express appetite regulating peptides such as galanin, nesfatin-1, CART and CCK (Gai, Geffen et al. 1990, Meister, Villar et al. 1990, Elias, Lee et al. 2001, Kohno, Nakata et al. 2008). SON neurons also express receptors for appetite regulating hormones including the leptin and MC4 receptors (Håkansson, Brown et al. 1998, Jacqueline, Unga et al. 2013).

C-Fos immune-like reactivity is observed in the SON following entrained feeding (Johnstone, Fong et al. 2006) and other feeding cues, including α-MSH (Sabatier, Caquineau et al. 2003), gastric administration of a high sugar load (Hume, Sabatier et al. 2017) and CCK administration (Caquineau, Douglas et al. 2010) . C-Fos expression in response to CCK is attenuated by leptin (Caquineau, Douglas et al. 2010). Moreover, even though α-MSH increases c-Fos activation in the SON and stimulates oxytocin dendritic release, it decreases the electrical excitability of SON neurons and the

secretion of oxytocin into the general circulation (Sabatier, Caquineau et al. 2003). In contrast, peripheral secretin increases SON neuronal firing (Velmurugan, Brunton et al. 2010).

SON neurons have also been suggested to function as glucose sensors as they express insulin receptors and glucokinase. Both insulin and glucose stimulate the secretion of oxytocin, when administered together, and vasopressin, when administered individually or in combination, into the SON (Song, Levin et al. 2014). This response is still present in DIO rats (Sladek, Stevens et al. 2015).

1.2.1.8. Caudal brainstem

The brainstem consists of the midbrain, pons and medulla oblongata and acts as a conduit for motor and sensory information travelling between the CNS and periphery. It is important in consciousness, gustation and the autonomic control of the cardiac, respiratory and digestive system.

The importance of the brainstem in appetite regulation is supported by the ability of decerebrated rats, that have the connection between the brain and brainstem severed, to become satiated and metabolically respond to starving conditions by, for example, decreasing their energy expenditure (Grill and Norgren 1978, Harris, Kelso et al. 2006).

The NTS, area postrema (AP) and dorsal motor nucleus (DMN) are three nuclei in the caudal brainstem important in the regulation of energy homeostasis. Together they form the dorsal-vagal complex (DVC) shown in figure 1.2.1.3. The DVC expresses a variety of receptors for factors involved in appetite regulation including the leptin receptor, insulin receptor, MC4 receptor, ghrelin receptor (growth hormone secretagogue receptor; GHSR) and CRH receptor (Skofitsch, Insel et al. 1985, Unger, Moss et al. 1991, Grill, Schwartz et al. 2002, Kishi, Aschkenasi et al. 2003, Zigman, Jones et al. 2006).

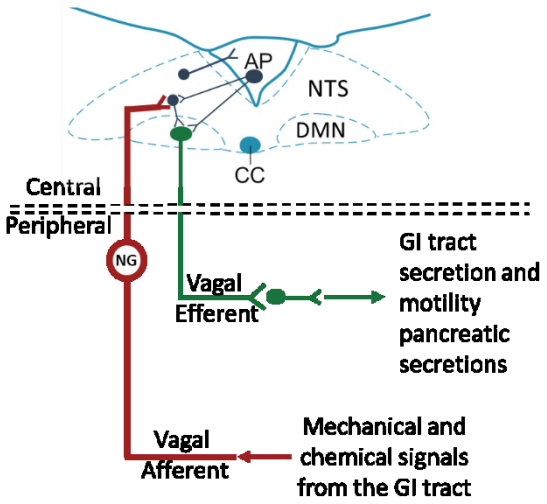


Figure 1.2.1.3 Schematic diagram showing dorsal vagal complex organisation and innervation.

Schematic coronal section of the rodent brainstem showing the organisation of the dorsal vagal complex and its innervation. Vagal sensory neurons innervate the gastrointestinal tract and project primarily to the nucleus tractus solitarius (NTS). Their cell bodies are located in the nodose ganglion (NG). The dorsal motor nucleus (DMN) contains vagal motor neurons that innervate the gastrointestinal tract and pancreas. There is extensive intercommunication between the NTS, DMN and AP. CC, central canal.

Leptin and urocortin (a CRH receptor endogenous agonist), administration into the DVC decreases food intake (Grill, Markison et al. 2000, Grill, Schwartz et al. 2002), as does MC4 receptor agonist administration into the 4th ventricle (Grill, Ginsberg et al. 1998). In contrast, intra-DVC administration of ghrelin increases food intake (Faulconbridge, Cummings et al. 2003)

The NTS forms connections with the GI tract, various nuclei in other regions of the brain involved in appetite regulation, and other nuclei in the brainstem. Centrally, it forms neuronal circuits with the amygdala, PVN, ARC and LHA, amongst others (van der Kooy, Koda et al. 1984). From the periphery, and relating to energy homeostasis, the NTS receives gustatory information from the tongue, via cranial nerves 7, 9 and 10, which is relayed to the parabrachial nucleus (PBN), also located in the brainstem (Katz, Nicolelis et al. 2000). It also receives mechanical and chemical sensory information from the gut via the vagal afferent nerve, cranial nerve 10, which is discussed in more detail in chapter 4. This sensory information is integrated in the NTS which projects to and regulates the DMN. The DMN contains the cell bodies of parasympathetic motor vagal efferent neurons that in turn control gastric and pancreatic function, mediating the vago-vagal reflex (Travagli, Hermann et al. 2006).

The NTS is the main source of GLP-1 in the brain, and one of the two central nuclei that contain POMC neurons (Wang, He et al. 2015, Holt, Richards et al. 2019). NTS GLP-1 knockdown does not affect normal feeding and body weight in mice. However, it does blunt stress induced hypophagia (Holt, Richards et al. 2019). NTS POMC knockdown doesn't affect food intake or body weight either

(Zhan, Zhou et al. 2013). However, NTS serotonin receptor-expressing POMC neurons are important in the acute anorectic effects of lorcaserin, an FDA approved anti-obesity medication (D'Agostino, Lyons et al. 2018). Moreover, NTS POMC neurons are activated by peripheral CCK, the appetite supressing effects of which are partly mediated by MC4R in the brain stem (Fan, Ellacott et al. 2004). CCK is also expressed centrally in the NTS in neurons that project to the PVN; their activation decreases food intake and body weight in mice (D'Agostino, Lyons et al. 2016).

In the brainstem, the AP forms prominent reciprocal projections with the NTS and PBN; centrally, it is mainly innervated by the PVN and DMH (Shapiro and Miselis 1985). It is a circumventricular organ and is thus able to sense factors in the general circulation. It was traditionally designated the 'chemoreceptor trigger zone' due to its role in emesis and detecting noxious substances (Miller and Leslie 1994). However, it is also important in metabolism (Price, Hoyda et al. 2008).

Neuronal activation, measured by the expression of c-Fos, is detected in the AP in response to feeding (Johnstone, Fong et al. 2006) and various satiety signals including CCK (Luckman, Hamamura et al. 1993), ghrelin (Lawrence, Snape et al. 2002), GLP-1 (Baumgartner, Pacheco-López et al. 2010) and PYY_{3-36} (Blevins, Chelikani et al. 2008), peptides that have corresponding receptors in the AP (Hill, Campbell et al. 1987, Richards, Parker et al. 2014, Cabral, Cornejo et al. 2017).

1.2.2. Peripheral energy homeostasis

The central circuitry regulating energy homeostasis integrates information from the periphery, including hormonal signals from the GI tract.

1.2.2.1. Enteroendocrine cells and gut hormones

Enteroendocrine cells (EECs) are specialised cells in the gut epithelium that secrete gut hormones. They account for less than 1% of the intestinal epithelial cell population and are typically classified according to the gut hormone they predominantly secrete (Spreckley and Murphy 2015, Gribble and Reimann 2016). For example, EECs that express the preproglucagon gene are classified as L-cells (Spreckley and Murphy 2015). However transcriptomic (Habib, Richards et al. 2012) and single-cell RNA- sequencing studies (Glass, Calero-Nieto et al. 2017) have shown that there is considerable overlap in the expression of gut hormones within EECs. For example, L cells in the proximal intestine can be grouped into three clusters that vary in the range of gut hormones and receptors they express (Glass, Calero-Nieto et al. 2017).

The existence of EECs that form synapses with neurons that directly innervate the brain suggest there are two modes by which EECs can transmit sensory information from the gut- one which is

relatively slow, through hormones, and another more rapid method- neuronal firing (Bohorquez, Shahid et al. 2015, Spreckley and Murphy 2015, Kaelberer, Buchanan et al. 2018). The existence of endocrine-like and neuron-like EECs may also explain the differences in EEC turnover observed. EEC turnover in the upper GI is estimated to be around 3-10 days, based on thymidine labelling studies (Gribble and Reimann 2016). However, in the distal gut, EECs living up to 60 days have been observed (Bohorquez, Shahid et al. 2015).

Gut hormones vary in their mode of action, target tissue, downstream effects and region of expression. They can have autocrine, paracrine and endocrine effects, and can modulate the activity of neurons such as the vagus nerve.

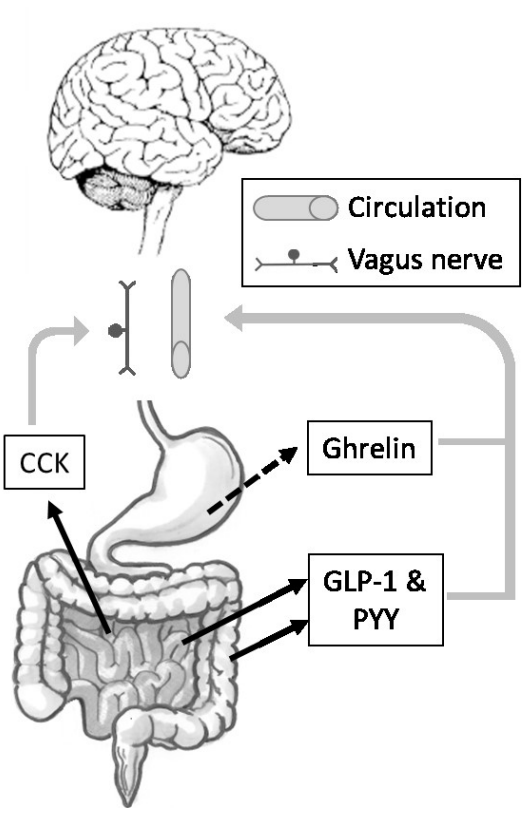


Figure 1.2.2.1 Illustration of the putative routes by which gut hormones can regulate appetite.

Anorectic (-) and orexigenic (- -) gut hormones secreted at different levels of the gut can signal to the central nervous system directly via the general circulation or indirectly via the vagus nerve. CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; PYY, peptide YY.

1.2.2.1.1. Ghrelin

Ghrelin is an orexigenic hormone that, in the gut, is produced by X/A-like cells in rodents, and P/D_1 type cells in humans. These cells are predominantly located in the stomach and upper GI tract (Date,

Kojima et al. 2000, Rindi, Necchi et al. 2002). Ghrelin is encoded by pre-proghrelin (117 aa) that is cleaved to produce ghrelin (28aa), a small signalling peptide and obestatin (23aa). Ghrelin is post-translationally modified by ghrelin O-acyl-transferase (GOAT) which acetylates ghrelin's serine 3 residue with an eight-carbon fatty acid producing the active form acyl-ghrelin, the endogenous ligand of the GHSR (Kojima, Hosoda et al. 1999, Yang, Brown et al. 2008). The liver-expressed antimicrobial peptide 2 (LEAP 2) was recently identified as an endogenous antagonist of this receptor (Ge, Yang et al. 2018).

In rodents, central and peripheral administration of ghrelin increases food intake and adiposity (Tschop, Smiley et al. 2000, Wren, Small et al. 2000). Ghrelin's central effects are thought to be mediated by NPY/AgRP neurons in the ARC; centrally administered ghrelin increases AgRP and NPY gene expression in the ARC (Kamegai, Tamura et al. 2001), and ghrelin's orexigenic effect is lost in AgRP and NPY knockout mice (Chen, Trumbauer et al. 2004). Ghrelin also increases food intake in humans when administered intra-venously, no gastric motility effects were seen in this study (Wren, Seal et al. 2001).

In addition to playing a role in appetite regulation, ghrelin has been found to supress sympathetic BAT nerve activity (Yasuda, Masaki et al. 2003); inhibit glucose induced insulin secretion in humans (Tong, Prigeon et al. 2010) and in rodents (Reimer, Pacini et al. 2003); promote adipogenesis (Thompson, Gill et al. 2004) and modulate gastric motility (Masuda, Tanaka et al. 2000, Levin, Edholm et al. 2006).

Even though most of the metabolic effects of ghrelin are thought to be mediated by the acetylated form, there is evidence to suggest des-acyl ghrelin may also have some biological activity in adipogenesis and glucose metabolism. However, these putative effects are controversial (Thompson, Gill et al. 2004, Zhang, Chai et al. 2008, Heppner, Piechowski et al. 2014).

Mice lacking ghrelin or GHSR are resistant to diet induced obesity, making ghrelin a potential target for anti-obesity therapies (Zigman, Nakano et al. 2005). However, neutralising ghrelin with oligonucleotides had short lived effects in rodents (Shearman, Wang et al. 2006), and neutralisation with antibodies, though successful in rodents (Zorrilla, Iwasaki et al. 2006), failed in human trials as an obesity therapy (CYT009-GhrQ cyto biotechnology vaccine). This may be because ghrelin signalling is disrupted during obesity, with the obese having decreased circulating levels of ghrelin (Tschop, Weyer et al. 2001) and loss of postprandial ghrelin suppression (le Roux, Patterson et al. 2005).

GHSR antagonists have also failed in the clinic, this may be due to GHSR's constitutive activity (Holst and Schwartz 2004). Inverse agonists are therefore considered a better approach. In humans, the inverse agonist F-05190457 was found to delay gastric emptying. However, it also increased heart rate, drowsiness and chronic administration resulted in tachyphylaxis, putting into question its efficacy as an anti-obesity therapy. No effect on food intake was investigated and no changes in post prandial insulin levels were observed (Denney, Sonnenberg et al. 2017).

1.2.2.1.2. Cholecystokinin (CCK)

In the gut CCK is secreted from I cells, predominantly found in the proximal intestine, in response to fats and proteins (Polak, Bloom et al. 1975, Liddle, Goldfine et al. 1985). CCK is encoded by preprocholecystokinin and can be metabolised into different sized CCK peptides ranging from 4 to 83 amino acids in length. These have different potencies, clearance rates and actions (Overduin, Gibbs et al. 2014). In rats, the most predominant form in the nervous system is CCK-8, whereas CCK-58 is the major circulating form (Reeve, Green et al. 2003).

Intravenous administration of CCK (-8 or -33) in lean and obese subjects decreases food intake (Kissileff, Pi-Sunyer et al. 1981, Pi-Sunyer, Kissileff et al. 1982, Lieverse, Jansen et al. 1994, Ballinger, McLoughlin et al. 1995). However, chronic administration of CCK leads to rapid behavioural tolerance (Crawley and Beinfeld 1983), in which CCK-8 infused rodents decrease their meal size but, in compensation, increase their meal frequency resulting in no change in body weight (West, Fey et al. 1984, West, Greenwood et al. 1987). CCK-58, compared to CCK-8, is more potent at supressing food intake, has a longer half-life and, unlike CCK-8, increases inter-meal intervals (Overduin, Gibbs et al. 2014). Increased satiety is also observed in rats following intraperitoneal administration of CCK-33, but not of CCK-8 (Washington, Coggeshall et al. 2011).

CCK's effects on food intake are thought to be regulated by the CCK-A (CCK-1R) receptor, rather than CCK-B receptor (CCK-2R). CCK-A knockdown in mice results in loss of exogenous CCK's anorectic effects, but these effects persist in CCK-B knockdown mice (Kopin, Mathes et al. 1999). Additionally, in rhesus monkeys, CCK-A antagonists increase food intake, whereas CCK-B antagonists do not (Moran, Ameglio et al. 1993). CCK-A antagonism also increases food intake in humans (Beglinger, Degen et al. 2001).

CCK-A is expressed on vagal terminals. Consequently, following vagotomy, the anorectic effects of CCK-8 are blunted or diminished in rats, depending on the CCK-8 dosage (Joyner, Smith et al. 1993). These residual effects at higher doses may be due to non-vagal mechanisms. CCK-A is also expressed in the pancreas, where it modulates pancreatic exocrine secretion (Varga, Kisfalvi et al. 1997), the

stomach, where activation decreases gastric emptying (Liddle, Morita et al. 1986, Scarpignato, Varga et al. 1993); and in the gall bladder, where it stimulates gall bladder contraction (Liddle, Goldfine et al. 1985, Tang, Biemond et al. 1996). However, it is worth noting that there are species differences in the expression of CCK-A and CCK-B in the pancreas (Monstein, Nylander et al. 1996, Tang, Biemond et al. 1996).

CCK agonists have failed to promote weight loss in obese patients (Jordan, Greenway et al. 2008) . Nonetheless, the ability of CCK to act in synergy with amylin and leptin may lead to it being useful as a combination therapy to treat obesity (Barrachina, Martinez et al. 1997, Bhavsar, Watkins et al. 1998, Matson, Reid et al. 2000).

1.2.2.1.3. Preproglucagon products: Glucagon-like Peptide 1 (GLP-1)

Preprogucagon (GCG) is expressed in α cells of the pancreas, L cells of the gut and in the NTS of the brainstem (Larsen, Tang-Christensen et al. 1997, Sandoval and D'Alessio 2015). The gene gives rise to a variety of peptides including glucagon, GLP-1 and oxyntomodulin, a GLP-1 and glucagon receptor dual agonist. Which peptide is made is partly dependent on which prohormone convertase (PC) enzyme predominates in that tissue.

In α-cells, PC2 is more predominant giving rise to glucagon, glicentin-related pancreatic peptide, intervening peptide 1 and the major proglucagon fragment. In the brainstem and in L-cells, PC1/3 is more predominant, leading to the production of GLP-1, GLP-2, oxyntomodulin, intervening peptide 1 and glicentin (Rouille, Martin et al. 1995). This is summarised in figure 1.2.2.2.

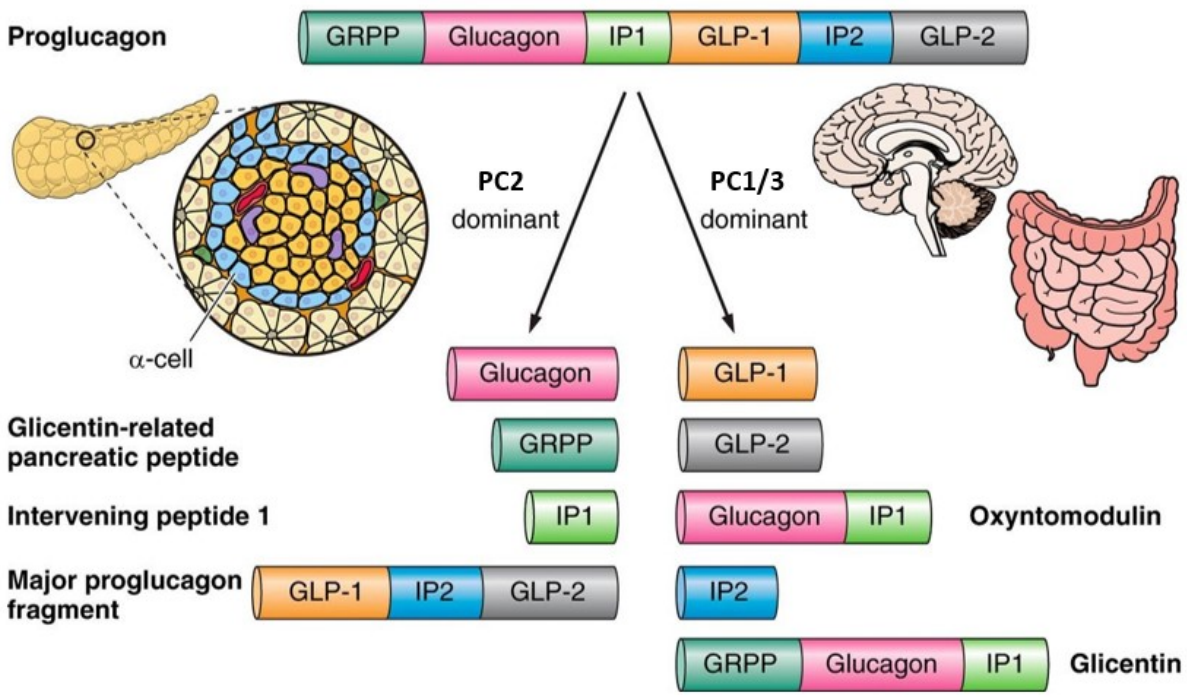


Figure 1.2.2.2 Schematic representation of proglucagon post-translational processing by pro-hormone convertase 1/3 and 2.

Proglucagon gives rise to different peptide products depending on which prohormone convertase it is cleaved by. Prohormone convertase 2 (PC2) is predominant in α-cells of the pancreas whereas prohormone convertase 1/3 (PC1/3) is predominant in L cells of the gut and in the brainstem. GLP-1: glucagon-like peptide 1, GLP-2: glucagon-like peptide 2, IP2: Intervening peptide 2. Image adapted from (Sandoval and D'Alessio 2015).

L-cells express a variety of nutrient sensors, allowing for the detection of ingested nutrients and subsequent GLP-1 release. In the gut, the density of GLP-1 immunoreactive cells increases from the proximal to the distal gut, with the greatest density in the rectum in humans and in the ileum in rats (Eissele, Goke et al. 1992).

GLP-1

GLP-1 is an anorectic gut hormone that has two major biologically active forms, amidated GLP-1_{7-36} and glicentin extended GLP-1_{7-37}. In the gut, GLP-1_{7-36} is four-fold more prevalent than GLP-1_{7-37}, with GLP-1_{7-36} rising more extensively than GLP-1_{7-37} in response to a meal (Orskov, Rabenhoj et al. 1994). Both forms are rapidly degraded by the serine protease dipeptidyl peptidase IV (DPP-IV) that recognises the alanine residue in position 2 at the amino terminus of the peptide, giving rise to GLP-

$1_{9\text{-}36/37}$, classically believed to be inactive. DPP-IV is expressed in endothelial cells adjacent to GLP-1 secreting EEC, in addition to other places including enterocytes and in circulation as a soluble plasma peptide. Consequently, GLP-1 has as a short plasma half-life of 1-2 minutes and only ~10% of secreted GLP-1 reaches the liver (Deacon, Pridal et al. 1996, Mulvihill, Varin et al. 2017). GLP-1 is also susceptible to proteolytic cleavage by neutral endopeptidase 24.11 (NEP24.11) (Hupe-Sodmann, McGregor et al. 1995). GLP-1 and its metabolites are rapidly cleared by the kidneys (Deacon, Pridal et al. 1996).

The GLP-1 receptor (GLP-1R) is a G-protein coupled receptor belonging the secretin family (class B), a class of receptors characterised by having a large extracellular ligand binding domain (Thorens 1992). GLP-1R is highly expressed in appetite regulating centres of the brain such as the brainstem and hypothalamus (ARC, PVN, DMH and amygdala) in non-human primates (Heppner, Kirigiti et al. 2015) and rodents (Bullock, Heller et al. 1996, Cork, Richards et al. 2015). Moreover, peripheral administration of GLP-1 leads to c-Fos activation in the PVN and brainstem (Baggio, Huang et al. 2004) suggesting it may mediate its effects on appetite via these pathways. Peripherally, it is expressed in the kidney, pancreas and gut, amongst other areas (Bullock, Heller et al. 1996).

Central administration of the GLP-1R agonist Exendin 4 or the GLP-1R antagonist $Exendin_{9\text{-}39}$ decreases or increases food intake and body weight in rodents, respectively (Meeran, O'Shea et al. 1999, Baggio, Huang et al. 2004). However, global or central and peripheral nervous system specific GLP-1R knock-down does not cause hyperphagia or obesity (Scrocchi, Hill et al. 2000, Sisley, Gutierrez-Aguilar et al. 2014).

GLP-1 is also an incretin hormone, potentiating insulin release following nutrient ingestion (Kreymann, Williams et al. 1987); and is involved in the ileal brake, decreasing gastric emptying and intestinal motility (Schjoldager, Mortensen et al. 1989, Wettergren, Schjoldager et al. 1993, Chelikani, Haver et al. 2005).

GLP-1's anorectic and incretin effects make it a valuable target in the clinic for the treatment of diabetes and obesity. There are currently a wide range of FDA approved therapies. They include DPP-IV inhibitors, which increase the amount of active GLP-1 in circulation, as well as other DPP-IV metabolites including the incretin GIP (Ahrén, Landin-Olsson et al. 2004, Nauck 2016); exenatide, a synthetic version of exendin-4, a DPP-IV resistant GLP-1 analogue that is found in the saliva of the gila monster (Eng, Kleinman et al. 1992); and other long acting GLP-1R agonists which are modified to prevent proteolytic cleavage and renal clearance (Deacon, Knudsen et al. 1998, Nauck 2016),

The most recently approved drug for the treatment of diabetes and obesity is semaglutide. Semaglutide shares 94% homology with human GLP-1, however it has amino acid substitutions that decrease its susceptibility to DPP-IV cleavage and other modifications that strengthen binding to albumin, which decreases renal clearance and proteolytic cleavage (Jensen, Helleberg et al. 2017). Semaglutide is more effective at promoting weight loss than liraglutide (O'Neil, Birkenfeld et al. 2018), while maintaining some of the cardiac benefits observed with liraglutide in the LEADER study (Marso, Bain et al. 2016). Interestingly, more recently an oral formulation of semaglutide was created that is currently undergoing clinical trials to test efficacy in comparison to current injectable therapies. These trials have been named the PIONEER trials. Initial studies have found semaglutide to be safe and effective when compared to placebo (Aroda, Rosenstock et al. 2018), and other therapies, including sitagliptin and 1.8mg liraglutide, according to press releases by Novonordisk (Nordisk 2018, Nordisk 2018); peer reviewed papers on the latter are pending.

These forms of medication still cause gastrointestinal side effects. There were some concerns about liraglutide causing pancreatitis but the LEADER study suggests this is not the case (Marso, Daniels et al. 2016).

1.2.2.1.4. Peptide YY (PYY)

PYY is a member of the PP fold family like NPY and PP. It is secreted by L cells in the gut and is found increasingly along the distal gut, with the highest concentration in the rectum (Adrian, Ferri et al. 1985, Ekblad and Sundler 2002).

PYY is secreted in response to a meal, approximately in proportion to the calories consumed (Adrian, Ferri et al. 1985). In circulation, it is found in two different forms: PYY_{1-36} and the truncated form PYY_{3-36} (Eberlein, Eysselein et al. 1989, Grandt, Schimiczek et al. 1994). PYY_{1-36} is an agonist for receptors Y_1, Y_2 and Y_5. The N-terminus truncated form is generated by hydrolysis of PYY_{1-36} by DPP-IV, which limits the activity of the resulting PYY_{3-36} to the Y_2 receptor (Mentlein, Dahms et al. 1993).

PYY_{3-36} is thought to be primarily responsible for PYY's anorectic effects. PYY_{3-36} administration in lean and obese humans and rodents supresses food intake (Batterham, Cowley et al. 2002, Batterham, Cohen et al. 2003, Challis, Pinnock et al. 2003) and inhibits gastric emptying (Savage, Adrian et al. 1987). Additionally, PYY knockdown mice are obese and hyperinsulinemic (Batterham, Heffron et al. 2006, Boey, Lin et al. 2006).

Peripheral administration of PYY_{3-36} upregulates POMC and supresses NPY mRNA levels in the ARC of rodents (Batterham, Cowley et al. 2002, Challis, Pinnock et al. 2003). Additionally, when

administered directly into the ARC, PYY_{3-36} decreases food intake and inhibits NPY neurons (Batterham, Cowley et al. 2002), suggesting AgRP/NPY neurons in the ARC play a role in PYY's anorectic effect. Interestingly, when administered centrally into the ventricles, PYY_{3-36} increases food intake (Clark, Sahu et al. 1987, Corp, McQuade et al. 2001).

PYY's anorectic effects are thought to be mediated by the Y_2 receptor as Y_2 receptor knockout mice do not show an anorectic response to PYY_{3-36} (Batterham, Cowley et al. 2002). Y_2 receptors in vagal afferents may also be important as Y_2 receptor expression in the vagus changes according to the feeding status of rodents, with this change being mediated via CCK (Burdyga, de Lartigue et al. 2008). However, vagotomy studies have found conflicting results regarding whether PYY_{3-36} requires an intact vagus to mediate its anorectic effects when peripherally administered (Abbott, Monteiro et al. 2005, Halatchev and Cone 2005, Koda, Date et al. 2005).

PYY can also be truncated at the C-terminus to generate $PYY_{1/3-34}$, forms which do not activate the Y_2 receptor in humans or pigs (Torang, Veedfald et al. 2015, Torang, Bojsen-Moller et al. 2016). This truncated form fails to modulate glucose induced insulin secretion in cells and supress appetite in mice (Lafferty, Flatt et al. 2018), suggesting that the PYY C terminus is important in mediating its physiological effects, and that assays binding to the N terminus may thus report inflated values of biologically active PYY (Torang, Veedfald et al. 2015).

$PYY_{1/3-34}$ is not the only PYY metabolite found in circulation (Olsen, Kofoed et al. 2016). PYY is also cleaved by metalloendoprotease meptin β, at multiple sites, and NEP24.11, at residues 29-30; and aminopeptidase P removes the tyrosine residue at the N terminus (Medeiros and Turner 1994, Addison, Minnion et al. 2011). The half-life of total PYY is thought to be ~9mins, with the half-life of PYY_{3-36} (~14.9mins) being longer than the half-life of PYY_{1-36} (Torang, Bojsen-Moller et al. 2016).

PYY's use as an anti-obesity therapy is limited by its rapid metabolism and nauseating side effects (le Roux, Borg et al. 2008). Such unwanted actions might be ameliorated through the generation of degradation resistant analogues, which prolong the half-life, and the use of PYY as part of combination therapies that allow for administration of a lower dosage.

1.1.1.1.1. Glucose-dependent insulinotropic peptide (GIP)

GIP secreting cells are highly concentrated in the upper segments of the small intestine (Mortensen, Christensen et al. 2003, Theodorakis, Carlson et al. 2006). Classically, GIP is thought to be secreted from EEC K-cells however, like with many other hormones, there is overlap, and sub-populations of L-cells also express GIP (Mortensen, Christensen et al. 2003, Theodorakis, Carlson et al. 2006). GIP is

also secreted by α-cells in the pancreas, however the major isoform synthesised in the pancreas is GIP_{1-30} whereas in the gut it is GIP_{1-42} (Fujita, Wideman et al. 2010). Like GLP-1, GIP is inactivated by DPP-IV cleavage and undergoes renal clearance (Kieffer, McIntosh et al. 1995).

Oral glucose is a potent stimulator of GIP secretion in humans and rodents (Cataland, Crockett et al. 1974, Yoder, Yang et al. 2010). In rats, GIP dose-dependently increases in response to intraluminal administration of carbohydrates and fats but not proteins (Yoder, Yang et al. 2009, Yoder, Yang et al. 2010). Absorption of glucose is thought to be important in mediating GIP secretion as mice lacking the sodium glucose co-transporter (SGLT1) do not secrete GIP in response to orally administered glucose (Gorboulev, Schürmann et al. 2012). Moreover, patients suffering from malabsorption have reduced postprandial GIP (Besterman, Cook et al. 1979).

The GIP receptor (GIP-R), like GLP-1R, belongs to the secretin receptor family (Yamada, Hayami et al. 1995). The GIP-R is expressed in various tissues including the pancreas, stomach, small intestine, brain and adipose tissue (Usdin, Mezey et al. 1993). In the pancreas, GIP acts as an incretin, potentiating glucose-induced insulin secretion (Dupre, Ross et al. 1973). Consequently, GIP-R deficient mice, caused by targeted mutations, have impaired glucose tolerance and glucose-induced insulin secretion (Miyawaki, Yamada et al. 1999). Interestingly, these mice are protected from DIO (Miyawaki, Yamada et al. 2002), an effect lost when GIP-R is re-expressed in adipocytes (Ugleholdt, Pedersen et al. 2011).

1.2.2.2. Adiposity signals

1.2.2.2.1. Leptin

Leptin, a hormone synthesised in adipocytes and encoded by the ob gene, is important in appetite regulation (Zhang, Proenca et al. 1994, Ahima, Prabakaran et al. 1996). Circulating leptin levels correlate with body adiposity (Maffei, Halaas et al. 1995, Considine, Sinha et al. 1996) and when administered exogenously it increases energy expenditure and decreases food intake and body weight (Halaas, Gajiwala et al. 1995, Weigle, Bukowski et al. 1995, Heymsfield, Greenberg et al. 1999).

The absence of leptin acts as an energy deficit/ starvation signal. Leptin deficient mice due to a mutation in the Ob gene are obese (ob/ob mice), severely hyperphagic and prone to diabetes. This phenotype is reversible by the exogenous administration of leptin (Campfield, Smith et al. 1995, Halaas, Gajiwala et al. 1995, Pelleymounter, Cullen et al. 1995).

Leptin binds to the leptin receptor (Tartaglia, Dembski et al. 1995). Leptin receptor deficiency also results in an obese phenotype (db/db mice) (Chen, Charlat et al. 1996, Lee, Proenca et al. 1996). In humans, leptin deficiencies or receptor abnormalities, although rare, are a cause of obesity (Montague, Farooqi et al. 1997, Farooqi, Wangensteen et al. 2007). In the case of leptin deficiency, the obese phenotype seen in humans, like in mice, can be cured by administering exogenous leptin (Farooqi, Jebb et al. 1999). However, exogenous leptin administration in other forms of obesity does not serve as a therapy as these individuals tend to be resistant to leptin and display elevated levels of the hormone (Considine, Sinha et al. 1996, Heymsfield, Greenberg et al. 1999).

Leptin is able to cross the blood brain barrier (BBB), gaining access to the CNS to relay information on peripheral adiposity (Schwartz, Seeley et al. 1996). Within the CNS, the leptin receptor is expressed in the ARC, in both POMC and AgRP/NPY neurons, the PVN, the DMN and VMN (Cheung, Clifton et al. 1997, Baskin, Schwartz et al. 1999). Within the ARC, leptin up regulates the activity of POMC neurons and inhibits that of AgRP/NPY neurons, having downstream effects on energy homeostasis (Schwartz, Seeley et al. 1996, Schwartz, Seeley et al. 1997, Wang, Bing et al. 1997). Moreover, leptin receptor neuron-specific knockout mice are obese and have increased levels of AgRP and NPY (Cohen, Zhao et al. 2001).

1.2.2.2.2. Adiponectin

Adiponectin is a hormone secreted from adipocytes (Scherer, Williams et al. 1995). However, unlike leptin, its secretion is negatively correlated to adiposity (Arita, Kihara et al. 1999).

Adiponectin's role in glucose homeostasis has been extensively investigated. It supresses glucose production, independently of insulin levels (Berg, Combs et al. 2001), and increases insulin sensitivity, by stimulating fatty acid combustion in skeletal muscle and by decreasing hepatic triglyceride content (Yamauchi, Kamon et al. 2001). Additionally, ob/ob mice overexpressing adiponectin display improved insulin sensitivity (Yamauchi, Kamon et al. 2003).

Adiponectin binds two receptors that have differential expression patterns: adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). AdipoR1, expressed more widely than AdipoR2, is abundant in skeletal muscle, but also found in other tissues including the brain, heart, kidney, liver and lungs. AdipoR2, in contrast, is mainly expressed in the liver (Yamauchi, Kamon et al. 2003). Mice lacking AdipoR1 and AdipoR2 are insulin resistant, have impaired glucose tolerance and increased tissue triglyceride content (Yamauchi, Nio et al. 2007).

Both adiponectin receptors are also expressed in appetite regulating centres of the brain, including the brainstem and hypothalamus (Qi, Takahashi et al. 2004, Hoyda, Fry et al. 2007). However,

adiponectin's role in energy homeostasis is unclear, as is how adiponectin gains access to the cerebral spinal fluid.

Qi et al. found that centrally administered adiponectin increases energy expenditure (it increases UCP-1 in BAT) and decreases body weight, without affecting food intake, in both lean and obese (ob/ob) mice (Qi, Takahashi et al. 2004). Similarly, Fruebis et al. found that mice fed a high-fat/sucrose diet treated with adiponectin lost weight without there being any changes in food intake (Fruebis, Tsao et al. 2001). On the other hand, (Kubota, Yano et al. 2007) found that adiponectin (i.v and i.c.v) stimulates food intake, as seen in (Yamauchi, Kamon et al. 2003), and decreases energy expenditure (decreased UCP-1 in BAT).

1.2.2.3. Enteric nervous system and vagus

The enteric nervous system (ENS) innervates the gut and is able to regulate gastrointestinal function independently of the CNS. It is comprised of two major ganglionic plexuses: the myenteric (Auberch's) plexus and the submucosal (Meissner's) plexus. The myenteric plexus is located between the circular and longitudinal muscle layers and found throughout the length of the gut, oesophagus and stomach. The submucosal plexus is located in the submucosa, between the mucosa and circular muscles, and predominates in the small and large intestine (Furness 2012).

The ENS sends projections to various tissues including the pancreas, gall bladder and sympathetic neurons, some of which are bidirectional. It plays an important role in regulating intestinal motility, fluid movement across the epithelium, blood supply to the gut and gastric and exocrine secretions (Furness 2012).

The vagus nerve is one of the major extrinsic branches of the ENS that is discussed in more detail in chapter 4.

1.3. Macronutrient diets

Energy homeostasis is thus regulated in part by the effect of peripheral signals on central circuits. These peripheral signals communicate different information regarding nutritional status. There is evidence that this information can include the particular amount or percentage of specific macronutrients in ingested food, and may explain the different effects of specific macronutrients on energy homeostasis and metabolism.

1.3.1. High protein diets

Diets exceeding the habitual recommended protein consumption of 15% of the total energy content are considered to be high in protein (Pesta and Samuel 2014). High protein diets (HPD) are more

effective, compared to diets rich in other macronutrients, at promoting satiety and weight loss/weight maintenance (Latner and Schwartz 1999, Westerterp-Plantenga, Lejeune et al. 2003, Hu 2004, Westerterp-Plantenga, Luscombe-Marsh et al. 2006). The exact mechanisms underlying these effects are still not fully understood. However, some suggested mechanisms include the thermic effect of protein ingestion, the stimulation of gluconeogenesis and the secretion of gut hormones.

1.3.1.1. Thermic effect

DIT is the energy expended in the processing of ingested nutrients. It is calculated by dividing the increase in energy expenditure from basal fasting levels by the energy content of the ingested food (Westerterp 2004).

Protein isn't stored, it is rapidly metabolised in various ways that are energy demanding, for example, protein synthesis, gluconeogenesis and urea genesis. Consequently, protein ingestion increases EE (Luscombe, Clifton et al. 2002) and has a greater DIT effect than other macronutrients (Westerterp 2004). The extent of protein induced energy expenditure is affected by the protein source. For example, protein originating from pork was found to induce a higher thermogenic response than soy protein, which might be due to the amino acid composition of the proteins and the proportion of essential amino acids present, which was higher in the pork (Mikkelsen, Toubro et al. 2000). However, the increased EE effect observed following protein intake is insufficient to account for the magnitude of weight loss observed following a HPD (Eisenstein, Roberts et al. 2002).

1.3.1.2. Gluconeogenesis

HPDs stimulate gluconeogenesis (Veldhorst, Westerterp-Plantenga et al. 2009, Veldhorst, Westerterp et al. 2012). This increased gluconeogenesis effect correlates with protein induced energy expenditure (Veldhorst, Westerterp-Plantenga et al. 2009). However, its role in appetite regulation is controversial. Veldherst et al. failed to find a correlation between appetite and gluconeogenesis in humans (Veldhorst, Westerterp et al. 2012). On the other hand, Mithieux et al. claim that intestinal gluconeogenesis is important in the satiating effects of HPDs; intestinal knockdown of glucose-6 phosphatase, an enzyme important in glucose production, made mice insensitive to protein-induced satiety (Mithieux, Misery et al. 2005, Penhoat, Mutel et al. 2011), even though the small intestine plays a small role in the gluconeogenic effects of HPDs (Azzout-Marniche, Gaudichon et al. 2007, Penhoat, Mutel et al. 2011).

1.3.1.3. Protein-induced satiety

Compared to other macronutrients, protein is acutely the most satiating (Batterham, Heffron et al. 2006). The sensing of amino acids may be important in mediating the effect as the timing of protein-induced satiety correlates with the appearance of amino acids in circulation (Veldhorst,

Nieuwenhuizen et al. 2009) and because plasma amino acid levels correlate with satiety (Mellinkoff, Frankland et al. 1956). However, there does not appear to be a specific amino acid driving the response; the appetite supressing effects of different protein sources are associated with the appearance of different amino acids in circulation. For example, whey satiety was associated with the appearance of serine, isoleucine, threonine and alanine (Veldhorst, Nieuwenhuizen et al. 2009), casein with the appearance of branched chain amino acids (Veldhorst, Nieuwenhuizen et al. 2008), and soy with the appearance of taurine (Veldhorst, Nieuwenhuizen et al. 2009). Different proteins can also affect gut hormone secretion differently. For example, whey triggers a greater GLP-1 and insulin response than casein and soy, and casein supresses active ghrelin to a greater extent than soy protein (Veldhorst, Nieuwenhuizen et al. 2009).

Differences in response to varying protein sources could be due to differences in digestion kinetics, affecting the rate of amino acid release and region of the gut exposed to the digesta. Differences in amino acid composition, and consequently the concentration of essential amino acids, may also affect satiety and feeding behaviour. Moreover, the method of protein delivery, e.g. mixed meal versus pure protein; the state of protein, e.g. solid versus liquid protein; the preload; and the amount of protein ingested are just some of the factors that need to be considered when designing and comparing studies.

The satiating effects of protein are also thought to be mediated, in part, through changes in gut hormone secretion. HPDs, compared to isocaloric high carbohydrate (HCD) and high fat diets (HFD), stimulate PYY secretion in obese and lean subjects most potently (Batterham, Heffron et al. 2006, van der Klaauw, Keogh et al. 2013). Moreover, PYY null mice are resistant to the satiating effects of HPDs (Batterham, Heffron et al. 2006). High protein meals, compared to high carbohydrate meals, also stimulated the secretion of CCK-8 and GIP, and supressed total ghrelin levels, though no differences in GLP-1 were observed in this study (Blom, Lluch et al. 2006). Prolonged acyl-ghrelin suppression following a HP meal is also observed in (Al Awar, Obeid et al. 2005, Tannous dit El Khoury, Obeid et al. 2006) but not (Batterham, Heffron et al. 2006, van der Klaauw, Keogh et al. 2013).

Findings regarding the effect of protein on GLP-1 have also found conflicting results. (Batterham, Heffron et al. 2006, Blom, Lluch et al. 2006, Veldhorst, Nieuwenhuizen et al. 2009) observed no change, whereas (Lejeune, Westerterp et al. 2006, Veldhorst, Nieuwenhuizen et al. 2009, Belza, Ritz et al. 2013, van der Klaauw, Keogh et al. 2013) found a significant increase in GLP-1 following a HPD compared to HFD, HCD (van der Klaauw, Keogh et al. 2013), and normal protein diets (NPD) (Lejeune, Westerterp et al. 2006, Veldhorst, Nieuwenhuizen et al. 2009, Belza, Ritz et al. 2013).

Appetite regulation is a complex multifactorial system. Differences in study design can greatly affect the results and may be the cause of inconsistent findings in the literature. For example, differences in the time of intervention and fasting, subject age and body composition, the palatability, quantity and physical state of the food, all affect food intake. Moreover, post-prandial changes in gut hormone levels are not always reflected in changes in satiety and/or hunger, further confounding findings (Heden, Liu et al. 2013, van der Klaauw, Keogh et al. 2013, Leidy, Clifton et al. 2015).

Centrally, HPDs activate POMC neurons in the ARC and neurons in the NTS (Faipoux, Tomé et al. 2008), and supresses the activity of orexins in the LHA (Journel, Chaumontet et al. 2012)

1.3.2. Protein digestion

Protein digestion can be divided into three phases: the gastric, pancreatic and intestinal phases. In the gastric phase, acid conditions denature proteins and pepsin, an endopeptidase active at low pH and generated from the precursor pepsinogen, cleaves the bond between aromatic and hydrophobic amino acids, producing different length peptide fragments (polypeptides, oligopeptides and free amino acids). However, this phase is not critical as patients that have undergone total gastrectomy are still able to assimilate proteins (H J Freeman and Kim 1978, Berg JM, Tymoczko JL et al. 2002).

In the pancreatic phase, peptide fragments from the gastric phase stimulate the secretion of CCK and secretin from the small intestine. These two hormones then stimulate the secretion of pancreatic juices, enzymes and protease precursors that are synthesised in pancreatic acinar cells (Meyer and Kelly 1976). Protease precursors, such as trypsinogen, are cleaved by enterokinase secreted in the intestine in response to the prior mentioned hormones, forming trypsin. This triggers autocatalysis and the formation of other active proteases that further breakdown the peptide fragments in the intestine (H J Freeman and Kim 1978, Berg JM, Tymoczko JL et al. 2002).

The intestinal phase includes reactions taking places within the lumen, at the brush border or within enterocytes. Within the lumen, the digesta is mixed up with secreted enzymes, including pancreatic enzymes, that break the peptide fragments down even further. At the brush border there are aminopeptidases associated with the intestinal epithelia that cleave di- and tri- peptides into free amino acids that are primarily absorbed in the jejunum. Alternatively, the di and tri-peptides can be transported into enterocytes for further cleavage, with relatively few oligopeptides making it to the portal circulation (H J Freeman and Kim 1978, Berg JM, Tymoczko JL et al. 2002).

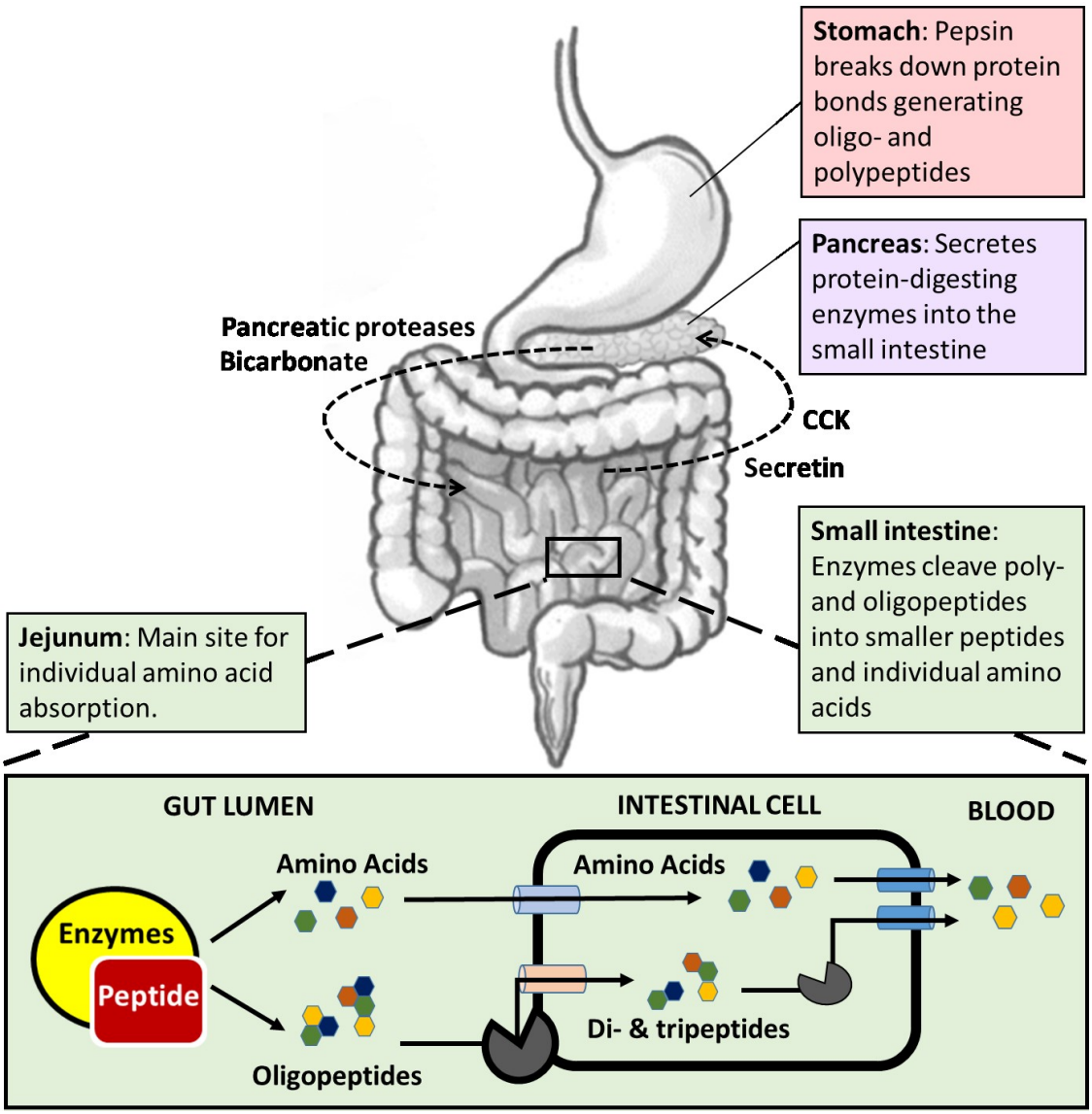


Figure 1.3.2.1 Schematic diagram summarising protein digestion in the GI tract.

The diagram shows a summary of the protein digestion process that takes place in the gut, leading to the eventual release of amino acids into circulation. Some amino acids are retained within the cell for different cellular process.

1.4. Amino acids

Proteins are broken down into amino acids in the gut. Amino acids then enter the circulation to reach other tissues where they form the building blocks of other proteins, act as a source of energy or act as precursors for various molecular pathways and molecules including neurotransmitters, nucleotides and hormones.

Amino acids can be classified as proteinogenic, naturally encoded and usable in protein synthesis, and non-proteinogenic. There are 20 proteinogenic amino acids that can be further categorised as

essential, i.e. only obtainable from the diet as the body is unable to synthesise them de novo, and non-essential, which can be synthesised by the body. In addition, there are conditionally essential amino acids, these must be obtained from the diet when de novo synthesis is insufficient to meet the body's requirements (Wu 2013). These are outlined in table 1.3.2.1.

Essential	Conditionally Essential	Non-essential
Histidine (His)	Arginine (Arg)	Alanine (Ala)
Isoleucine (Iso)	Cysteine (Cys)	Asparagine (Asn)
Leucine (Leu)	Glutamine (Gln)	Aspartate (Asp)
Lysine (Lys)	Glycine (Gly)	Glutamate (Glu)
Methionine (Met)	Proline (Pro)	Serine (Ser)
Phenylalanine (Phe)		Tyrosine (Tyr)
Threonine (Thr)		
Tryptophan (Trp)		
Valine (Val)		

Table 1.3.2.1 Essential, conditionally essential and non-essential amino acids with their three letter codes.

Note that different amino acids can be conditionally essential under different conditions, and different sources propose different amino acids to be conditionally essential (Jaksic, Wagner et al. 1991, Wu 2009, Wang, Wu et al. 2013, Wu 2013).

Amino acids are made up of an amino group, a carboxyl group and a variable organic side chain depicted in figure 1.3.2.1.

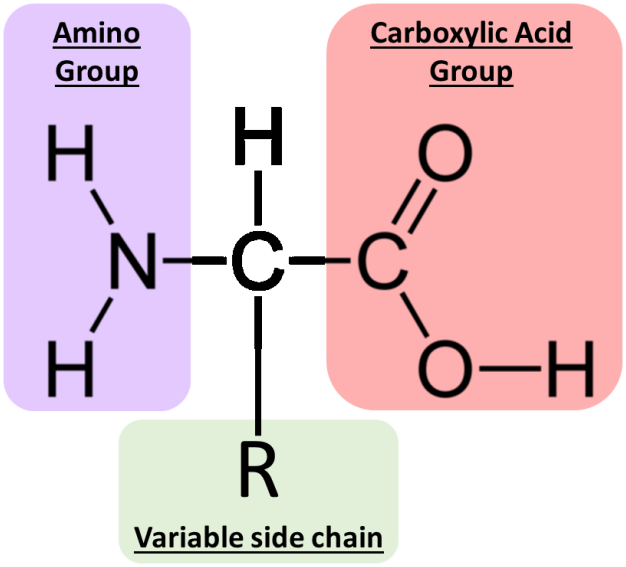


Figure 1.3.2.1 Illustration depicting the basic structure of an amino acid.

Consequently, proteinogenic amino acids can also be classified according to the chemical properties of their side chains as indicated in table 1.3.2.2.

Acidic	Aliphatic	Amidic	Aromatic	Basic	-S or –OH containing
Aspartate Glutamate	Alanine Glycine Proline Isoleucine* Leucine* Valine*	Asparagine Glutamine	Histidine Phenylalanine Tryptophan Tyrosine	Arginine Lysine	Cysteine Methionine Serine Threonine

Table 1.3.2.2 Amino acid categories based on side chain.

***** Branched-chain amino acids- amino acid that have an aliphatic side-chain with a branch.

1.4.1. Amino acids and central mechanisms of appetite regulation

Amino acids are able to cross the BBB through Na^+-dependent transporters, located on the anti-luminal side to allow for active export of amino acids from the brain, or via facilitative transporters (Betz and Goldstein 1978). Alternatively they can enter the CNS through circumventricular organs of

the brain where the BBB is incomplete (Hawkins, DeJoseph et al. 1995). Uptake by facilitative transporters is competitive and saturable (Battistin, Grynbaum et al. 1971). Consequently, the ratio of amino acids in circulation can greatly affect which amino acids and how much of them cross the BBB. Tracking of radiolabelled amino acid entry into the brain of rats revealed that essential amino acids enter the brain to a greater extent than non-essential amino acids (Oldendorf 1971).

Within the CNS, amino acids act as precursors for neurotransmitters that are important in appetite regulation. For example: tryptophan is a precursor for serotonin, tyrosine for dopamine and histidine for histamine, which have all been implicated in energy homeostasis (Heisler, Jobst et al. 2006, Volkow, Wang et al. 2011, Tabarean 2016). Consequently, diet composition can affect the synthesis of certain neurotransmitter and brain function (Fernstrom 1977, Wurtman 1983). Moreover, amino acids themselves can act as neurotransmitters, for example glutamate is a major excitatory neurotransmitter (Meldrum 2000).

Amino acids can also be detected centrally to regulate appetite. Branched-chain amino acids such as leucine can decrease food intake and body weight by activating intracellular mTORC1 (Cota, Proulx et al. 2006, Blouet, Jo et al. 2009, Blouet and Schwartz 2012). This mechanism is discussed in further detail in section 1.4.3.3.1.

1.4.2. Amino acids and peripheral mechanisms of appetite regulation

In the periphery, amino acids can stimulate the secretion of appetite regulating hormones via amino acid sensors. These can be in the form of extracellular receptors, for example promiscuous G-protein coupled receptors, transporters and intracellular sensors as displayed in figure 1.4.2.1. In addition, the gut microbiota can metabolise specific amino acids to form other metabolically active molecules, some of which can also activate endogenous receptors in the gut and other regions of the body (Chimerel, Emery et al. 2014).

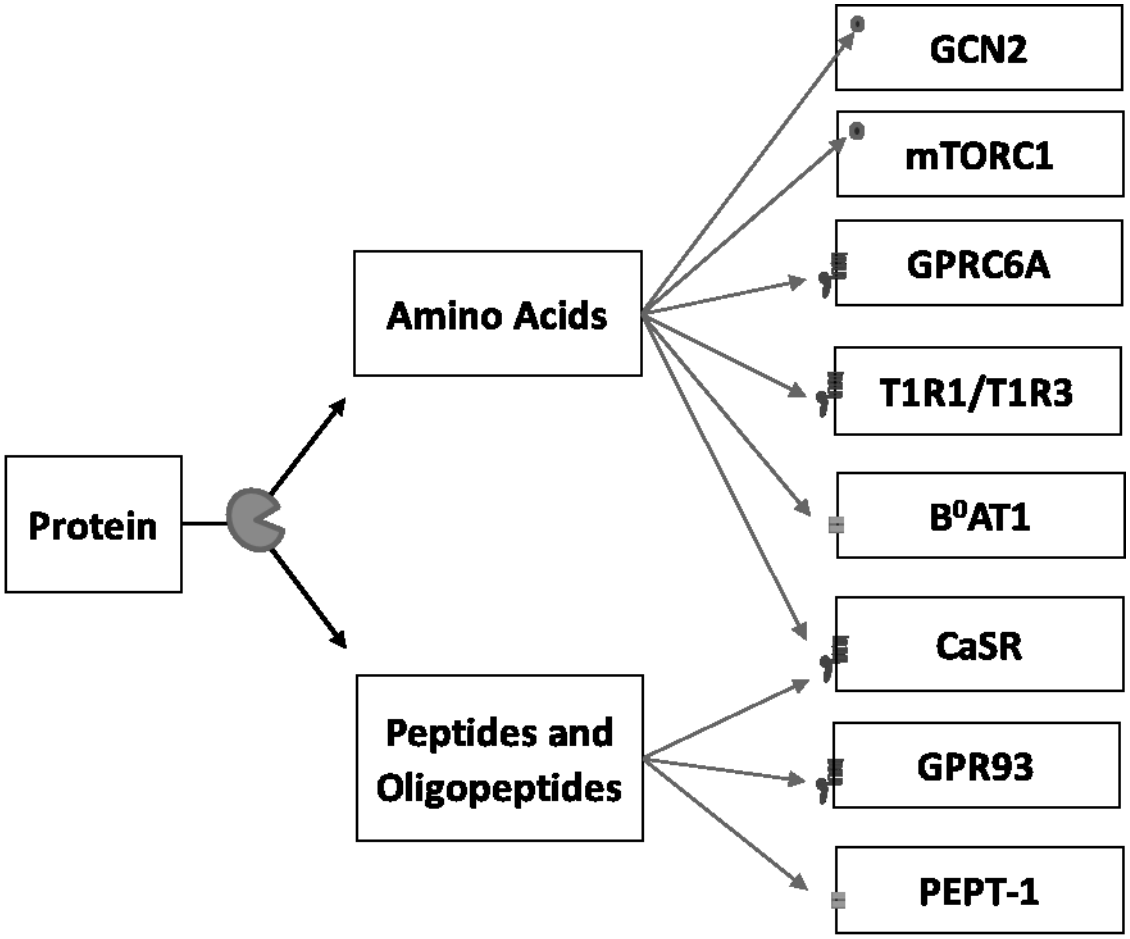


Figure 1.4.2.1 Overview of protein breakdown product sensors.

Proteins are broken down into amino acids, peptides and oligopeptides by bacterial, gastrointestinal and pancreatic enzymes, which can be detected by G-protein coupled receptors, transporter systems and intracellular sensors. GCN2, general control non-repressed 2 protein; mTORC1, mammalian target of rapamycin complex 1; GPRC6A, G protein-coupled receptor family C group 6 member A; T1R1/T1R3, taste receptor type 1 member 1/taste receptor type 1 member 3; B0AT1, neutral amino acid transporter; CaSR, calcium sensing receptor; GPR93, G protein-coupled receptor 93; PEPT-1, peptide transporter 1. Adapted from (Norton and Murphy 2017).

G-protein coupled receptors can activate several intracellular pathways that lead to the secretion of gut hormones. For example, the GPRC6A can recruit G_q protein that triggers the release of intracellular Ca^{2+} stores and subsequent GLP-1 secretion in response to L-ornithine via a PLC and IP_3 mediated pathway *in vitro* (Oya, Kitaguchi et al. 2013). They can be expressed on the apical surface of EECs or other gut epithelial cells, directly detecting nutrients in the lumen, or on the basolateral surface of cells, detecting absorbed amino acids.

Amino acids, di- and tri peptides can enter cells through proton coupled transporters. The simultaneous entry of protons, such as Na^+, with the amino acid can depolarise the cell, triggering the opening of voltage gated Ca^{2+} channels. The influx of Ca^{2+} into the cell then triggers the exocytosis of vesicles containing gut hormones (Reimann, Williams et al. 2004). Amino acid ligand gated ion channels, for example glycine ionotropic receptors can also stimulate hormonal secretion through cellular depolarization via Cl^- currents (Gameiro, Reimann et al. 2005). Alternatively, once within the cell, amino acids can activate intracellular sensors such as mTORC1.

1.4.3. Amino acid sensing receptors

Amino acids have been shown to stimulate the secretion of gut hormones *in vivo* and *in vitro* via transporter systems, intracellular sensors and extracellular G-protein coupled receptors (GPCR).

1.4.3.1. Amino acid extracellular sensing receptors

Of the promiscuous amino acid-sensing G-protein coupled receptors, there are three well characterised Class C GPCRs: G-protein-coupled receptor family C group 6 member A (GPRC6A), taste receptor 1 member 1 and taste receptor 1 member 3 (T1R1/T1R3) heterodimer and the calcium sensing receptor (CaSR). Recently another class A receptor has been characterised: G-protein-coupled receptor 142 (GPR142) (Lizarzaburu, Turcotte et al. 2012, Lin, Efanov et al. 2016, Rudenko, Shang et al. 2019). It is also worth noting that there are peptone (GPR93) (Choi, Lee et al. 2007) and amino acid metabolite sensors (GPR35 and FPR1) (Wang, Simonavicius et al. 2006, Husted, Trauelsen et al. 2017) which are not discussed further in this thesis.

Non-orphan class C GPCRs share in common a large bi-lobed extracellular ligand-binding venous fly trap (VFT) motif that forms dimers (Rondard, Goudet et al. 2011, Reimann, Tolhurst et al. 2012). This VFT is connected to the seven transmembrane domain (7TM) by a cysteine rich domain. The 7TM is common to all GPCRs and mediates downstream G protein signalling. The conformation of the VFT (open or closed) and orientation (resting or active) are important in the activation mechanism, as is the conformation of the 7TM.

Within Class C GPCRs there is a cluster of receptors that have varying affinities for L-α-amino acids, these include the previously mentioned CaSR, T1R1/T1R3 and GPRC6A. The CaSR, for example, responds most potently to aromatic amino acids, particularly L-Tryptophan and L-Phenylalanine, followed by aliphatic and polar amino acids (figure 1.4.3.1) (Wellendorph, Johansen et al. 2009).

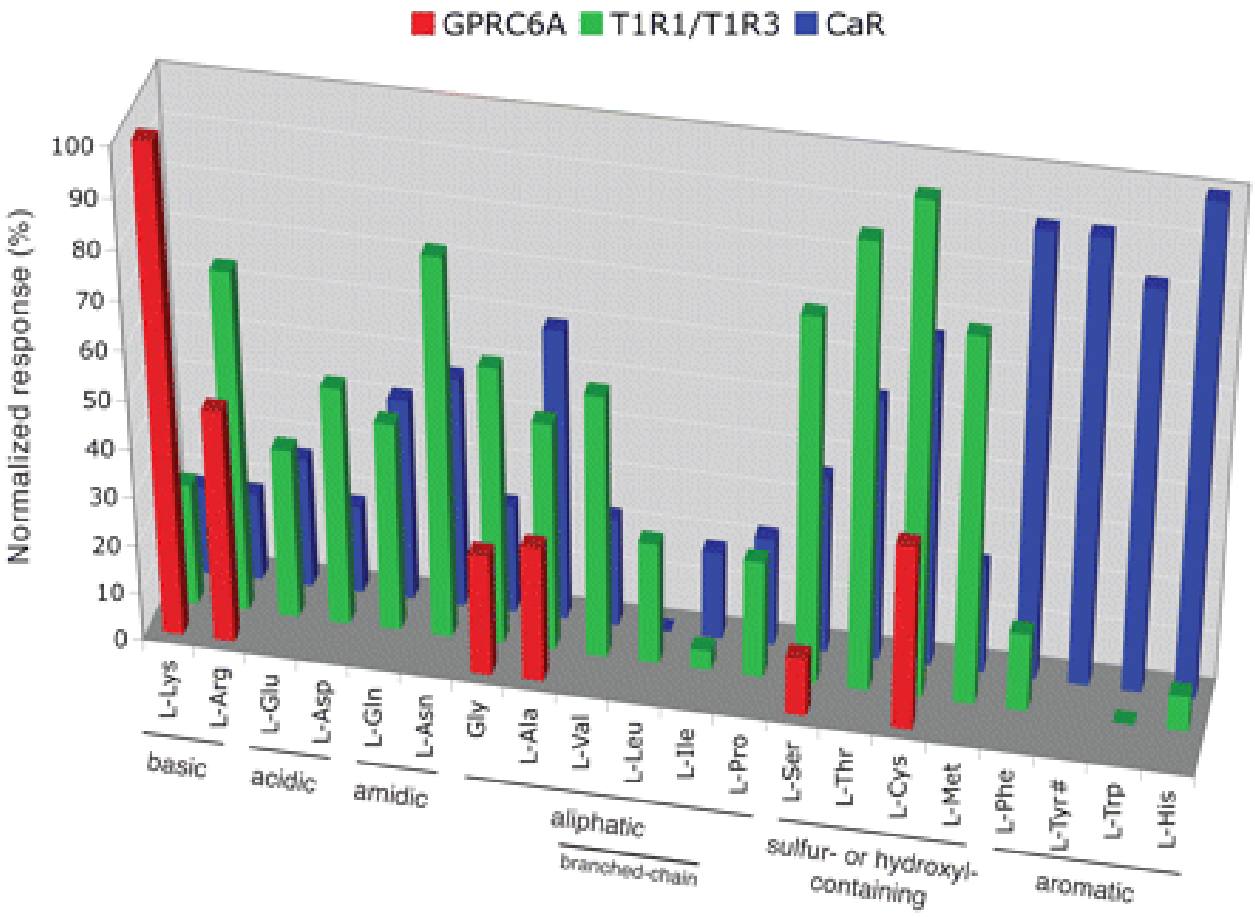


Figure 1.4.3.1 Response of GPPCR class C L-amino acid sensors to different amino acids.

The response to CaSR was normalised to L-Histidine and calculated by comparing the potentiating effects of the 20 different amino acids at a concentration of 10mM on the Ca^{2+} response (Conigrave, Quinn et al. 2000). The response to T1R1/T1R3 was normalised to L-Cysteine and measured in the presence of IMP 2mmol/L for all amino acids except L-Tyrosine (#)(Nelson, Chandrashekar et al. 2002). The response to GPRC6A was measured in the presence of 1mmol/L of Ca^{2+} and Mg^{2+}, normalised to L-Lysine, and based on the reported EC50 of the mouse GPRC6A (Christiansen, Hansen et al. 2007). Image taken from (Wellendorph, Johansen et al. 2009).

1.4.3.1.1. T1R1/T1R3

There are three type 1 taste receptor subunits that have been identified to date: T1R1, T1R2 and T1R3. These subunits form the heterodimers T1R1/T1R3 and T1R2/T1R3 that detect umami (savoury) and sweet tastes, respectively. Type 2 taste receptors detect bitter tastes (Chandrashekar, Mueller et al. 2000); they are more like class A GPCRs than class C, unlike type 1 taste receptors.

In humans, T1R1/T1R3 is particularly sensitive to the amino acid monosodium L-Glutamate (Li, Staszewski et al. 2002). In contrast, in rodents, T1R1/T1R3 is activated by a variety of amino acids,

with L-Cysteine being the most potent (Li, Staszewski et al. 2002, Nelson, Chandrashekar et al. 2002, Daly, Al-Rammahi et al. 2013). These differences in receptor agonist selectivity may be due to rodents and humans only sharing ~70% sequence homology in T1Rs (Nelson, Hoon et al. 2001). T1R1/T1R3's response is potentiated by guanosine nucleotides and the 5' ribonucleotide inosine monophosphate (IMP) (Nelson, Chandrashekar et al. 2002) (Daly, Al-Rammahi et al. 2013). Additionally, T1R3 responds to calcium (Tordoff, Alarcón et al. 2012).

T1R1/T1R3 was originally found to be expressed in the tongue, particularly in the fungiform taste buds. It was later identified in various other tissues including the brain, GI tract, pancreas and skeletal muscle (Wauson, Zaganjor et al. 2012). However, its role in these extra-gustatory tissues is unclear.

In the STC-1 immortalised mouse intestinal enteroendocrine cell line, amino acids stimulate the secretion of CCK, an effect reduced by T1R1/T1R3 knockdown. Additionally, in intestinal explants, amino acid induced CCK secretion was potentiated by IMP, a known potentiator of T1R1/T1R3 response, and attenuated by Gurmarin, a T1R3 inhibitor (Daly, Al-Rammahi et al. 2013). In pigs, high protein diets up-regulate signalling components of T1Rs *in vivo* (De Giorgio, Mazzoni et al. 2016). However, whether human T1R1/T1R3s can mediate gut hormone secretion requires further investigation.

Taste cells from T1R1 or T1R3 whole coding sequence knock-out models still respond in part to umami taste, this may be due to the detection of L-amino acids and IMP by metabotropic glutamate receptors (Yasumatsu, Manabe et al. 2015, Pal Choudhuri, Delay et al. 2016), suggesting there is redundancy in the system. Additionally, T1R knockout mice appear to have a normal body weight (Zhao, Zhang et al. 2003), which puts into question the involvement and importance of T1R1/T1R3 in energy homeostasis.

1.4.3.1.2. GPRC6A

GPRC6A is an L-amino acid sensor (Wellendorph, Hansen et al. 2005). In humans, there are three prevalent isoforms that differ in their expression and amino terminal sequence length. The effect this has on receptor function remains to be investigated. The first isoform is the most abundant. It is widely expressed and has the longest structural sequence. The second isoform is abundantly found in the kidney and the third isoform, the least abundant, is found in the brain, kidneys and skeletal muscle (Wellendorph and Brauner-Osborne 2004).

GPRC6A has various agonists and allosteric modulators including: divalent cations, calcimimetics, osteoclastin and L-amino acids, particularly basic and small aliphatic amino acids (Pi, Faber et al. 2005, Wellendorph, Hansen et al. 2005, Christiansen, Hansen et al. 2007). However, the potency of amino acids at the GPRC6A is species dependent. For example, in humans, L-Arginine is the most potent amino acid activator of the GPRC6A, whereas in rodents it is L-Ornithine (Clemmensen, Smajilovic et al. 2014).

GPRC6A is expressed in the kidneys, pancreas and GI tract, hinting at its role in various physiological functions, including energy and glucose homeostasis (Wellendorph and Brauner-Osborne 2004). However, its physiological function in such remains unclear after the conflicting findings from knockout models.

Initial exon 2 knockout models targeted the receptors VFT motif. Exon 2 knockout did not affect the body weight of mice but did result in various metabolic abnormalities including impaired glucose tolerance, hyperglycaemia and insulin resistance, suggesting a potential role in glucose homeostasis, disrupted adiposity and renal clearance of calcium and phosphorus, as well as feminisation of males and impaired bone mineralisation (Pi, Chen et al. 2008).

Conversely, GPRC6A exon 6 deletion, targeting the 7 transmembrane domain and intracellular C terminus, did not affect bone mineralisation (Wellendorph, Johansen et al. 2009), glucose tolerance or adiposity (Smajilovic, Clemmensen et al. 2013) in mice on a normal chow diet. However, when on a HFD, exon 6 knockout mice were more prone to diet induced obesity, hyperphagic and less active; as well as hyperglycaemic and glucose intolerant (Clemmensen, Smajilovic et al. 2013).

In the gut, GPRC6A is expressed in somatostatin and gastrin producing cells of the antral glands (Haid, Widmayer et al. 2011), and at a low level in L-cells of the small intestine (Oya, Kitaguchi et al. 2013). Activation by basic amino acids can trigger GLP-1 secretion *in vitro* in the GLUTag L-cell line (Wellendorph, Hansen et al. 2005), supporting a role in appetite regulation. However, this effect does not appear to occur in primary cultures of the mouse small intestine (Oya, Kitaguchi et al. 2013), putting into question its physiological importance. Moreover, global GPRC6A knockout mice suggest GPRC6A is not crucial for the anorectic effects of HPDs and L-Arginine; nor for the GLP-1 secretagogue effect of L-Arginine and L-Ornithine (Kinsey-Jones, Alamshah et al. 2015, Alamshah, McGavigan et al. 2016, Clemmensen, Jorgensen et al. 2017).

1.4.3.1.3. CaSR

The CaSR is important in calcium homeostasis and other cellular processes, including differentiation, proliferation and hormone secretion. Its main ligand is calcium, but its activity is also modulated by changes in pH, divalent cations, oligopeptides, aromatic amino acids and, less potently, aliphatic amino acids. The CaSR is normally expressed on the surface of cells as a homodimer (Ward, Brown et al. 1998), however it can also form heterodimers (Chang, Tu et al. 2007).

The CaSR can signal via various intracellular pathways both G-protein-coupled and G-protein-independent, with certain agonists being biased for certain pathways. For example, polyamines are biased towards ERK1/2 signalling (Thomsen, Hvidtfeldt et al. 2012). Its importance in calcium homeostasis is evident from the lethal phenotypes arising from CaSR knockdown (Ho, Conner et al. 1995). However, the CaSR is also expressed in tissues that are not normally associated to calcium homeostasis.

In the pancreas, the CaSR is found in islets of Langerhans, where it is thought to regulate glucagon and insulin secretion, and in duct cells and acinar cells (Squires, Harris et al. 2000, Racz, Kittel et al. 2002). Moreover, mice with a gain-of-function CaSR mutation had impaired glucose tolerance and hypocalcaemia (Babinsky, Hannan et al. 2017), suggesting a role for the CaSR in glucose homeostasis.

In the gut the CaSR is involved in gastric acid secretion (Busque, Kerstetter et al. 2005), fluid transport across the epithelium (Cheng, Okuda et al. 2002, Cheng, Geibel et al. 2004) and potentially gut motility (Muramatsu, Hira et al. 2014). Moreover, the CaSR acts as a nutrient sensor and is expressed in various EECs, including gastrin secreting G cells (Ray, Squires et al. 1997), cholecystokinin secreting I cells (Liou, Sei et al. 2011), somatostatin producing D cells (Haid, Jordan-Biegger et al. 2012), enterochromaffin cells (Symonds, Peiris et al. 2015) and L cells (Diakogiannaki, Pais et al. 2013).

In vitro studies suggest the CaSR mediates the secretion of various gut hormones, in response to certain amino acids. In I cells, CaSR mediates the secretion of CCK in response to L-Phenylalanine and L-Tryptophan (Liou, Sei et al. 2011, Wang, Chandra et al. 2011). In rat small intestinal loops L-Phenylalanine, L-Tryptophan, L-Glutamine, L-Asparagine, L-Arginine stimulate the secretion of GLP-1, PYY and GIP, an effect blunted or blocked, depending on the amino acid, by CaSR antagonism or Ca^{2+} depletion (Mace, Schindler et al. 2012). Additionally, CaSR activation in colonic and small intestinal primary cell cultures, and L cells, stimulates the secretion of GLP-1, an effect that may be mediated by TRP channels (Diakogiannaki, Pais et al. 2013, Pais, Gribble et al. 2015).

In rats, intra-ileal administration of L-Phenylalanine suppressed food intake, an effect blocked by CaSR antagonism (Alamshah, Spreckley et al. 2017). Additionally, oral administration of R-568, a CaSR agonist, decreased food intake in mice (Wu, Zhou et al. 2017), but at high concentrations led to emesis in mink (Wu, Zhou et al. 2017).

1.4.3.2. Amino acid and peptide transporter systems

A variety of promiscuous amino acid transporters exist, each with differing, yet overlapping amino acid specificity. They are expressed on the apical and basolateral membrane of cells and can also be located on the membrane of intracellular organelles, for example lysosomes, which may play a role in intracellular amino acid sensing.

Amino acid and peptide transporters have also been found to act as nutrient sensors mediating the secretion of gut hormones. Examples include peptide transporter 1 (PEPT-1) and the sodium-dependent transporter of neutral amino acids B^0AT1 and SNAT2, which are enriched in L-cells (Joshi, Tough et al. 2013).

In STC-1 cells, SNAT2 mediates the increase in intracellular calcium ions stimulated by L-Proline (Young, Rey et al. 2010). In GLU-tag and primary L-cells, glutamine stimulates GLP-1 secretion by raising intracellular calcium levels, which may be mediated by SNAT2 (Tolhurst, Zheng et al. 2011) . However, further investigation is required to determine the physiological role of SNAT2 in gut hormone secretion.

PEPT-1 is a H^+ coupled di- and tri- peptide transporter expressed on the apical surface of enterocytes in the gut. Coupling of peptide transport with H^+ depolarises the cell membrane, opening L-type calcium channels. The resultant increase in intracellular Ca^{2+} triggers the release of GLP-1 in primary L-cells (Diakogiannaki, Pais et al. 2013) and organoids (Zietek, Rath et al. 2015), as confirmed using PEPT-1 knockout models. However it may also indirectly mediate the secretion of gut hormones such as CCK (Liou, Chavez et al. 2011). Expression and activity of the transporter is influenced by dietary protein content (Shiraga, Miyamoto et al. 1999) and may influence food intake in response to a HPD (Nässl, Rubio-Aliaga et al. 2011).

B^0AT1 is found on the apical membrane of the intestinal epithelium and in the kidney. Mice lacking B^0AT1 fed a high fat diet gain less weight, have less abdominal white adipose tissue, increased basal levels of GIP and GLP-1, and improved glycaemic control. This phenotype may reflect the reduced absorption of the products of protein digestion and consequently increased luminal amino acid levels. These animals are also slightly hyperphagic, perhaps in an attempt to meet their amino acid requirements (Jiang, Rose et al. 2015). Human mutations in the gene encoding B^0AT1 result in

Hartnup disorder, characterised by amino acid insufficiency which is usually overcome by eating higher protein levels, but suggesting that B^0AT1 isn't an optimal target for anti-obesity agents (Cheng, Shah et al. 2017).

1.4.3.3. Intracellular sensing mechanisms

1.4.3.3.1. Mammalian target of rapamycin complex 1 (mTORC1)

MTORC1 is a serine/threonine protein kinase that acts as a regulator in metabolism, growth and protein synthesis. Its activity is regulated by growth factors, insulin, oxidative stress, nutrient availability and amino acids, particularly branched chain amino acids (Blouet, Ono et al. 2008).

A major effector molecule of mTOR is p70 S6 kinase 1 (SK6). Adeno viral modulation of SK6 expression in the mediobasal hypothalamus (MBH) affects food intake and body weight bidirectionally, with overexpression of SK6 decreasing meal size, energy expenditure and body weight (Blouet, Ono et al. 2008). Activation of mTORC1 in the MBH using leucine also decreases meal size, an effect mediated via a neuronal circuit consisting of POMC ARC neurons that project to oxytocin neurons in the PVN which in turn send projections to the NTS ($ARC^{POMC} \rightarrow PVN^{oxytocin} \rightarrow NTS$) (Blouet, Jo et al. 2009). Additionally, administration of leucine into the NTS decreases food intake and body weight via mTOR activation in NTS POMC neurons as part of a circuit that remains to be fully characterised (Blouet and Schwartz 2012).

MTORC1 inhibition in AgRP neurons supresses HF feeding induced BAT thermogenesis. In accordance, mice transitioning to a high fat diet and overexpressing an mTORC1 effector in ARC AgRP neurons display increased energy expenditure. However, mTORC1 effector molecule overexpression had no effect on AgRP function in chow fed mice. This suggests mTORC1 plays a role in the AgRP regulated adaptive BAT thermogenic response to nutrient availability (Burke, Darwish et al. 2017).

MTORC1 is activated by Rheb, downstream of growth factor signalling. However, this is dependent on the recruitment of mTORC1 to the surface of lysosomes, which is thought to be dependent on nutrient availability, particularly amino acids and glucose (Wolfson and Sabatini 2017).

Rag GTPases form the Rag A/B_{GTP} – Rag C/D_{GDP} complex which recruits mTORC1 to the surface lysosomal membranes in the presence of nutrients. These Rags are linked to the lysosomal membrane via the Ragulator complex. The amino acid transporter SLC38A9 also binds to the Ragulator and acts as a positive regulator of mTORC1 pathway. Leucine sensing sestrin-2 also modulates the pathway by binding to GATOR2, a positive modulator of the mTOR pathway, in the absence of leucine. Binding of sestrin-2 to GATOR2 in the absence of leucine inhibits the mTOR

pathway (Wolfson and Sabatini 2017, Wyant, Abu-Remaileh et al. 2017). These recent findings are starting to hint at how amino acids may activate mTORC1, though much work remains to be done.

1.4.3.3.2. General control non-repressed 2 protein (GCN2)

As previously mentioned, animals must obtain essential amino acids from the diet. Studies have shown that rats reject diets lacking in individual amino acids, an effect thought to be mediated by the anterior piriform cortex (APC) (Koehnle, Russell et al. 2004).

GCN2 detects changes in the availability of amino acids by binding to uncharged tRNAs, which accumulate when there is an amino acid deficiency. This activates GCN2 which in turn phosphorylates eukaryotic initiation fator-2 α (eIF2α). Mice lacking GCN2 as a whole or specifically in the CNS fail to reject diets lacking in the essential amino acid leucine or threonine, suggesting central CGN2 is essential in the aversive response to amino acid deficient meals (Hao, Sharp et al. 2005, Maurin, Jousse et al. 2005). Moreover, they fail to increase phosphorylation of elf2α in the APC, supporting a role for this signalling pathway in this adaptive eating behaviour (Maurin, Jousse et al. 2005). However, Leib et al. were unable to replicate these studies and suggest that a GCN2-independent mechanism is responsible for the alterations in feeding behaviour following the ingestion of EAA-deficient foods (Leib and Knight 2015).

Elf2α phosphorylation supresses protein synthesis and positively regulates the translation of activating transcription factor 4 (ATF4) in amino acid deprived cells (Harding, Novoa et al. 2000). ATF4 targets genes involved in amino acid biosynthesis, amino acid transport and protein degradation, amongst others (Baird and Wek 2012). ATF4 knockdown in mice decreases intracellular amino acid concentration, increases energy expenditure, blunts DIO and results in hypoglycaemia (Seo, Fortuno et al. 2009).

1.5. Hypothesis and aims

Current anti-obesity therapies are either ineffective, or highly invasive and difficult to provide on a global scale. Therefore, there is a growing need for new treatments. High protein diets are effective at promoting weight loss, but they are difficult to adhere to. By understanding the mechanism by which they promote satiety and weight loss, we can potentially exploit the system to identify new therapeutic targets.

Amino acids are important in mediating the effects of HPDs. Work in our group has identified various appetite supressing amino acids including L-Cysteine (McGavigan, O'Hara et al. 2015), L-Arginine (Alamshah, McGavigan et al. 2016) and L-Phenylalanine (Alamshah, Spreckley et al. 2017). L-

Phenylalanine's anorectic effects require further investigation to determine the mechanism underlying its effects on energy homeostasis.

Amino acid sensors in the gut have been identified to mediate the secretion of appetite regulating hormones. However, more recently, a limited subset of nutrient sensors, including the CaSR, have been identified in vagal sensory neurons. Their role in energy homeostasis remains unexplored.

Hypotheses

- Chronic exposure to high protein diets modulates the expression of appetite regulating gut hormones and nutrient sensors which may present novel therapeutic targets.
- L-Phenylalanine's appetite regulating effects are mediated via gut hormone dependent mechanisms that are modulated centrally.
- The vagus nerve has nutrient sensing capabilities which modulate energy homeostasis.

General aims

- To investigate the effects of chronic high protein diets on gut hormones and nutrient sensors.
- To examine the mechanism underlying L-Phenylalanine's effects on food intake.
- To determine the role of the calcium sensing receptor in the nodose ganglia in energy and glucose homeostasis.

Chapter 2: The Effects of High Protein Diets on Appetite

2.1. Introduction

2.1.1. High protein diets

The 'Committee on Medical Aspects of Food Policy' estimate an average requirement of 0.6g/Kg/day of protein in adults (19-50 year olds) (Policy 1991). Diets exceeding the habitual recommended protein consumption of 15% of the total energy content are considered to be high in protein (Pesta and Samuel 2014).

Numerous studies have examined the effects of HPDs on food intake, hormone secretion and circulating amino acid levels in humans and rodents, but the lack of standardisation in studies has resulted in discrepant observations. Variable factors include the source of protein, protein quantity, and route of administration, subject age and BMI and time of intervention. Nonetheless, increasing the amount of protein in the diet is thought to increase satiety, decrease food intake, improve body composition and stimulate greater DIT (Batterham, Heffron et al. 2006, Westerterp-Plantenga, Luscombe-Marsh et al. 2006, Potier, Darcel et al. 2009).

The acute effects of high protein loads on the secretion of gut hormones and DIT are covered in more detail in chapter 1.

2.1.2. Long term effects of high protein diets

Human studies examining the effects of prolonged (>1 year) increased dietary protein intake mainly focus on changes in body weight and body composition. These studies tend to show improved weight maintenance and body composition, with high dropout rates and subjects failing to meet prescribed protein levels (Leidy, Clifton et al. 2015). The latter highlights the difficulty of sustaining a HPD.

The largest study to date contrasting low and high protein diets for weight maintenance found increased protein intake to promote weight loss maintenance. This study, denoted the 'diet, obesity and genes' (Diogenes) study was a multi-centre study comprised of 938 adults and 253 children (Larsen, Dalskov et al. 2010). Moreover, a meta-analysis comparing the effectiveness of weight loss interventions varying in protein content, with a minimum follow up of a year, found weight loss in diets with an increased protein content to be ~0.4kg greater than contrasting diets (fat mass loss ~0.44kg) (Clifton, Condo et al. 2014). Failure to meet prescribed protein intake is a common problem in weight loss interventions. Stratification of the data according to protein intake at the end of the study, calculated using urine measures and diet diaries, revealed fat mass loss to be three-fold greater, when the difference in protein content between the contrasting diets, at the end of the

study, was >5% (Clifton, Condo et al. 2014). This suggests HPDs are more effective than diets rich in other macronutrients at promoting weight loss, if adhered to.

The effects of prolonged protein intake have also been examined in different animal models. In rats (Jean, Rome et al. 2001) and mice (Batterham, Heffron et al. 2006, Kinsey-Jones, Alamshah et al. 2015, Vu, Luong et al. 2017) prolonged increased protein intake supresses chronic food intake, but not consistently (Zhou, Keenan et al. 2011, Schwarz, Tomé et al. 2012, Cui and Kim 2018). It also reduces body weight/weight gain and fat mass (Batterham, Heffron et al. 2006, Zhou, Keenan et al. 2011, Kinsey-Jones, Alamshah et al. 2015, Vu, Luong et al. 2017).

Unlike in humans, the effects of prolonged HPDs on gut hormones have been investigated in various animal models by examining both their expression and secretion. However, these findings, summarised in table 2.1.2.1, have been inconsistent.

In pigs, 30 days of HPD feeding resulted in increased numbers of serotonin and ghrelin immunoreactive cells in the pyloric mucosa, but no changes in PYY immunoreactive cells (De Giorgio, Mazzoni et al. 2016). On the other hand, in mice, prolonged HPD intake for 16 weeks, did not affect the expression ghrelin in the stomach but did increase PYY expression in the ileum and colon (Batterham, Heffron et al. 2006). Differences in animal species may be the cause of inconsistent findings. In rodents, PYY expression is mainly found distally, whereas in pigs it is frequently found in the duodenum (Gribble and Reimann 2016).

The secretion of hormones in response to a prolonged HPD intake has also resulted in confounding results. In mice GLP-1 plasma levels were lower following a chronic HPD (Vu, Luong et al. 2017), unlike in rats where GLP-1 levels were raised (Zhou, Keenan et al. 2011). Similarly, changes in circulating levels of PYY are inconsistent. In mice, prolonged HPD intake resulted in increased circulating levels of PYY in the fed and post-prandial state (Batterham, Heffron et al. 2006). This was not observed in rats (Stengel, Goebel-Stengel et al. 2013) or other studies in mice (Vu, Luong et al. 2017). This suggests the choice of animal model is not the only reason for inconsistencies in the literature. In the prior mentioned studies, the ratio of macronutrients in the diet and protein load were all different (Batterham, Heffron et al. 2006, Zhou, Keenan et al. 2011, Stengel, Goebel-Stengel et al. 2013, Vu, Luong et al. 2017).

Gut hormones are secreted downstream of nutrient sensors, the expression of which has been reported to change in response to dietary interventions. In the gut, there are nutrient sensors which are well characterised for their role in detecting protein breakdown products. These include peptone sensors such as GPR92, and amino acid sensors such as the CaSR, GPRC6A and T1R1/T1R3. In

response to a prolonged HPD a temporary increase in the expression of GPR92 in the stomach of mice (Rettenberger, Schulze et al. 2015) and an increase in the number of α-transducin and α-gustducin immunoreactive cells in the pyloric mucosa of pigs has been observed (De Giorgio, Mazzoni et al. 2016). A-transducin and α-gustducin are involved in taste receptor intracellular signalling. On the other hand, no difference in GPRC6A expression were found in response to a chronic HPD in mice (Kinsey-Jones, Alamshah et al. 2015). The expression of the CaSR in response to a HPD has not been investigated.

Even though findings in the literature are inconsistent, it would appear HPDs are able to modulate gut hormone secretion and expression in the long run. The mechanism underlying these changes, and whether they drive weight loss or changes in body composition is not clear. Furthermore, whether changes in the expression of nutrient sensors correlate to changes in appetite and/or gut hormone secretion/expression would be interesting to explore.

Study	Species	Duration	Protein content	FI, BW and body composition effect	Fed state	Hormonal change in HPD versus NPD group
(Vu, Luong et al. 2017)	Mice	8 weeks	HPD: 60% protein Control: 20% protein	Decreased food intake. Temporary weight loss and improved body composition (NMR method).	Fasted overnight	- Acyl ghrelin: circulating ↓ Leptin: circulating - Insulin: circulating ↓ GLP-1: circulating - PYY: circulating - Glucagon: circulating
					Post-prandial: overnight fast followed by 1-hour re-feeding	- Acyl ghrelin: circulating ↓ Leptin: circulating - Insulin: circulating ↓ GLP-1: circulating - PYY: circulating ↑ Glucagon: circulating
(De Giorgio, Mazzoni et al. 2016)	Pig	30 days	HPD: 35% protein NPD: 14.5% protein	No change in food intake or body weight vs control.	Unclear	↑ Serotonin IR cells in the pyloric mucosa - Ghrelin IR cells in the pyloric mucosa - CCK IR cells in the duodenum and jejunum - PYY IR cells in colon
(Stengel, Goebel-Stengel et al. 2013)	Rats	32 days	HPD: 52% protein Control: 15% protein	Monitored for 14 day: decreased food intake and increased weight loss in HPD groups. Improved body composition in HPD groups (NMR method).	Light phase fed *ad libitum*	- GIP: circulating ↓ Insulin: circulating - Leptin: circulating - PYY: circulating - PP: circulating - Amylin: circulating
					2 hour dark phase feeding	↓ GIP: circulating - Insulin: circulating - Leptin: circulating - PYY: circulating - PP: circulating - Amylin: circulating

(Zhou, Keenan et al. 2011)	Rats	10 weeks	HPD-W: 38% protein (whey) HPD-S: 38% protein (soy) Control: 14% protein (egg white)	Decreased weight gain vs control. Decreased cumulative food intake in HPD-W but not HPD-S rats. Decreased fat mass detectable by weight but not by NMR technology.	Fed *ad libitum* culled in early light phase.	↑ Total GLP-1: circulating - CCK: circulating - PYY: circulating - Leptin: circulating
(Batterham, Heffron et al. 2006)*	Mice	16 weeks	HPD: 40% protein NPD: 15% protein	Decreased body weight gain and inguinal fat. Food intake data is from acute, not chronic studies.	Fed	↑ PYY: mRNA expression in the colon and ileum - Ghrelin: mRNA expression in stomach
					Fasted	↑ PYY: mRNA expression in the ileum - Ghrelin: mRNA expression in stomach
(Lacroix, Gaudichon et al. 2004)	Rats	3 and 6 months	HPD: 50% protein NPD: 14% protein	Mean energy intake at 6 months lower in HPD group. Decreased in body weight and fat mass in HPD group	Fasting	↓ Leptin: circulating ↓ Insulin: circulating ↓ Cortisol: circulating
(Green, Levan et al. 1986)	Rats	2 weeks	75% casein vs 5% casein	No change in food intake at 2 weeks. No body composition data.	Fed *ad libitum* decapitated in early light phase	- CCK: circulating

Table 2.1.2.1 Hormonal response to prolonged increased dietary protein in different species.

↑, increase; ↓, decrease; - no difference; HPD vs NPD. * Circulating plasma hormone levels are from acute rather than chronic high protein intake and therefore are not included in the table. Additionally, Batterham et al. found a decrease in leptin levels in mice fed a HPD chronically, but do not report whether the mice were fed or fasted. FI, food intake; BW, body weight; HPD, high protein diet; NPD, normal protein diet; NMR, nuclear magnetic resonance.

2.1.3. Hypothesis and aims

Hypothesis

Prolonged intake of a HPD will promote weight loss, improve body composition and affect the expression of gut hormones and nutrient sensors in the gut.

Aims

To investigate the effect of long term HPD intake in mice on:

1. Food intake, body weight and body composition.
2. Circulating hormone levels.
3. Expression of anorectic GLP-1 and PYY in the gut.
4. Expression of nutrient sensing CaSR in the gut.

2.2. Methods

2.2.1. Animals

Six to seven week old C57BL/6 mice were purchased from Charles River and singly housed in individually ventilated cages under controlled conditions (temperature: 21-23°C; light cycle: 12hour light/12 hour dark) for the duration of the study. Mice had *ad libitum* access to food unless otherwise stated.

All animal work was performed according to the UK Home Office Animals Scientific Procedures Act 1986 (project license number: 70/8068) and approved by the Central Biomedical Service at the Hammersmith Campus in Imperial College London.

At the end of the study, *ad libitum* fed mice were decapitated during the light cycle. Trunk blood was collected, their body composition was assessed and then the carcass was further dissected.

2.2.2. Diet

Mice were fed isocaloric high fat diets that varied in protein and carbohydrate content as summarised in table 2.2.2.1. Further details on the composition of the diets can be found in appendix 1.

In brief, there were three treatment groups (n=12) that received either a high fat high protein diet (HPD), a high fat normal protein diet (NP) or a NPD pair fed to the HPD group for 50 days. Diets were differentiated using tasteless dyes and purchased from Research Diets (New Jersey, USA). The pair-fed group was administered the median food intake of the HPD group from the previous day, before

the onset of the dark phase. However, they were given a NPD instead to control for the food intake effects of HPDs.

The diet selected in this study was the same as the one used by Batterham et al. as they found prolonged protein intake to modulate the expression of PYY in the gut (Batterham, Heffron et al. 2006).

	Fat (kcal %)	Protein (kcal %)	Carbohydrate (kcal %)
HPD	45	40	15
NPD	45	15	40

Table 2.2.2.1: **Macronutrient composition of mouse high protein and normal protein diet**. HPD, high protein diet; NPD, normal protein diet.

2.2.3. The effect of a long-term high protein diet on body composition in mice

Lean mass and fat mass, expressed as a percentage of total body weight, was assessed by nuclear magnetic resonance imaging using 'Echo MRI-100 body composition analyser' (Echo medical systems, Huston, Texas) post mortem. This system is based on the concept that hydrogen atoms contain a proton whose spin varies according to its surrounding environment. This allows for the differentiation and quantification of hydrogen atoms across different tissues.

Muscle tissue mass containing water but not fat nor bone minerals is detected as lean mass. Fat mass is expressed as equivalent weight to canola oil. The system used does not detect substances such as claws and hair. Fat mass and lean mass were calculated as a percentage of total body weight, taking into consideration that weight (g) = Lean + Fat + Free Water + Undetectable substances + Systematic Errors.

This technique has been shown to correlate well with the chemical carcass analysis of body composition. However, post mortem Echo MRI values slightly overestimate live values (Nixon, Zhang et al. 2010).

2.2.4. The effect of a long-term high protein diet on plasma hormone levels in *ad libitum* fed mice

Trunk blood was collected in EDTA K3 coated tubes (SARSTEDT, Leicester, UK) containing ~200 Killakrein inhibitor units of aprotinin per ml of blood (Nordic Pharma, Reading, UK), 1mg/ml of the serine proteases inhibitor pefabloc SC (Sigma,Poole, UK) and 100µmol/L of DPP-IV inhibitor (Millipore, Molsheim, France). Blood was then centrifuged for 10 minutes at 3000g. The plasma was

separated, aliquoted, frozen on dry ice and then stored at -80°C until further use. One of the aliquots was acidified using HCl to achieve a final concentration of 0.05N in case the serine protease inhibitor was insufficient to maintain the stability of active ghrelin for further analysis (Blatnik and Soderstrom 2011).

Levels of active GLP-1, acyl ghrelin, PYY, insulin, GIP, leptin and pancreatic polypeptide (PP) were simultaneously assayed using MILLIPLEX$_{MAP}$ mouse metabolic magnetic bead panel (Millipore, Molsheim, France) and the MAGPIX platform with xPONENT software (Luminex xMAP technology).

The technology utilises colour coded microspheres coated with specific antibodies. The antibodies bind to their respective gut peptide antigens and the signal is detected using a biotinylated detection antibody and streptavidin-phycoerythrin reporter conjugate. The data is acquired using the MAGPIX platform which identifies each individual microsphere based on their colour and quantifies the bioassay based on the fluorescent signal from the reporter conjugate.

The data was processed using a 5-parameter logistic curve on GraphPad Prism software (Prism 7.02, GraphPad Software Inc, CA, USA). Glucagon plasma levels were quantified using the Cisbio sandwich ELISA as per manufacturer's indications (Cisbio Bioassay, Codolet, France).

2.2.5. The effect of a long-term high protein diet on mRNA expression in mice

Jejunum, ileum and colon samples were dissected, snap frozen in liquid nitrogen and stored at -80°C. The jejunum was identified as the mid 4 cm region of the GI tract; the ileum as the 4 cm of GI tract proximal to the caecum; and colon as 4 cm of GI tract distal to the caecum.

RNA was extracted using PureLinkRNA mini kit (Thermofisher, Paisley, UK) according to manufacturer's instructions. RNA was eluted using RNase free water and quantified in triplicates using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Purity was examined using the A_{260}/A_{280} and A_{260}/A_{230} ratios. The A_{260}/A_{280} ratio indicates whether the sample is composed of RNA or DNA. An A_{260}/A_{280} of ~2 considered pure RNA. The A_{260}/A_{230} ratio can be used as a marker of contamination of, for example, phenol and guanidine. The A_{260}/A_{230} ratio should be ~2.0-2.2.

Samples were treated with RQ1 RNase-free DNase kit (Promega, Southampton, UK) to remove contaminant genomic DNA. Then, 1ug of RNA was reverse transcribed to cDNA using High Capacity cDNA reverse transcription kit (Thermofisher, Paisley, UK), as per manufacturer's instructions. The resulting cDNA was diluted one in five using RNase and DNase free water.

Preproglucagon (GCG) and PYY mRNA expression levels were examined using a SYBR green mix (Sigma, Poole, UK) and quantified relative to β-actin by singleplex. Primer sequences were obtained

from (Symonds, Peiris et al. 2015). The sequence was run on the NCBI BLAST service to ensure non-target genes were not being amplified with the selected primer sequence. Additionally, an efficiency standard curve was performed, and the qPCR end products were run on an agarose gel.

Validated CaSR (Mm00443375_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Mm03024075_m1) primers were purchased from thermofisher scientific and multiplexed using TaqMan gene expression assay (Life Technologies, Paisley, UK). Real time quantitative PCR was performed in the CFX 384 Real-Time PCR detection System (Biorad, Singapore).

Relative mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). B-actin was used as the reference gene for GCG and PYY expression, whereas HPRT-1 was found to be a more appropriate internal control when examining CaSR mRNA expression as their Ct cycles were more similar in the gut.

2.2.6. Statistical analysis

Data is expressed as mean ± standard error of the mean. Overall significance in chronic food intake was analysed using the generalised estimating equation (GEE) with STATA9 software. All other analysis was performed using GraphPad Prism software (Prism 7.02, GraphPad Software Inc, CA, USA). Body composition and gut hormone studies were analysed by performing a one-way ANOVA with Tukey's *post hoc* test. Analysis of changes in gene expression, within a tissue across different diets, was also performed using a one-way ANOVA with Tukey's post hoc test. P values less than <0.05 were considered statistically significant.

2.3. Results

2.3.1. The effect of a long-term high protein diet on cumulative food intake and body weight in mice

Chronic intake of a HPD, compared to a NPD, slightly decreased cumulative food intake in mice over 50 days non-significantly, according to GEE analysis. Correspondingly, a non-significant trend for decreased weight gain was observed in the HPD group, compared to the NPD group. On the other hand, mice on NPD pair-fed to a HPD did show a significant overall decrease in weight gain in comparison to mice on a HPD and NPD according to GEE analysis.

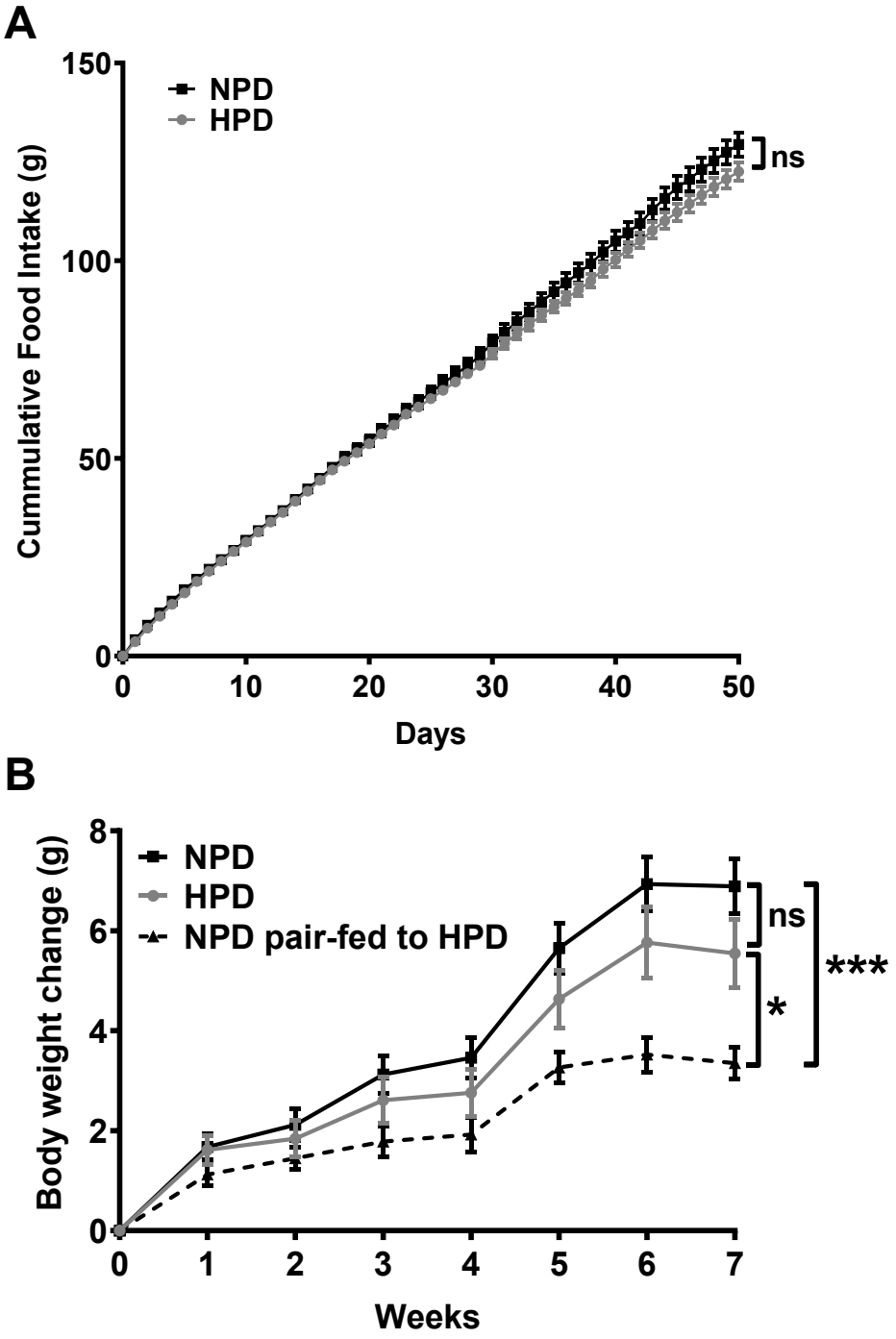


Figure 2.3.1.1 The effect of long-term high protein consumption on cumulative food intake and body weight in mice over 50 days

A) Cumulative food intake of male mice fed a NPD Vs a HPD. B) Body weight change of mice fed a HPD, NPD or a NPD pair-fed to a HPD. Data is expressed as mean ± SEM and analysed using GEE (n=12). *p<0.05, ***p<0.001.

2.3.2. The effect of a long-term high protein diet on body composition in mice

Post mortem, mice fed a HPD for 50 days had a non-significant trend for decreased fat mass and increased lean mass, expressed as a percentage of total body weight, in comparison to mice fed a NPD. Mice fed a NPD pair-fed to a HPD followed a similar trend to mice fed a HPD.

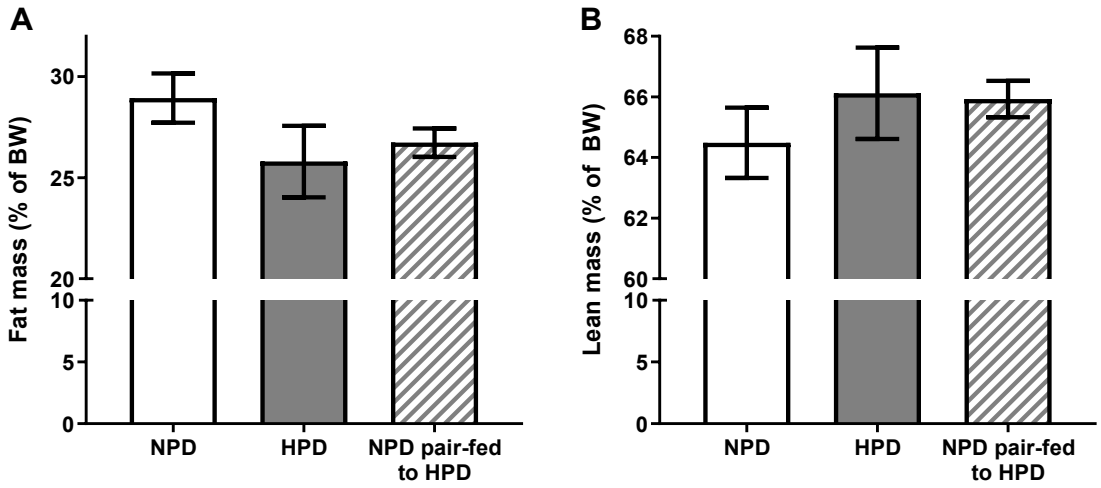


Figure 2.3.2.1 The effects of a prolonged high protein diet on body composition in mice.

The body composition of mice fed a NPD, HPD and NPD pair-fed to a HPD for 50 days was examined post mortem. A) Fat mass and B) lean mass were calculated as a percentage of total body weight. Data is expressed as mean ± SEM (n= 12).

2.3.3. The effect of a long-term high protein diet on plasma gut hormone levels in *ad libitum* fed mice

Long term changes in dietary protein content did not affect plasma acyl-ghrelin, active GLP-1, PYY or glucagon levels in *ad libitum* fed mice. However, mice fed a HPD did show a non-significant decrease in PP levels compared to pair-fed and NPD mice, and a significant decrease in leptin levels (NPD: 29068 ± 3090 pg/ml vs HPD: 15172 ± 2290 pg/ml, $p<0.01$), mirrored by the pair-fed group (NPD: 29068 ± 3090 pg/ml vs NPD pair-fed to HPD: 18317 ± 2333 pg/ml $p<0.05$). The pair fed group also had significantly lower circulating GIP compared to the NPD (NPD: 66.4± 8.8 pg/ml vs NPD pair-fed to HPD: 12.4 ± 8.2 pg/ml, $p<0.01$) and HPD fed mice (HPD: 61.8 ± 16.2 pg/ml vs NPD pair-fed to HPD: 12.4 ± 8.2 pg/ml, $p<0.05$). There was a trend for decreased insulin in the pair-fed group in comparison to all other groups.

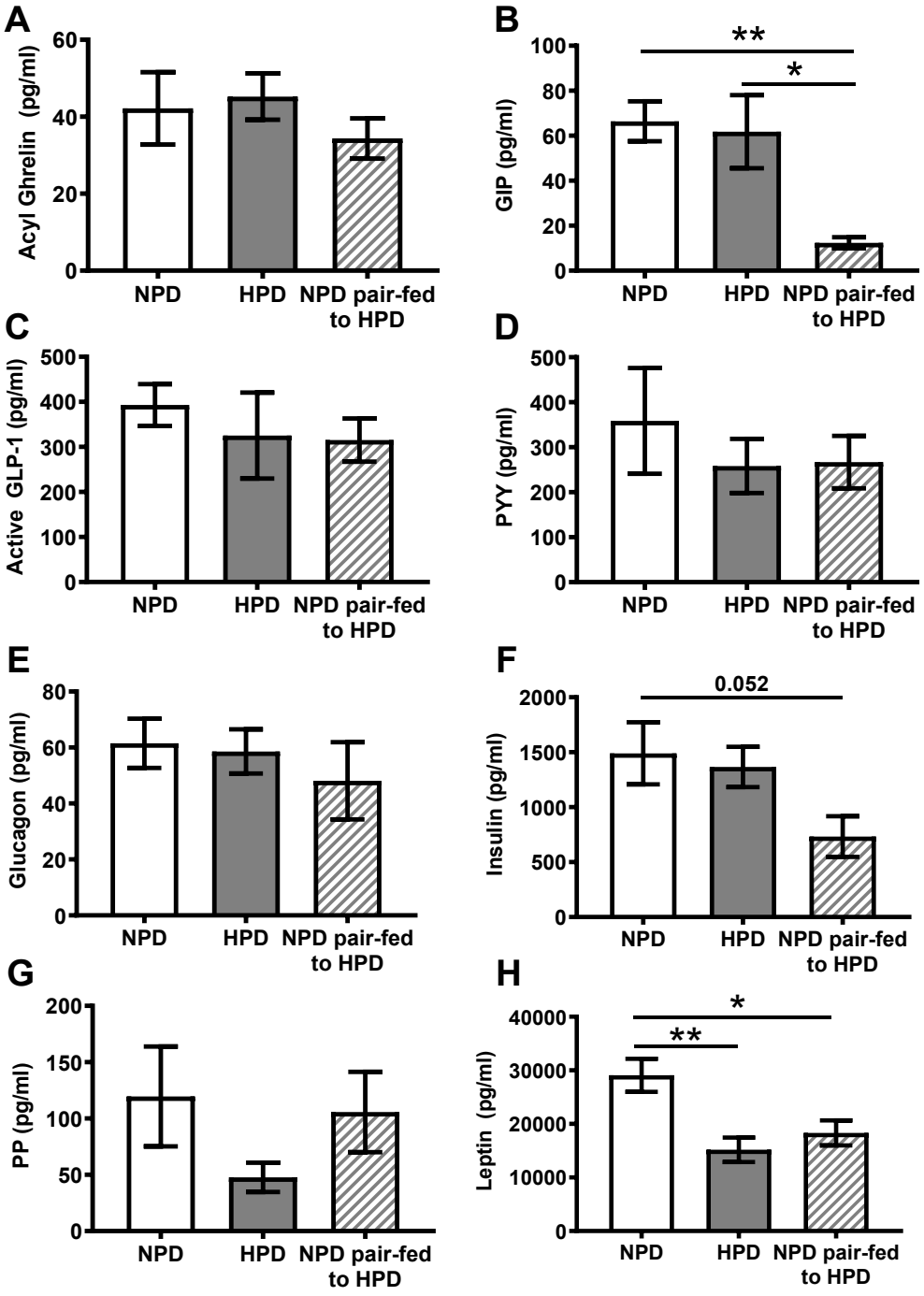


Figure 2.3.3.1 The effects of a prolonged high protein diet on plasma hormone levels.

The effect of increased dietary protein on circulating levels of A) acyl ghrelin (n=6-10), B) GIP (n=10-12), C) active GLP-1 (n=3-5), D) PYY (n=4-5), E) glucagon (n=7-11), F) insulin (n=11-12), G) PP (n=8) and H) leptin (n=11-12) was examined in mice. Data presented as mean ± SEM. *P<0.05, **p<0.01.

2.3.4. The effect of a long-term high protein diet on PYY expression in the gut of *ad libitum* fed mice

Ad libitum fed mice on a HPD for 50 days did not display any significant changes in PYY mRNA expression, at different levels of the gut, in comparison to NPD fed mice. However, PYY mRNA expression was elevated in the jejunum of HPD fed mice significantly in comparison to pair-fed mice (HPD: 1.348 ± 0.247 AU vs NPD pair-fed to HPD: 1.348 ± 0.247 AU), and non-significantly in comparison to mice on a NPD. Levels of PYY mRNA expression increased from the proximal to the distal gut in all mice. Its expression was particularly elevated in the colon.

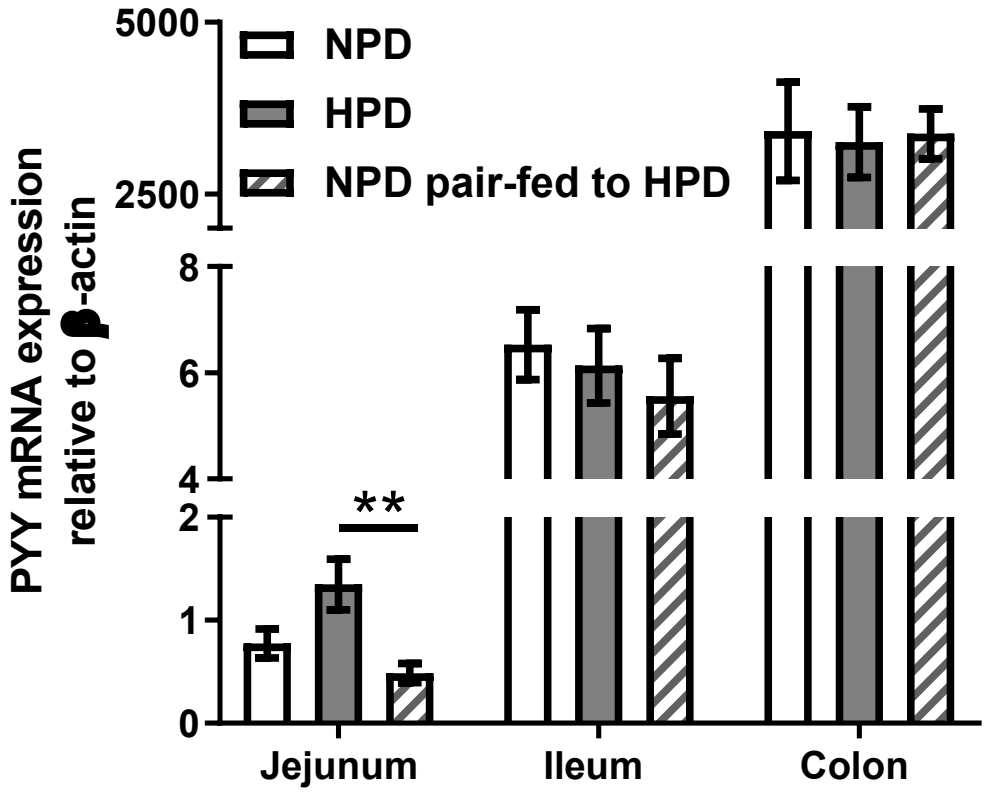


Figure 2.3.4.1 The effects of a prolonged high protein diet on PYY mRNA expression at different levels of the gut in *ad libitum* fed mice.

Relative PYY mRNA expression in different region of the GI tract of male mice fed a HPD, NPD or a NPD pair-fed to a HPD for 50 days. Data is presented as mean ± SEM (n=10-12). **p<0.01.

2.3.5. The effect of a long-term high protein diet on GCG expression in the gut of *ad libitum* fed mice

Ad libitum fed mice on a HPD for 50 days did not display any significant changes in GCG mRNA expression, at different levels of the gut, in comparison to NPD fed mice. However, GCG mRNA expression was significantly supressed in the pair-fed mice, compared to the NPD fed mice, in the jejunum (NPD pair-fed to HPD: 0.606 ± 0.113 AU vs. NPD: 1.309 ± 0.166 AU), ileum (NPD pair-fed to HPD: 2.977 ± 0.732 AU vs. NPD: 6.156 ± 0.895 AU) and colon (NPD pair-fed to HPD: 5.343 ± 1.107 AU vs. NPD: 10.540 ± 1.956 AU). It was also supressed compared to mice on a HPD in the jejunum (NPD pair-fed to HPD: 0.606 ± 0.113 AU vs. HPD: 1.473 ± 0.243 AU) and ileum (NPD pair-fed to HPD: 2.977 ± 0.732 AU vs. HPD: 5.386 ± 1.037 AU). Levels of GCG mRNA expression increased from the proximal to the distal gut in all mice.

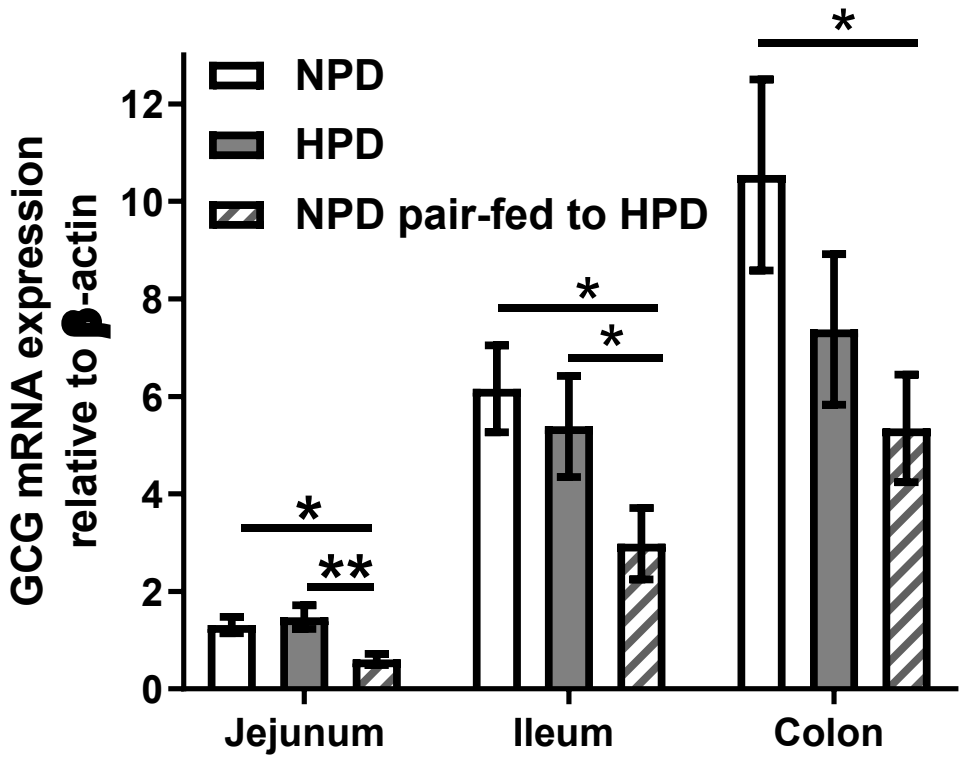


Figure 2.3.5.1 The effects of a prolonged high protein diet on GCG mRNA expression at different levels of the gut in *ad libitum* fed mice.

Relative GCG mRNA expression in different region of the GI tract of male mice fed a HPD, NPD or a NPD pair-fed to a HPD for 50 days. Data is presented as mean ± SEM (n=10-11). *p<0.05, **p<0.01.

2.3.6. The effect of a long-term high protein diet on CaSR expression in the gut of a*d libitum* fed mice

Ad libitum fed mice on a HPD for 50 days did not display any significant changes in CaSR mRNA expression, at different levels of the gut, in comparison to NPD and NPD pair-fed to HPD. CaSR mRNA expression was similar in the colon and ileum, which was greater than expression in the jejunum.

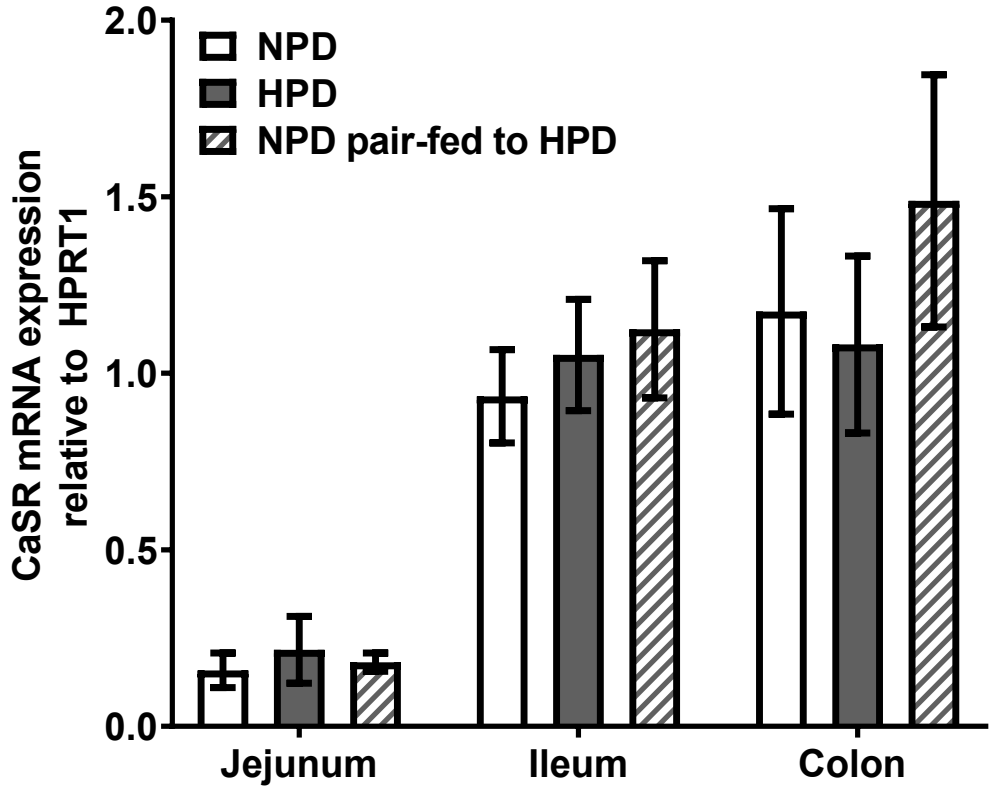


Figure 2.3.6.1 The effects of a prolonged high protein diet on CaSR mRNA expression at different levels of the gut in *ad libitum* fed mice.

Relative CaSR mRNA expression in different region of the GI tract of male mice fed a HPD, NPD or a NPD pair-fed to a HPD for 50 days. Data is presented as mean ± SEM (n=7-11).

2.4. Discussion

This chapter aimed to examine the effect of prolonged protein intake on the food intake, body weight, body composition and circulating hormone levels of mice. The expression of these hormones and of the CaSR in the gut were also investigated in response to a HPD. No significant effects were found in response to prolonged protein intake, other than a decrease in circulating levels of leptin in HPD fed mice.

Changes in food intake and body weight observed in mice administered a prolonged HPD compared to a NPD were non-significant. The lack of food intake effect is in accordance with some mice studies (Schwarz, Tomé et al. 2012) but in contrast to others (Kim, Park et al. 2012, Vu, Luong et al. 2017). Differences in the macronutrient ratio of the studies may be one of the reasons for the discrepant findings. Vu et al. used a diet with a lower fat content (10% vs 45%), a greater overall protein content (60% vs 40%) and a larger protein increment when comparing the NPD to HPD (40% vs 25%), than this study (Vu, Luong et al. 2017). Kim et al. also used a lower fat content, however the overall protein content and protein increment used is similar to this study (Kim, Park et al. 2012). This would suggest that the difference in fat content may be a driving factor. However, Schwarz et al. controlled for fat content, using both high fat and low fat diets varying in protein content, and saw no difference in cumulative food intake in any of the groups (Schwarz, Tomé et al. 2012). Studies in rats also result in different effects on cumulative food intake as seen in table 2.1.2.1.

Protein source has also been suggested to affect satiety in the long run (Zhou, Keenan et al. 2011). Rats on a 10-week high protein diet showed a decrease in cumulative food intake when the source of protein originated from whey but not soy, even though the total protein content was the same (Zhou, Keenan et al. 2011). The primary source of protein in this study was casein, the same as the previously mentioned studies (Kim, Park et al. 2012, Schwarz, Tomé et al. 2012, Vu, Luong et al. 2017), and is therefore unlikely to be the source of discrepancy.

The diet used in this study was based on a previous study that found a chronic HPD to modulate PYY expression in the distal gut (Batterham, Heffron et al. 2006). Unfortunately, Batterham et al. does not present chronic food intake data for comparison. However, Batterham et al. did find prolonged protein intake to decrease weight gain in mice in accordance with (Schwarz, Tomé et al. 2012, Vu, Luong et al. 2017) but not (Kim, Park et al. 2012). The decrease in body weight observed by Vu et al. was only temporary (Vu, Luong et al. 2017).

The pair-fed group in this study gained significantly less weight than the HPD and NPD groups. A pair-fed group was included to isolate the energy intake effects from other effects the HPD may be

having on the mice. Pair-fed mice alter their eating behaviour and tend to rapidly eat the allotted meal following its administration, resulting in periods of caloric restriction (Ellacott, Morton et al. 2010). Pair-feeding was performed once daily, before the onset of the dark phase, during which the mice were weighed. Consequently, the BW of pair-fed mice may have been confounded by their "fasted state", making the difference in BW observed more pronounced. Ideally, the mice would have been yoked fed by an automated feeding system (Ellacott, Morton et al. 2010). This was not available and therefore, in future, meal allocation in the pair-fed cohort should be divided into two or more allotments.

There was a non-significant trend for decreased fat mass in the HPD treated mice. Improvements in body composition following prolonged protein intake are observed more consistently in the literature (Lacroix, Gaudichon et al. 2004, Batterham, Heffron et al. 2006, Blouet, Mariotti et al. 2006, Zhou, Keenan et al. 2011, Schwarz, Tomé et al. 2012, Stengel, Goebel-Stengel et al. 2013, Vu, Luong et al. 2017). However, Vu et al. found the decrease in fat mass to be temporary (Vu, Luong et al. 2017). In this study, the trend for a decrease in fat mass was mirrored by a more pronounced decrease in circulating leptin levels, suggesting that this is a real effect.

Two of the few studies that have a pair-fed control group found no difference in body weight between HPD and NPD pair-fed to HPD rats, suggesting the decreased energy intake in chronically HPD fed rats is responsible for the weight loss effect (Jean, Rome et al. 2001, Blouet, Mariotti et al. 2006). However, both studies found differences in the body composition between HPD and pair-fed rats, suggesting the effects of HPD are not limited to changes in food intake. This was supported by findings that HPD fed rats have reduced hepatic lipogenesis (Blouet, Mariotti et al. 2006). Similarly, decreased lipid accumulation in the liver of mice (Schwarz, Tomé et al. 2012) and reduced lipogenesis in the BAT of HPD adapted rats (Moura, Kawashita et al. 2001) has been observed. This study did not find any changes in the lean to fat mass ratio between HPD mice and pair-fed mice, though this may have been masked by the relatively higher weight loss of the pair-fed group.

The effect of prolonged protein intake on energy expenditure has been examined in mice following a 12 week HPD (Kim, Park et al. 2012, Vu, Luong et al. 2017) and in rats following a 2 week HPD (Stepien, Gaudichon et al. 2011). Mice fed a HPD expended less energy than mice fed a NPD (Kim, Park et al. 2012, Vu, Luong et al. 2017) but not a low protein diet (9.8%) (Kim, Park et al. 2012). The difference observed by Kim et al. was not statistically significant, probably due to the smaller sample size (Kim, Park et al. 2012). This is in disagreement with studies in rats (Stepien, Gaudichon et al. 2011), which unlike in mice, were performed at temperatures closer to thermoneutrality. None of the prior mentioned studies accounted for energy lost in faeces. Moreover, considering there tends

to be a difference in the lean to fat mass ratio between NPD and HPD fed rodents, normalising energy expenditure data by body mass may be inappropriate as the contribution of fat mass to metabolism is different to that of lean mass. Therefore, it is difficult to conclude what effects prolonged increased protein intake may have on energy expenditure and whether this is a factor contributing to the weight loss and decreased food intake observed in certain studies.

Changes in food intake observed in the literature may be due to increased satiety, mediated by variations in appetite-regulating hormones. However, as demonstrated by the variable results shown in table 2.1.2.1, there is little consensus regarding the effect of a prolonged HPD on gut hormone secretion. In this study, no significant changes in food intake were observed. Nevertheless, changes in gut hormone can take place independently of food intake effects. For example, although rats adapted to a high soy protein diet did not show differences in food intake, compared to controls, they did have higher circulating levels of GLP-1 (Zhou, Keenan et al. 2011). Therefore, in this study the secretion and synthesis (mRNA levels), of different hormones was examined in response to prolonged HPD intake.

No significant changes between NPD and HPD fed mice were observed in the secretion of hormones other than leptin, as previously mentioned, but there was a trend for decreased levels of PP, an anorectic hormone secreted by the endocrine pancreas that indirectly inhibits pancreatic secretion, in HPD fed mice. The pair-fed mice did however display decreased levels of GIP and insulin in comparison to NPD and HPD fed mice. Mice were pair-fed in the afternoon before the onset of the dark phase. By the time the mice were culled for tissue extraction the following morning, the majority had no food left. Therefore, the decreased levels of GIP and insulin in pair-fed mice was a likely consequence of intermittent caloric restriction arising from pair-fed feeding behaviour previously mentioned. Pair-fed mice did not however show increased levels of ghrelin. This may be because the mice become entrained to receive their food in the afternoon, therefore at the time the tissues and blood samples were taken, an increase in anticipatory ghrelin, thought to take place 2 hours prior to the expected meal in rats, had not taken place yet (Drazen, Seeley et al. 2006).

In this study, mice were fed *ad libitum* prior to tissue extraction in the light phase, where food intake is naturally lower. This was because HPD induced changes in mRNA expression were more pronounced in the fed, rather than the fasted state in (Batterham, Heffron et al. 2006). Unlike, Batterham et al., this study did not find HPD fed mice to have increased mRNA levels of PYY in the colon or ileum, compared to NPD fed mice (Batterham, Heffron et al. 2006). There was, however, a trend for increased PYY expression in the jejunum, which is normally the main site of protein absorption. This may indicate the importance of amino acids in mediating the effects of HPDs, which

has previously been suggested (Mellinkoff, Frankland et al. 1956, Veldhorst, Nieuwenhuizen et al. 2009). PYY mRNA levels in the jejunum of mice fed a prolonged HPD were not reported by Batterham et al. (Batterham, Heffron et al. 2006). The change in PYY expression observed is relatively small when compared to expression levels in the colon, but it is not known how this translates in terms of circulating hormone levels. It may be that this small difference in expression has significant ramifications for circulating PYY levels during the early phases of digestion. This study did not find differences in circulating levels of PYY, however, the tissue and blood samples were collected during the light phase when food intake is low. Therefore, changes in PYY secretion in response to early phase digestion would not have been detected.

This study also investigated changes in the expression of pre-proglucagon (GCG). GCG gives rise to various peptide, in a tissue specific manner. In the gut, this includes the anorectic hormone GLP-1 (Rouille, Martin et al. 1995), which regularly colocalises with PYY in the distal gut. However, in the proximal gut, where changes in PYY mRNA expression were observed, GLP-1 expression is more abundant (Svendsen, Hartmann et al. 2015). Therefore, this study was interested in determining whether a chronic HPD leads to changes in GCG expression in the gut. No significant difference was observed in GCG mRNA levels between NPD and HPD fed mice, at any level of the gut. GCG levels were however significantly lower in pair-fed mice. This is probably due to changes in the feeding behaviour of the pair-fed cohort and further highlights the need to change the pair-feeding protocol.

The expression of nutrient sensors, which are able to modulate the secretion of hormones, have been shown to change in response to dietary intervention. There are three well characterised amino acid sensor found in the gut: GPRC6A, T1R1/T1R3 and the CaSR. The expression of GPRC6A does not appear to be altered by prolonged protein intake in mice (Kinsey-Jones, Alamshah et al. 2015). However, an increase in α-transducin and α-gustducin immunoreactive cells in the pyloric mucosa of pigs has been observed in response to a prolonged HPD (De Giorgio, Mazzoni et al. 2016). A-transducin and α-gustducin are involved in taste receptor intracellular signalling. Therefore, this study was interested in examining the effects of a prolonged HPD on the expression of the CaSR. No significant difference was observed in CaSR mRNA levels in response to chronic changes in protein intake. This would suggest the CaSR is not important in driving the effects of a HPD, however, it does suggest that, if targeted to stimulate the secretion of anorectic hormones therapeutically, it would be unlikely to lose sensitivity. This is supported by evidence suggesting the CaSR undergoes agonist-driven insertional signalling, making it highly resistant to functional desensitisation and thus a promising pharmacological target (Grant, Stepanchick et al. 2011).

Although no changes in hormone secretion or synthesis were observed, there are still other variables that could be examined in future studies. For example, the secretion of other hormones involved in appetite regulation; changes in the activity of enzymes that inactivate hormones, for example DPP-IV which is susceptible to inhibition by products of protein digestion (Tulipano, Sibilia et al. 2011, Lacroix and Li-Chan 2014); and changes in hormone receptor expression, which are influenced by changes in diet, for example, the orexin receptor which is down-regulated in the nodose ganglion of mice chronically fed a HPD (Journel, Chaumontet et al. 2012). Additionally, changes in the CNS and gut microbiome could be investigated. However, considering the lack of food intake and body composition phenotype, repeating the study with different parameters is recommended. Alternatively, stratifying the data according to mice who did and did not respond to the HPD, based on body composition, or other markers, could also be interesting.

Overall this study aimed to examine the effects of chronic HPDs on food intake, body weight, body composition, gut hormone secretion and synthesis, and CaSR expression. No significant changes were found in any of the examined parameters, except for a decrease in circulating levels of leptin in HPD fed mice, which supported a trend for decreased fat mass in these mice, and a trend in increased PYY synthesis in the jejunum. Further work is required to understand the mechanism underlying the beneficial effects of HPDs.

Chapter 3: The Effects of L-Phenylalanine on Energy Homeostasis and Pancreatic Hormone Secretion

3.1. Introduction

3.1.1. L-Phenylalanine

L-Phenylalanine is an essential aromatic amino acid that is non-polar due to the benzyl ring in its side chain. It is commonly found in soy, breast milk, meat, eggs and nuts. As humans are unable to synthesise L-Phenylalanine, a daily intake of 25-27mg/kg/day is recommended in adults. L-Tyrosine can considerably spare L-Phenylalanine requirements to ~9-15mg/kg/day, depending on the amount of L-Tyrosine intake (WHO 2007, MacLeod and Ney 2010).

Tyrosine is made through the hydroxylation of Phenylalanine by the phenylalanine hydroxylating system which consists of the enzyme phenylalanine hydroxylase (PAH), the coenzyme tetrahydrobiopterin (BH_4) and enzymes involved in replenishing BH_4. Tyrosine can then be further metabolised to generate catecholamines, tricarboxylic acid cycle (TCA) intermediates or carbon dioxide and water (Kaufman 1999) figure 3.1.1.1.

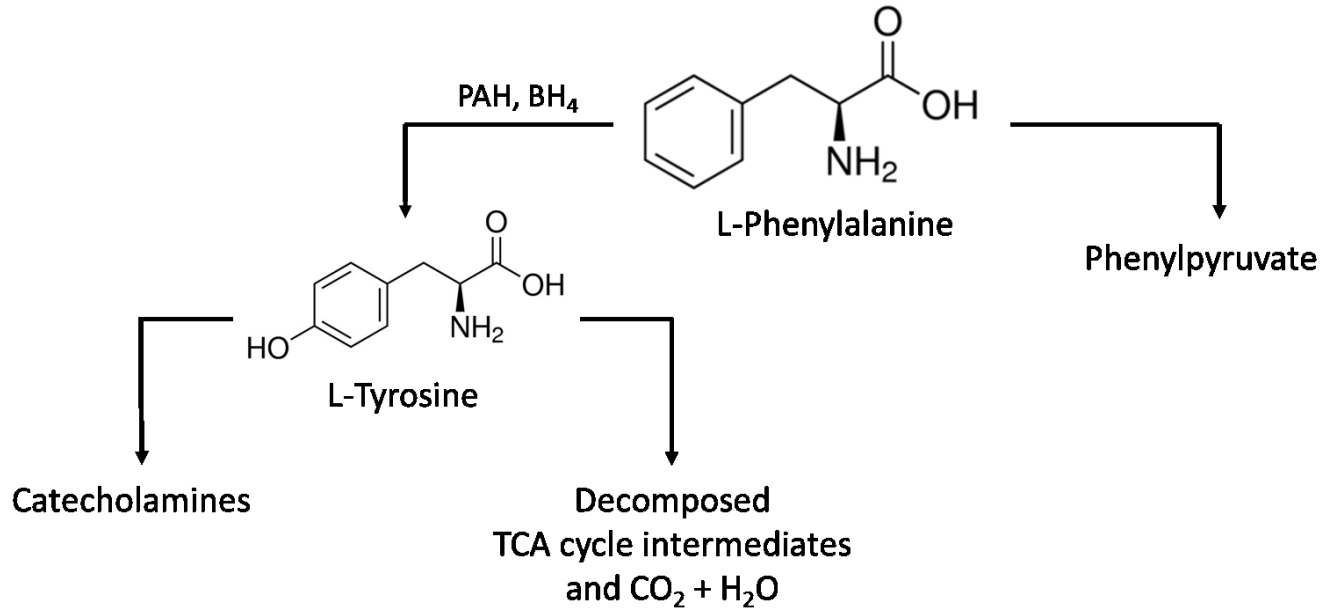


Figure 3.1.1.1 A schematic representation of L-Phenylalanine metabolism in the body.

L-Phenylalanine is hydroxylated into L-Tyrosine by the enzyme phenylalanine hydroxylase (PAH) in combination with the coenzyme tetrahydrobiopterin (BH_4). Tyrosine can then be further metabolised to generate catecholamines, tricarboxylic acid (TCA) cycle intermediates or carbon dioxide (CO_2) and water (H_2O). Adapted from (Kaufman 1999).

3.1.2. CaSR

The CaSR is primarily known for its role in the regulation of calcium homeostasis through its actions on the parathyroid gland and kidneys (Alfadda, Saleh et al. 2014). As mentioned in the introduction, it is also important in other cellular processes including differentiation, proliferation and hormone secretion. Its main ligand is calcium, but its activity is also modulated by changes in pH, divalent cations, oligopeptides and amino acids.

The CaSR can be found in the gut and pancreas, where it has been implicated in energy and glucose homeostasis (Alfadda, Saleh et al. 2014). In the pancreas the CaSR is found in both alpha and beta cells in the islets of Langerhans, where it is thought to regulate glucagon and insulin secretion, and in duct cells and acinar cells (Squires, Harris et al. 2000, Racz, Kittel et al. 2002). In the GI tract it is expressed in various EECs including G cells (Ray, Squires et al. 1997), I cells (Liou, Sei et al. 2011), D cells (Haid, Jordan-Biegger et al. 2012), enterochromaffin cells (Symonds, Peiris et al. 2015) and L cells (Diakogiannaki, Pais et al. 2013).

3.1.3. CaSR and the gut

The CaSR has been found in cells throughout the gut. In the stomach it is expressed in G cells and parietal cells. In G cells in humans (Ray, Squires et al. 1997) it is found on both the apical and basolateral membrane of the cell, allowing it to detect absorbed and luminal ligands . Activation of the CaSR in G cells stimulates gastrin secretion (Buchan, Squires et al. 2001). In parietal cells it is found on the basolateral membrane where its activation is thought to stimulate acid secretion via gastric H^+K^+-ATPases (Dufner, Kirchhoff et al. 2005). Gastric acid secretion is also stimulated by L-amino acids, including L-Phenylalanine, via the CaSR (Busque, Kerstetter et al. 2005).

In the small intestine the CaSR is expressed in the prior mentioned EECs and at the base of villi, and at the apex and base of crypts (Chattopadhyay, Cheng et al. 1998, Cheng, Okuda et al. 2002). *In vitro* it has been associated with the secretion of CCK (Liou, Sei et al. 2011, Wang, Chandra et al. 2011), GLP-1, PYY and GIP (Mace, Schindler et al. 2012, Diakogiannaki, Pais et al. 2013, Pais, Gribble et al. 2015), as discussed in the introduction. Therefore, it is possible that the CaSR also mediates the secretion of gut hormones in response to L-Phenylalanine, and that these hormones in turn regulate appetite and metabolism.

The CaSR is also expressed in nerves that innervate the gut: vagal sensory neurons (Egerod, Petersen et al. 2018) and the submucosal and myenteric plexus of the enteric nervous system (Cheng, Qureshi et al. 1999). Outside its potential role in appetite regulation, in the gut, the CaSR is important in salt and fluid balance, intestinal barrier integrity and cell growth and differentiation (Geibel and Hebert 2009, Tang, Cheng et al. 2016).

3.1.4. C-Fos as a marker of neuronal activation

To investigate how modulation of the CaSR in the gut might influence central neuronal circuits which regulate appetite, it is helpful to investigate neuronal activation in response to CaSR ligands. C-fos is an early gene transcription factor commonly used as a marker of neuronal activity. Its expression in quiescent neurons is low or undetectable; following neuronal activation its expression is transient, even if the stimulus is continuous (Durchdewald, Angel et al. 2009). Its ability to act as a marker of neuronal activation in various neuroendocrine systems was examined extensively by Hoffman et al (Hoffman, Smith et al. 1993).

C-Fos levels can be quantified by measuring expression at the mRNA and protein level. C-Fos mRNA levels peak 30 minutes after acute stimulation and maximal protein levels are reached 60-90 minutes post administration, persisting for ~5 hours (Sharp, Sagar et al. 1991, Verbalis, Stricker et al. 1991). The delay in protein expression provides more flexibility when choosing methods to euthanize experimental animals, an important consideration when measuring circulating hormones. However, c-Fos is inadequate for studying prolonged stimuli (Hoffman and Lyo 2002).

The expression of c-Fos is induced by several signal transduction pathways, including increased levels of cyclic AMP and intracellular calcium. Therefore, although c-Fos expression correlates well with neuronal depolarisation, the two can also be dissociated, for example, via the activation of the DNA serum response element in response to growth factors (Hoffman and Lyo 2002). The Fos protein can form a heterodimeric complex with the Jun protein family. This complex is able to recognise the TPA-responsive element, allowing it to modulate various genes involved in a range of physiological processes (Durchdewald, Angel et al. 2009). However, the regulation and role of c-Fos is complex and there are still gaps in our knowledge regarding such.

3.1.5. Hypothesis and aims

Hypothesis

L-Phenylalanine supresses food intake by stimulating the secretion of appetite regulating hormones via the CaSR in the GI tract and by modulating neuronal activity in the hypothalamus and brainstem.

Aims

To investigate:

1. The effect of oral and colonic administration of L-Phenylalanine on food intake in rodents.

2. The effect of oral administration of L-Phenylalanine on behaviour and energy expenditure in rats.
3. The effect of L-Phenylalanine on gut and pancreatic hormone secretion *in vivo.*
4. The role of the CaSR in mediating L-Phenylalanine-induced gut hormone secretion *in vitro*.
5. The effect of L-Phenylalanine on neuronal activation in appetite regulating centres of the brain

3.2. Methods

3.2.1. Animals

All animals were housed in individually ventilated cages under controlled standard light (12 hour light: 12 hour dark cycle) and temperature (21-23ºC) conditions with *ad libitum* access to rat and mouse 1 diet (RM1; Special Diet Services Ltd, Witham, Essex, UK) and water, unless otherwise stated.

All animal work was performed according to the UK Home Office Animals Scientific Procedures Act 1986 (project license number: 70/8068) and approved by the central biomedical service at the Hammersmith Campus in Imperial College London.

3.2.2. The effect of L-Phenylalanine oral administration on energy expenditure and food intake in rats in the early dark phase

Wistar rats purchased from Charles River (Margate, Kent, UK) weighing ~250-300g were individually housed in end feeder Comprehensive Lab Animal Monitoring System cages (CLAMS; Colombus instruments). Following a 24-hour acclimatisation period, *ad libitum* fed rats were orally gavaged with 6mmol/kg L-Phenylalanine or vehicle (3ml of 10% tween20, water) at the onset of the early dark phase. The study was repeated independently following a 72-hour wash-out period (non-cross over).

Metabolic parameters, oxygen consumption (VO_2) and carbon dioxide production (VCO_2), were measured by indirect calorimetry, normalised for body weight and used to calculate the respiratory exchange ratio (RER), an indirect indicator of the main substrate used during metabolism. Activity was determined from the number of consecutive beam breaks. Measurements of these parameters were taken every 17 minutes.

3.2.3. The effect of oral administration of L-Phenylalanine on behaviour in rats in the early light phase

Following oral gavage of 6mmol/kg L-Phenylalanine or vehicle (3ml of 10% Tween20, water), *ad libitum* fed rats from energy expenditure studies were observed for two hours in their home cages. Their behaviour was monitored every 5 minutes, for three consecutive five second intervals, resulting in 72 observations over the course of two hours. Behaviours were categorised according to table 3.2.3.1.

Behaviour category	Behaviour sub category	Definition
Feeding	Feeding	Feeding from food hopper or feeding from food held in paws
Drinking	Drinking	Licking at water spout
Locomotion	Locomotion	Moving around the cage with all four legs moving
	Rearing	Front paws raised off the ground and rear paws touching the ground
	Climbing	Front paws raised against side of the cage, cage furniture or suspended from cage lid
	Bed-making	Moving bedding with paws
Resting	Stationary	No paws moving
	Sleeping	Visibly asleep
Grooming	Grooming	Scratching, biting or licking itself
Discomfort	Pica	Consuming/chewing non-nutritive substances
	Head Down/Hunched	Abnormal body posture
	Tremor	Involuntary movements

Table 3.2.3.1 Classification and description of behaviours assessed in rats orally gavaged with L-Phenylalanine or Vehicle. An adaptation from (McGavigan, O'Hara et al. 2015).

3.2.4. The effect of oral administration of L-Phenylalanine on c-Fos-like immunoreactivity in rats

Following an overnight fast, rats used in energy expenditure and behavioural studies, weighing ~330-430g were randomised by body weight and orally gavaged with 6mmol/kg L-Phenylalanine or vehicle (3ml of 10% Tween20, water).

3.2.4.1. Tissue Collection

Ninety minutes post-administration rats were terminally anaesthetised with a 3ml intraperitoneal injection of pentobarbital sodium (Euthatal, Merial animal health Ltd, Essex, UK). Following loss of pedal reflex, rats were transcardially perfused with heparinised 0.01M PBS (appendix 2) to exsanguinate the animal and then with 4% paraformaldehyde (PFA) made in 0.01M PBS to fix the tissue (appendix 2). Once fixed, brains were extracted, further fixed overnight in 4% PFA at 4°C, dehydrated in 40% (w/v) (appendix 2) sucrose for cryoprotection, frozen in -80°C isopentane and stored at -80°C.

Forty micrometre thick coronal slices were cut using a freezing sled microtome (Shandon Southern products, LTD., Runcorn, Cheshire, UK) and stored in antifreeze solution (appendix 2) at -20°C as free-floating sections (McGavigan, O'Hara et al. 2015).

3.2.4.2. C-Fos immunohistochemistry

Free floating slices were blocked for endogenous peroxidase activity (0.6% H_2O_2 in methanol) for 15 minutes and non-specific binding for 2 hours (3% goat serum, 0.25% Triton-X in 0.01M PBS) prior to an overnight incubation with rabbit anti-c-Fos antibody ABE457 which targets the N-terminus of the c-Fos protein (1:15000, Merck, UK). The primary antibody was diluted in the blocking solution. In between each step the slices were washed with 0.01M PBS three times for five minutes.

Slices were then washed in 0.01M PBS 0.05% Tween20 and incubated for 2 hours at room temperature with biotinylated anti-rabbit IgG (1:400; VectorLabs, Peterborough, UK) made in secondary antibody diluent. The signal was amplified using an avidin-biotin horse radish peroxidase complex from Vector Laboratories (Vectastain Elite ABC-HRP Kit, UK) and visualised using 1% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, Poole, UK) solution, that is oxidised by the peroxidase enzyme in the presence of H_2O_2 to produce a brown pigment.

Slices were then washed in 0.01M PBS, mounted onto poly-lysine-coated slides, dried overnight, dehydrated in rising concentrations of ethanol and cleared/delipidated in xylene before coverslipping with DPX mounting media. Solutions are outlined in appendix 2.

Slides were visualised using a ZessAxioObserver widefield microscope. C-Fos counting was performed bilaterally in matched slices by individuals blinded to the treatments. C-Fos reactivity was quantified both manually, using the cell counter plug in in FIJI, and in an automated manner using a macro written by Steve Rothery (appendix 3). In brief, the macro would determine c-Fos reactive cells based on their staining intensity and size. Brain regions were identified using Paxino's and Watson's rat brain atlas (Paxinos and Watson 2006).

3.2.5. The effect of L-Phenylalanine administration on c-Fos-like immunoreactivity in mice

C57BL/6 male mice purchased from Charles River (Margate, Kent, UK) were individually housed and used to examine c-Fos-like immunoreactivity in response to orally and colonically administered L-Phenylalanine at the age of 10 and 14-16 weeks, respectively.

Mice were individually housed, fasted for four hours and then orally gavaged with 12mmol/Kg L-Phenylalanine, a dose known to decrease food intake, or vehicle control (0.3ml, 10% Tween20, water) (Alamshah, Spreckley et al. 2017). Ninety minutes post administration, mice were transcardially perfused as described in section 3.2.4.1.

In a separate study, conscious free moving mice colonically treated with 10mM of L-Phenylalanine (0.1ml, water), a dose determined from feeding studies and prior literature (Mace, Schindler et al. 2012, Alamshah, Spreckley et al. 2017), were transcardially perfused 100 minutes post administration. Tissue collection and staining was performed as described in section 3.2.4. However, in addition to the hypothalamus and brainstem, following publications that suggest the paraventricular thalamus also plays an important role in energy homeostasis (Ong, Liu et al. 2017, Zhang and van den Pol 2017), c-Fos-like immunoreactivity was also examined in this region of the brain. Brain regions were identified using Paxino's and Franklin's mouse brain atlas (Franklin and Paxinos 2007).

3.2.6. The effect of oral administration of L-Phenylalanine on hormone secretion

CaSR floxed mice were kindly donated by Chang (Chang, Tu et al. 2008) and bred in house. During breeding, the mice were group housed and fed rat and mouse 3 diet (RM3; Special Diet Services Ltd, Witham, Essex, UK). There on after, unless otherwise state, the mice were fed RM1 diet (Special Diet Services Ltd, Witham, Essex, UK).

CaSR floxed mice were used for experiments investigating tissue specific knockout of the CaSR (see chapter 4) by injecting AAV coding for cre recombinase in specific tissues. Without the presence of cre recombinase, these animals should be similar to wild types, and can thus be used as controls.

Ad libitum fed group housed CaSR floxed mice were orally gavaged with 0.3ml of 12mmol/kg L-Phenylalanine or vehicle control (10% Tween20, water) at the onset of the early dark phase. Twenty minutes post administration mice were decapitated from live.

3.2.6.1. Blood collection

Trunk blood was collected in chilled EDTA K3 coated tubes (SARSTEDT, Leicester, UK) containing ~200 Killakrein inhibitor units of aprotinin per ml of blood (Nordic Pharma, Reading, UK). The plasma was centrifuged for 15 minutes at 3000g, separated, aliquoted, frozen on dry ice and stored at -80C until further use.

3.2.6.2. GLP-1 radioimmunoassay

GLP-1 secretion was measured using a previously established in-house radioimmunoassay (Kreymann, Williams et al. 1987). The GLP-1 antibody used was raised in rabbits and specifically binds to the amidated forms of GLP-1; however, it does not cross-react with the glycine extended form (GLP-1_{1-37} and GLP-1_{7-37}) or other known gut hormones. ^{125}I-GLP-1, prepared by James Minion and Paul Bech using the Iodogen method (Wood, Wachter et al. 1981) and purified by high pressure liquid chromatography (HPLC), was used as the radioactive antigen tracer. The standard curve was constructed by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of 0.125pmol/ml GLP-1. The antibody, label and standard curve were made in 0.06M phosphate buffer supplemented with 0.3% BSA and 0.2% Tween 20. One hundred microliters of plasma were assayed and the reaction was brought to a final volume of 350µl using the supplemented buffer.

Following a four day incubation at 4°C, free and antibody bound GLP-1 was separated by dextran-coated charcoal adsorption (appendix 2). Two hundred and fifty µl of dextran-coated charcoal was added to the samples, the samples were then centrifuged at 1500g for 20mins at 4°C. The bound GLP-1, located in the supernatant, was aspirated and transferred to a fresh collection tube, leaving

the free GLP-1 located in the pellet. Both the free and the bound fraction were counted for four minutes using a gamma scintillation counter (LB2111 Multi Crystal Gamma Counter, Berthold Technologies, Bad Wildbad, Germany). The concentration of GLP-1 in the samples was determined using a non-linear plot on GraphPad Prism 7.02 (GraphPad Software INC, CA, USA).

3.2.6.3. Glucagon, insulin and glucose levels

Glucagon and insulin levels were quantified using mouse insulin and glucagon sandwich ELISA bioassays (Cisbio Bioassays, Codolet, France). Assays were performed according to manufacturer's instructions.

Glucose levels were quantified using colorimetric enzymatic glucose assay (Randox, UK, GL364). In brief, 2.5µl of sample were added to 250µl of buffer mix and incubated for 20 minutes at room temperature. The buffer mix contains glucose oxidase, an enzyme that oxidises glucose producing gluconic acid and hydrogen peroxide. The hydrogen peroxide further reacts with phenol and 4-aminophenazone producing a red-violet quinoneimine dye that is proportional to the initial amount of glucose in the sample. Glucose levels were quantified by comparing the absorbance of test samples to the absorbance of a calibrator sample (known amount of glucose). Absorbance was measured at 540nm using a biotek microplate reader (ELx808) following the 20 minute incubation.

3.2.7. The effect of colonic administration of L-Phenylalanine on food intake in mice in the early dark phase

CaSR floxed mice were bred as described above in section 3.2.6. Singly housed, free moving, *ad libitum* fed CaSR floxed mice aged 10-13 weeks were colonically administered 0.1ml of L-Phenylalanine or water at the onset of the early dark phase. Their food was replaced with pre-weighed standard chow and their food intake was monitored at 1, 2, 4 and 24 hours post-administration.

3.2.8. The effect of colonic administration of L-Phenylalanine on plasma hormones levels in mice in the early light phase

Group housed CaSR floxed mice aged 20-21 weeks were fasted for 5 hours prior to colonic administration of 0.1ml of 10mM L-Phenylalanine or water control. Mice were immediately returned to their cages and 30 minutes later they were decapitated from live.

Trunk blood was collected in EDTA K3 coated tubes (SARSTEDT, Leicester, UK) containing ~200 Killakrein inhibitor units of aprotinin per ml of blood (Nordic Pharma, Reading, UK), 1mg/ml of Pefabloc SC (Sigma, UK) and 100µmol/L of DPP-IV inhibitor (Millipore, Molsheim, France). Blood was then centrifuged for 10 minutes at 3000g. The plasma was separated, aliquoted, frozen on dry ice and then stored at -80°C until further use. One of the aliquots was acidified using HCl to achieve a final concentration of 0.05N in case the serine protease inhibitor was insufficient to maintain the stability of acyl ghrelin for further analysis (Blatnik and Soderstrom 2011).

3.2.8.1. Hormone MILLIPLEX MAGPIX assay

Levels of acyl ghrelin, PYY, insulin, glucagon, GIP and pancreatic polypeptide (PP) were simultaneously assayed using MILLIPLEX$_{MAP}$ mouse metabolic magnetic bead panel (Millipore, Molsheim, France) and the MAGPIX platform with xPONENT software (Luminex xMAP technology) as outlined in chapter 2 section 2.2.4.

3.2.9. The effect of colonic administration of L-Phenylalanine on GLP-1 secretion in mice in the early light phase

CaSR floxed mice were bred with PPG Cre-ER$_{T2}$ heterozygous (C57B6/J background) mice kindly donated by Fiona Gribble. PPG Cre-ER$_{T2}$ mice express tamoxifen-inducible cre recombinase under the expression of pre-proglucagon. In the absence of tamoxifen, cre recombinase is retained in the cytoplasm preventing the recombination of LoxP sites. Therefore, these mice should in theory be similar to wild types and were thus used as controls.

Group housed PPG Cre-ER$_{T2}$ $^{+/-}$ x CaSR floxed mice aged 7 weeks old were fasted for four hours prior to colonic administration of 0.1ml of 10mM L-Phenylalanine or glass distilled water control. Mice were immediately returned to their cages and 30 minutes later they were decapitated from live. Trunk blood was collected as described in cooled EDTA K3 coated tubes (SARSTEDT, Leicester, UK) containing ~200 Killakrein inhibitor units of aprotinin per ml of blood (Nordic Pharma, Reading, UK). Blood was then centrifuged for 15 minutes at 3000g. The plasma was separated, aliquoted, frozen on dry ice and then stored at -80°C until further use. Circulating GLP-1 levels were measured as described in section 3.2.6.2.

3.2.10. The role of the calcium sensing receptor in mediating L-Phenylalanine induced gut hormone release from murine primary colonic cells

3.2.10.1. Colonic crypt isolation

Colonic crypt extraction was performed using a modified version of the Psichas et al., protocol (Psichas, Tolhurst et al. 2017). Briefly, 4-8-week-old CaSR floxed mice were overdosed with pentobarbital administered intraperitoneally. Upon loss of pedal reflex and cessation of heart beat, mice were cervically dislocated and their colons were dissected. Any macroscopically visible connective tissue or fat on the colon was disposed of. Using a glass pipette, gut content was removed by flushing the colon with ice cold Leibovitz's L-15 media (Sigma, Poole, UK). The colon was then cut longitudinally, rinsed and further sectioned into $1mm^2$ squares. The chopped colon was washed with Leibovitz's L-15 media, until the media was clear of debris, and then rinsed with pre-warmed Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Poole, UK).

An initial 5 minute incubation in digestion media (appendix 2) at 37°C was performed to remove any cellular debris. Following three gentle shakes, the supernatant was disposed of and the chopped colon was incubated for a further 15 minutes in fresh pre-warmed digestion media, with intermittent vigorous shaking every 5 minutes. The chopped colon was allowed to settle; the supernatant was collected, centrifuged at 1000g for 5 minutes and the remaining pellet was re-suspended in complete pre-warmed DMEM (appendix 2) (digest 1). The procedure was repeated to obtain a total of four digests. When all digests were complete, they were combined, centrifuged at 1000g for 5 minutes, re-suspended in 7ml of complete DMEM containing 0.01M Y-2763 (biotechne, Abingdon, UK) to prevent anoikis (cell death from detachment to extracellular matrix) and filtered through a sterile nylon mesh (250µm pores). Filtered cells were plated onto 24 well plates, that were previously coated with 2.1% basement membrane matrix (Corning, The Netherlands), and incubated overnight at 37°C, 5% CO_2/95% O_2.

3.2.10.2. Secretion assay

Following an overnight incubation, the media was removed and wells were washed with secretion buffer (appendix 2) twice, before addition of 300µl of pre-treatment of control or antagonist solution made in secretion buffer. Following a 10 minute incubation, the pre-treatment was removed and 300µl of treatment made up in secretion buffer were added. Plates were then incubated for 2 hours at 37°C, 5% CO_2/95% O_2. The supernatant was collected, centrifuged at 1000g for 3 minutes to remove debris and stored at -20°C. Then, 250µl of lysis buffer (appendix 2) was added to each well to release intracellular peptide content and the plates stored at -80°C overnight

to be scraped the next day. Lysis content was transferred to eppendorfs and wells were washed with 250µl of secretion buffer that was also collected as part of the lysate. Lysate samples were stored at -20°C and gut hormone content was examined using the in house radioimmunoassay. Peptide secretion was expressed as a percentage of the total peptide content, i.e. secreted peptide/ (secreted peptide + lysate peptide content) x 100.

GLP-1 levels were measured using the in-house radioimmunoassay described in section 3.2.6.2. However, one hundred microlitres of supernatant and twenty microlitres of lysate were assayed. The reaction was brought to a final volume of 350µl using the supplemented buffer.

3.2.10.3. PYY radioimmunoassay

PYY levels were measured using a previously established in-house radioimmunoassay (Adrian, Ferri et al. 1985). The antiserum (Y21) was raised in rabbits against synthetic porcine PYY coupled to BSA by glutaraldehyde, and cross reacts with both the biologically active forms of PYY (PYY_{1-36} and PYY_{3-36}). Cross reactivity with other known gut hormones was not detected. ^{125}I-PYY, prepared by James Minion and Paul Bech using the Iodogen method (Wood, Wachter et al. 1981) and purified by HPLC, was used as the radioactive antigen tracer. The standard curve was constructed by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of 0.5pmol/ml PYY. The antibody, label and standard curve were made in 0.06M phosphate buffer supplemented with 0.3% BSA and 0.2% tween 20. One hundred and fifty microlitres of supernatant and forty microlitres of lysate were assayed. The reaction was brought to a final volume of 350µl using the supplemented buffer.

Following a three-day incubation at 4°C, free and antibody bound PYY was separated by immunoprecipitation. One hundred µl of sheep anti-rabbit antibody (Pharmacia Diagnostics, Uppsala, Sweden) was added to the samples. Following an hour incubation at room temperature, 500µl of 0.01% Triton X-100 (Sigma, UK) and 100 µl of 10% poly ethylene glycol (PEG) (Sigma, Poole, UK) were added to the samples. These were then centrifuged at 1500g for 30mins at 4°C, and free and bound radioactivity was quantified using gamma scintillation counters (LB2111 Multi Crystal Gamma Counter, Berthold Technologies, Bad Wildbad, Germany).

3.2.11. Statistical analysis

Data is expressed as mean ± standard error of the mean, except for behavioural and c-Fos studies that are expressed as median, interquartile range and total range.

Cumulative food intake and energy expenditure studies were analysed by calculating the area under the curve and performing a two-tailed unpaired t-test. RER was analysed using a repeated measures

two-way ANOVA with Sidak's multiple comparison test. The behavioural study and c-Fos studies were analysed using multiple tests, controlled for repeated measures using the Bonferroni-Dun method. All other studies were analysed by performing two-tailed unpaired t-tests except for the food intake experiment following colonic administration of L-Phenylalanine, which was paired.

3.3. Results

3.3.1. The effect of oral administration of L-Phenylalanine on cumulative food intake in rats in the early dark phase

Orally gavaging rats with 6mmol/kg L-Phenylalanine, compared to vehicle control (10%Tween20, water), significantly decreased cumulative food intake at 0-2 hours, as indicated by the area under the curve (AUC) in figure 3.3.1.1. B) (Vehicle: 280.31 ± 50.78 vs. L-Phenylalanine 153.86 ± 28.41; $p<0.05$). At 2-3hrs, rats administered L-Phenylalanine, increased their food intake to match that of the vehicle control group figure 3.3.1.1 A) (Vehicle: 4.56 ± 0.58g Vs. L-Phenylalanine: 4.71 ± 0.56g).

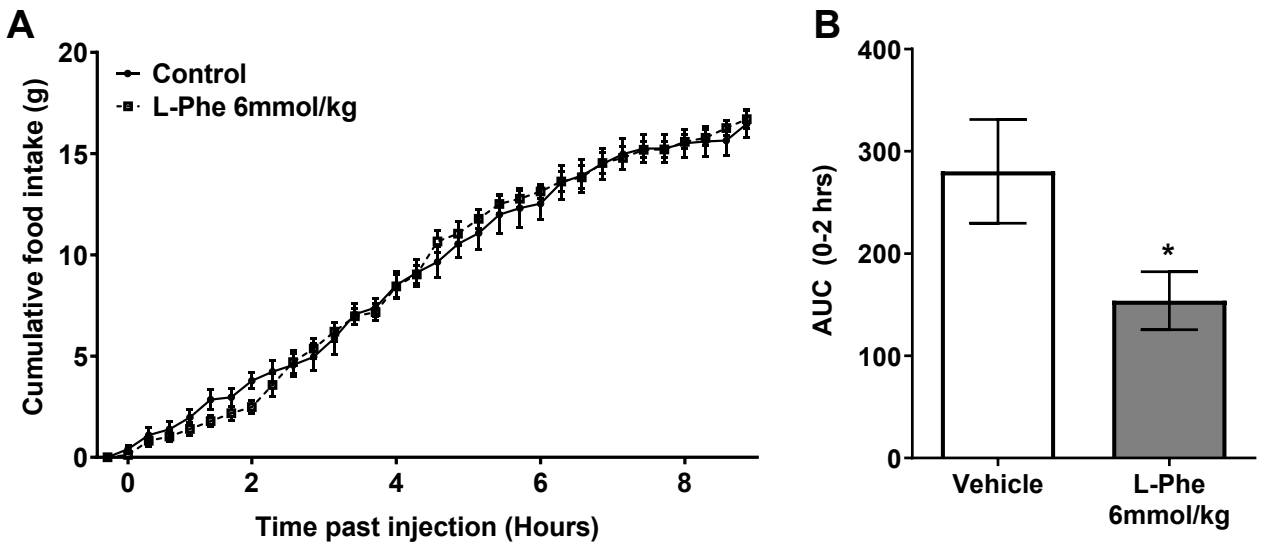


Figure 3.3.1.1 The effect of orally administered L-Phenylalanine on food intake in rats in the early dark phase

The effect of 6mmol/kg L-Phenylalanine oral gavage on cumulative food intake in *ad libitum* fed male rats at the onset of the early dark phase for A) 0-8 hours post administration. B) Area under the curve of 0-2 hour cumulative food intake. Data is expressed as mean ± SEM (n=7-8) *p<0.05.

3.3.2. The effect of oral administration of L-Phenylalanine on energy expenditure in rats in the early dark phase

Ad libitum fed rats were orally gavaged with 6mmol/kg L-Phenylalanine or vehicle control (10% Tween20, water) at the onset of the early dark phase. Energy expenditure was examined using CLAMS metabolic cages. At 0-2hours post administration, VO_2 was significantly higher in rats administered L-Phenylalanine, as indicated by the area under the curve (Vehicle: 10384 ± 365 n=8 Vs. L-Phenylalanine: 12142 ± 629, n=9; p<0.05). There was a trend for decreased respiratory exchange ratio and increased VCO_2 and activity at 0-2 hours in the L-Phenylalanine treated rats, but this did not reach significance.

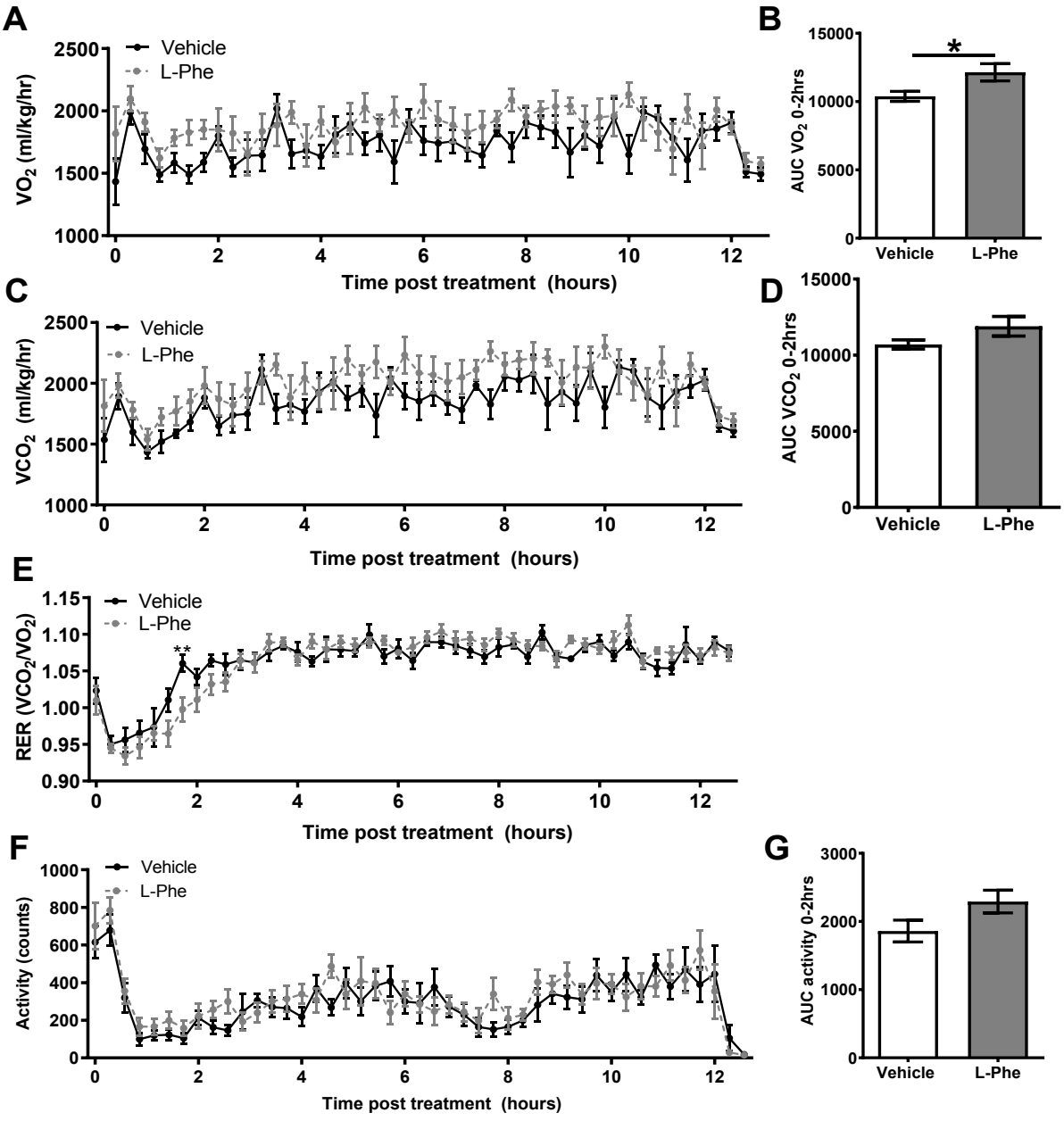


Figure 3.3.2.1 The effect of orally administered L-Phenylalanine on energy expenditure in rats in the early dark phase

The effect of 6mmol/kg L-Phenylalanine oral gavage in *ad libitum* fed male rats at the onset of the early dark phase on A) Oxygen consumption (VO_2) 0-12 hours post treatment and at B) 0-2 hours (AUC) C) Carbon dioxide production (VCO_2) 0-12 hours post treatment and at D) 0-2 hours (AUC), E) Respiratory exchange ratio (RER) 0-12 hours post treatment, F) Activity based on beam break counts 0-12 hours post treatment and G) 0-2hours post treatment (AUC). Data is expressed as mean ± SEM (n=8-9). *p<0.05, **p<0.01.

3.3.3. The effect of oral administration of L-Phenylalanine on behaviour in rats in the early light phase

Oral administration of 6mmol/kg L-Phenylalanine significantly increased locomotor activity in mice at 0-2 hours post administration, compared to vehicle control mice (L-Phenylalanine: median: 11.50, IQR: 9.25-15.50; Vs. Vehicle: median: 7.00, IQR: 6.00-8.50, $p<0.01$). There was a trend for decreased feeding and increased grooming, but this did not reach statistical significance.

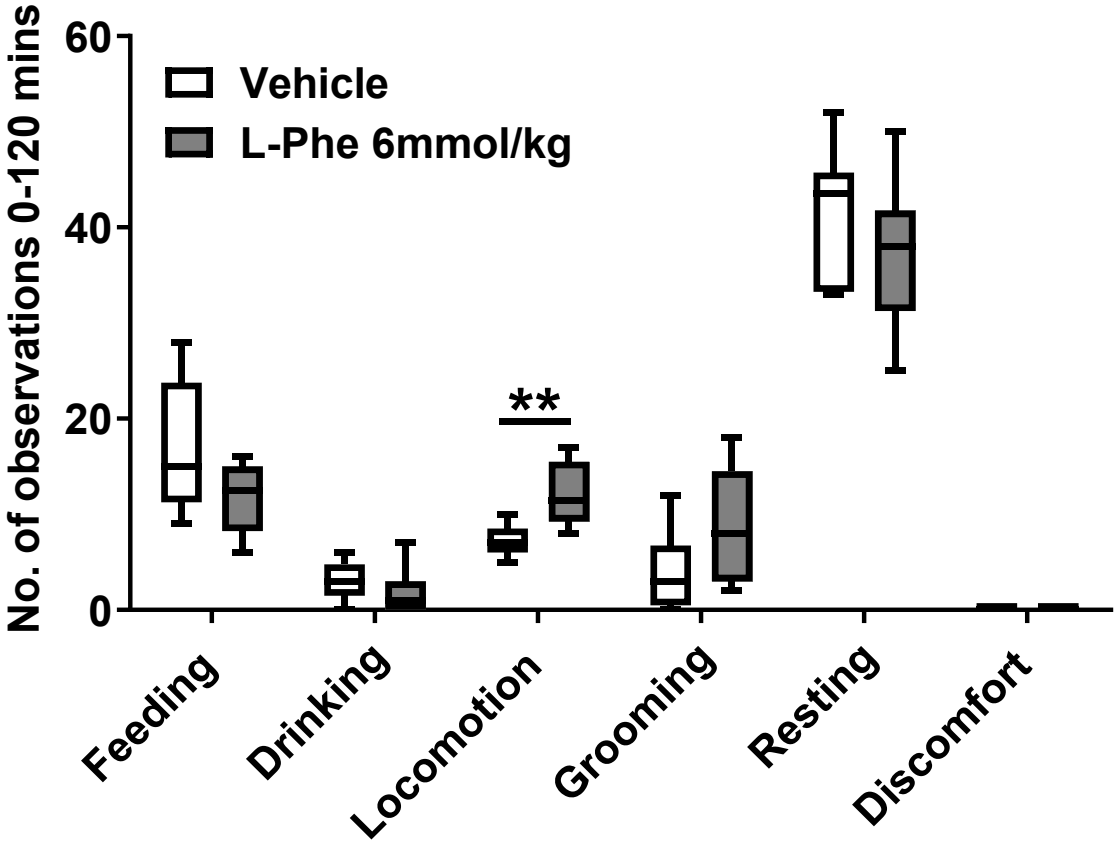


Figure 3.3.3.1 The effects of orally administered L-Phenylalanine on the general behaviour of rats.

The behaviour of rats administered 6mmol/kg L-Phenylalanine or vehicle control (10% Tween20, water) was monitored for two hours post administration. Data expressed as median, interquartile and total range (n=8) **adjusted $p<0.01$.

3.3.4. The effect of oral administration of L-Phenylalanine on c-Fos-like immunoreactivity in rats, quantified manually

Oral administration of 6mmol/kg L-Phenylalanine in rats resulted in a significant increase in c-Fos like immunoreactivity in the AP (L-Phenylalanine: median: 23, IQR: 17-36.5; Vs. Vehicle: median: 9, IQR: 5-13.5, adjusted $p<0.05$). There was a non-significant trend for increased activation in the NTS. No changes in neuronal activation in the hypothalamus were observed.

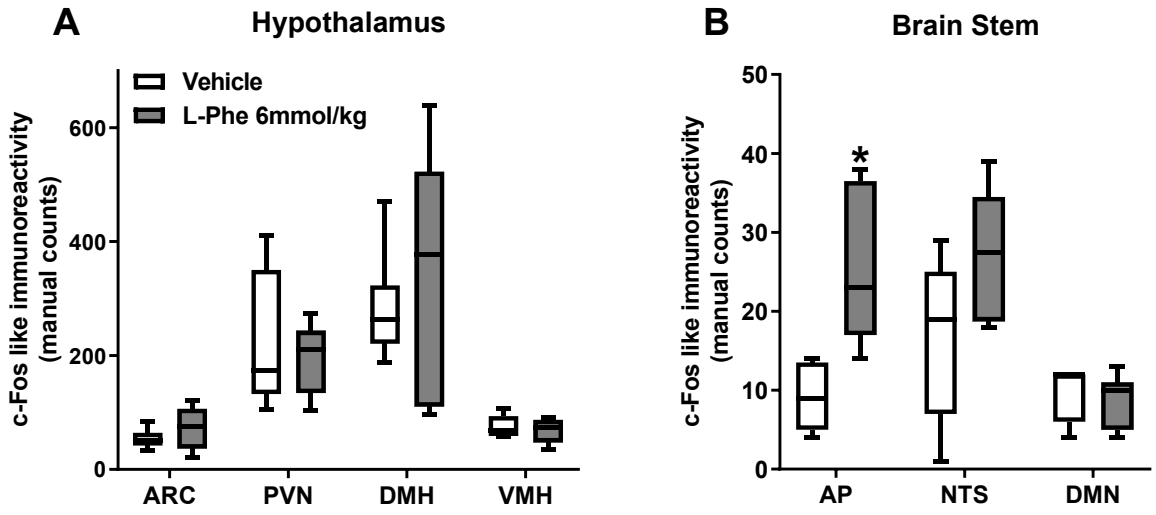


Figure 3.3.4.1 The effects of orally administered L-Phenylalanine on c-Fos like immunoreactivity in the brain of rats, quantified manually.

C-Fos like immunoreactivity in the A) hypothalamus (n=5-6) and B) brainstem (n=5-7) of rats orally administered 6mmol/kg L-Phenylalanine in the light phase was quantified manually. Data is expressed as median ± interquartile and total range. ARC, arcuate nucleus; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; AP, area postrema; NTS, nucleus tractus solitarius; DMN, dorsal motor nucleus.*adjusted p value <0.05.

3.3.5. The effect of oral administration of L-Phenylalanine on c-Fos-like immunoreactivity in the hypothalamus, thalamus and brainstem of mice, quantified manually

Oral administration of 12mmol/kg L-Phenylalanine, a dose known to decrease food intake in mice (Alamshah, Spreckley et al. 2017), resulted in a significant increase in c-Fos like immunoreactivity in the NTS (L-Phenylalanine: median: 56, IQR: 49.5-82; Vs. Vehicle: median: 16, IQR: 12.5-25, $p<0.01$), DMN (L-Phenylalanine: median: 13.5, IQR: 12.25-16.25; Vs. Vehicle: median: 7, IQR: 2-8, $p<0.05$) and AP (L-Phenylalanine: median: 62, IQR: 47.75-85.25; Vs. Vehicle: median: 18.5, IQR: 12-20.5, $p<0.05$) of the brainstem. There was a trend for increased activation in the VMH and PVT but this did not reach statistical significance.

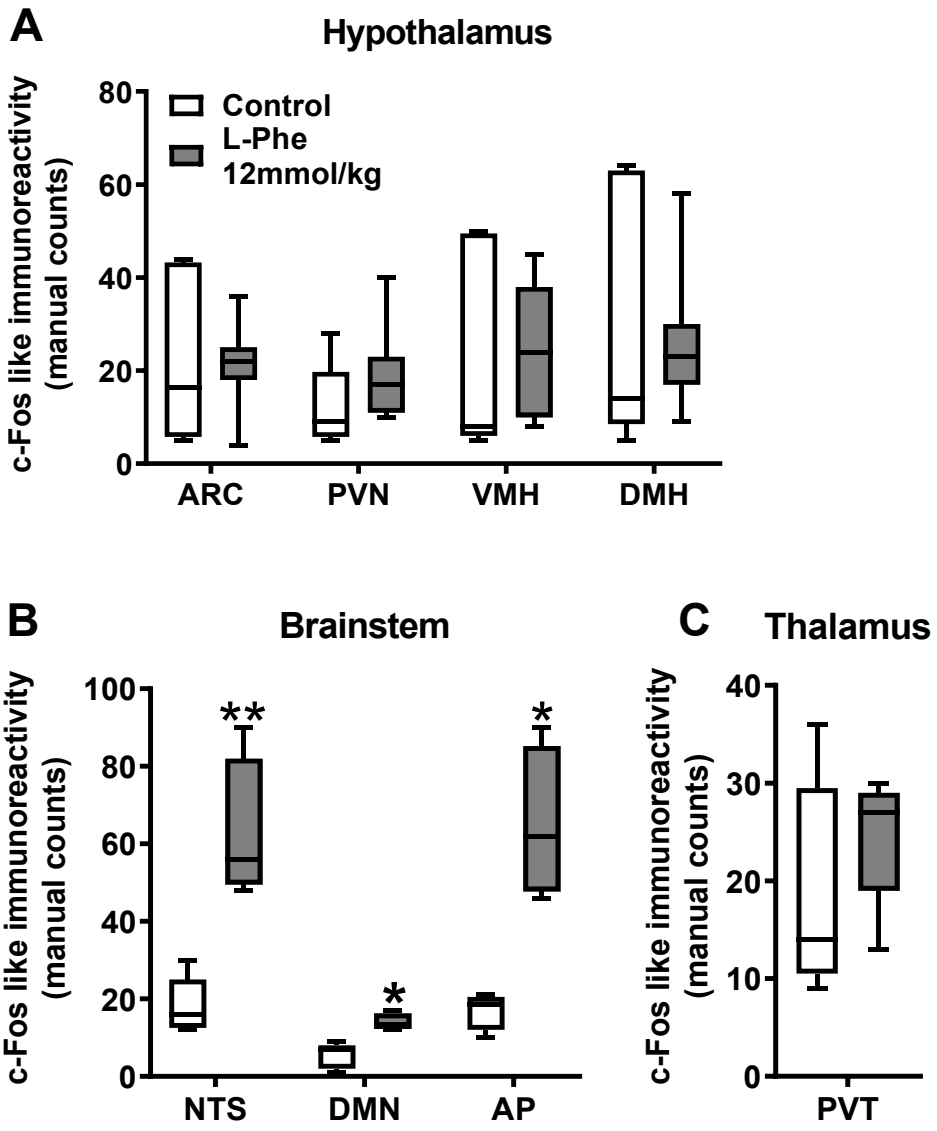


Figure 3.3.5.1 The effects of orally administered L-Phenylalanine on c-Fos like immunoreactivity in the brain of mice, quantified manually.

C-Fos like immunoreactivity in the A) hypothalamus (n=5-7), B) brainstem (n=4-5), and C) thalamus (n=5-7) of mice orally administered 12mmol/kg L-Phenylalanine in the light phase was quantified manually. Data is expressed as median ± interquartile and total range. ARC, arcuate nucleus; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; NTS, nucleus tractus solitarius; DMN, dorsal motor nucleus; AP, area postrema; PVT, paraventricular nucleus of the thalamus. *adjusted p value <0.05, **adjusted p value <0.01.

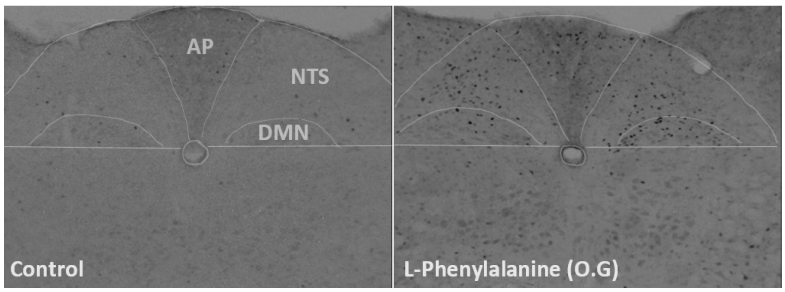


Figure 3.3.5.2 Representative figure of c-Fos staining in the brainstem of control and L-Phenylalanine treated mice

3.3.6. The effect of oral administration of L-Phenylalanine on c-Fos-like immunoreactivity in the hypothalamus, thalamus and brainstem of mice, quantified using an automated system

Oral administration of 12mmol/kg L-Phenylalanine resulted in a significant increase in c-Fos like immunoreactivity in the NTS (L-Phenylalanine: median: 137.5, IQR: 66.75-175.30; Vs. Vehicle: median: 33, IQR: 32.50-38.00, $p<0.05$) and AP (L-Phenylalanine: median: 111.5, IQR: 69-127; Vs. Vehicle: median: 21.5, IQR:13.75-33.75, $p<0.01$) of the brainstem. There was a trend for increased activation in the PVT.

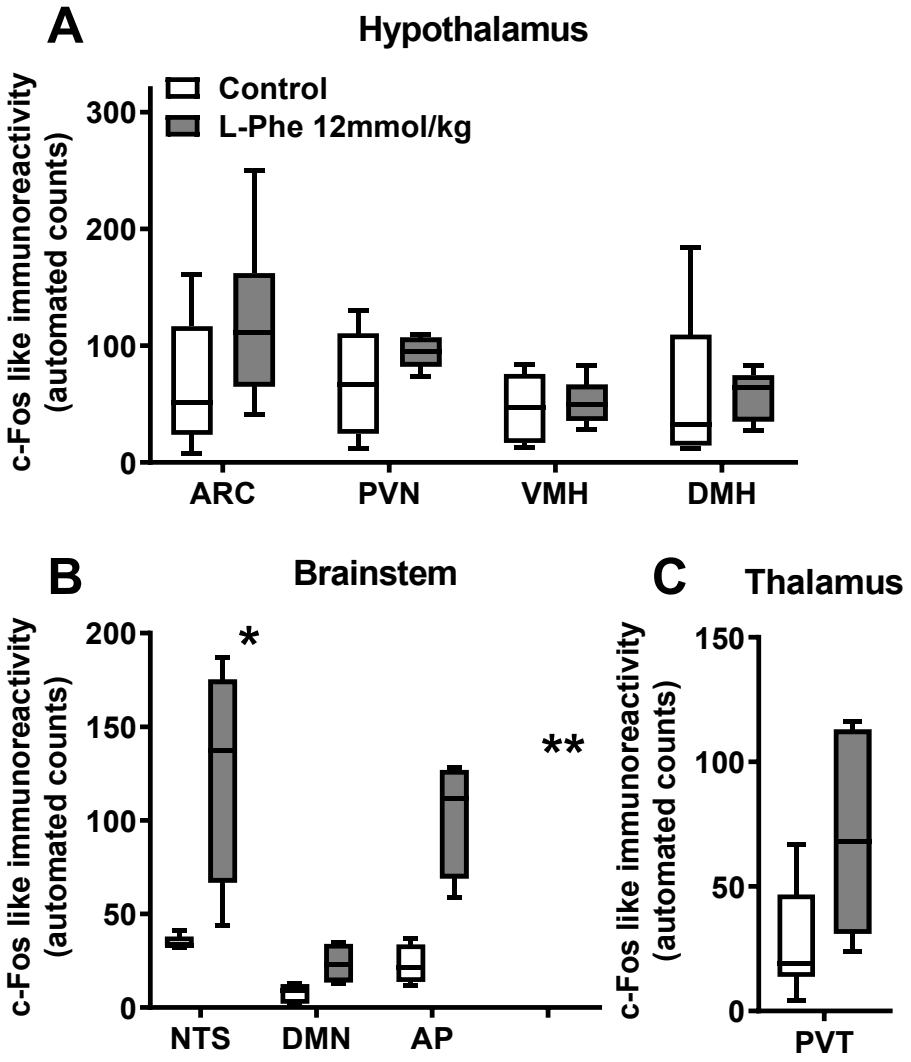


Figure 3.3.6.1 The effects of orally administered L-Phenylalanine on c-Fos like immunoreactivity in the brain of mice, quantified using an automated system.

C-Fos like immunoreactivity in the A) hypothalamus (n=5-7), B) brainstem (n=4-5), and C) thalamus (n=6-7) of mice orally administered 12mmol/kg L-Phenylalanine in the light phase was quantified using an automated system. Data is expressed as median ± interquartile and total range. ARC, arcuate nucleus; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; NTS, nucleus tractus solitarius; DMN, dorsal motor nucleus; AP, area postrema; PVT, paraventricular nucleus of the thalamus. *adjusted p value <0.05, **adjusted p value <0.01.

3.3.7. The effect of oral administration of L-Phenylalanine on GLP-1 secretion in mice

Ad libitum fed mice orally administered L-Phenylalanine in the onset of the early dark phase had a trend for increased GLP-1 secretion but this was not statistically significant. GLP-1 RIA intra-assay variability and limit of detection was 2.3% and 0.44pmol/L respectively.

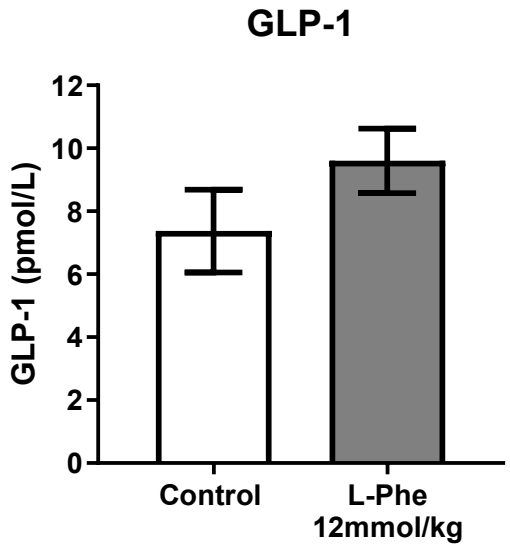


Figure 3.3.7.1 The effects of orally administered L-Phenylalanine on GLP-1 plasma levels in mice.

GLP-1 plasma levels in *ad libitum* fed mice orally administered 12mmol/kg L-Phenylalanine or vehicle control (10% Tween20) were quantified 20 minutes post-administration using the in-house radioimmunoassay. Data presented as mean ± SEM. *$p<0.05$ (n=5-6).

3.3.8. The effect of oral administration of L-Phenylalanine on pancreatic hormone secretion in mice

Ad libitum fed mice orally administered L-Phenylalanine in the onset of the early dark phase had significantly raised glucagon levels 20 minutes post administration (Control: 49.61 ± 6.28 pg/ml Vs. L-Phenylalanine: 141.7 ± 19.77 pg/ml; $p<0.05$). There was a trend for decreased insulin secretion, but this was not statistically significant.

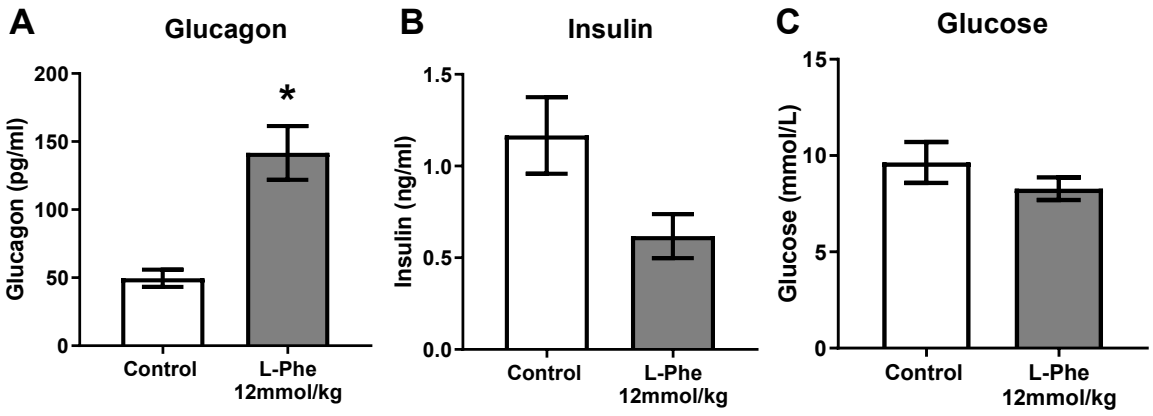


Figure 3.3.8.1 The effects of orally administered L-Phenylalanine on glucagon, insulin and glucose plasma levels in mice.

A) Glucagon (n=5-6), B) insulin (n=4-5) and C) glucose (4-6) plasma levels in *ad libitum* fed mice orally administered 12mmol/kg L-Phenylalanine or vehicle control (10% Tween20) were quantified 20 minutes post-administration. Data presented as mean ± SEM. *p<0.05.

3.3.9. The effect of colonic administration of L-Phenylalanine on food intake in mice in the early dark phase

Colonic administration of 10mM L-Phenylalanine led to a significant decrease in food intake in *ad libitum* fed mice in the early dark phase at 0-1 (Control: 0.475 ± 0.040g Vs. L-Phenylalanine: 0.328 ± 0.040g; p<0.05) , 2-4 (Control: 0.803 ± 0.040g Vs. L-Phenylalanine: 0.678 ± 0.04g; p<0.0001) and 0-4 hours (Control: 1.682 ± 0.066g Vs. L-Phenylalanine: 1.484 ± 0.084g; p<0.05) post administration compared to water treated controls (n=15-16, cross over study). After 24hours there was a trend for decreased food intake following colonic administration of L-Phenylalanine compared to control mice.

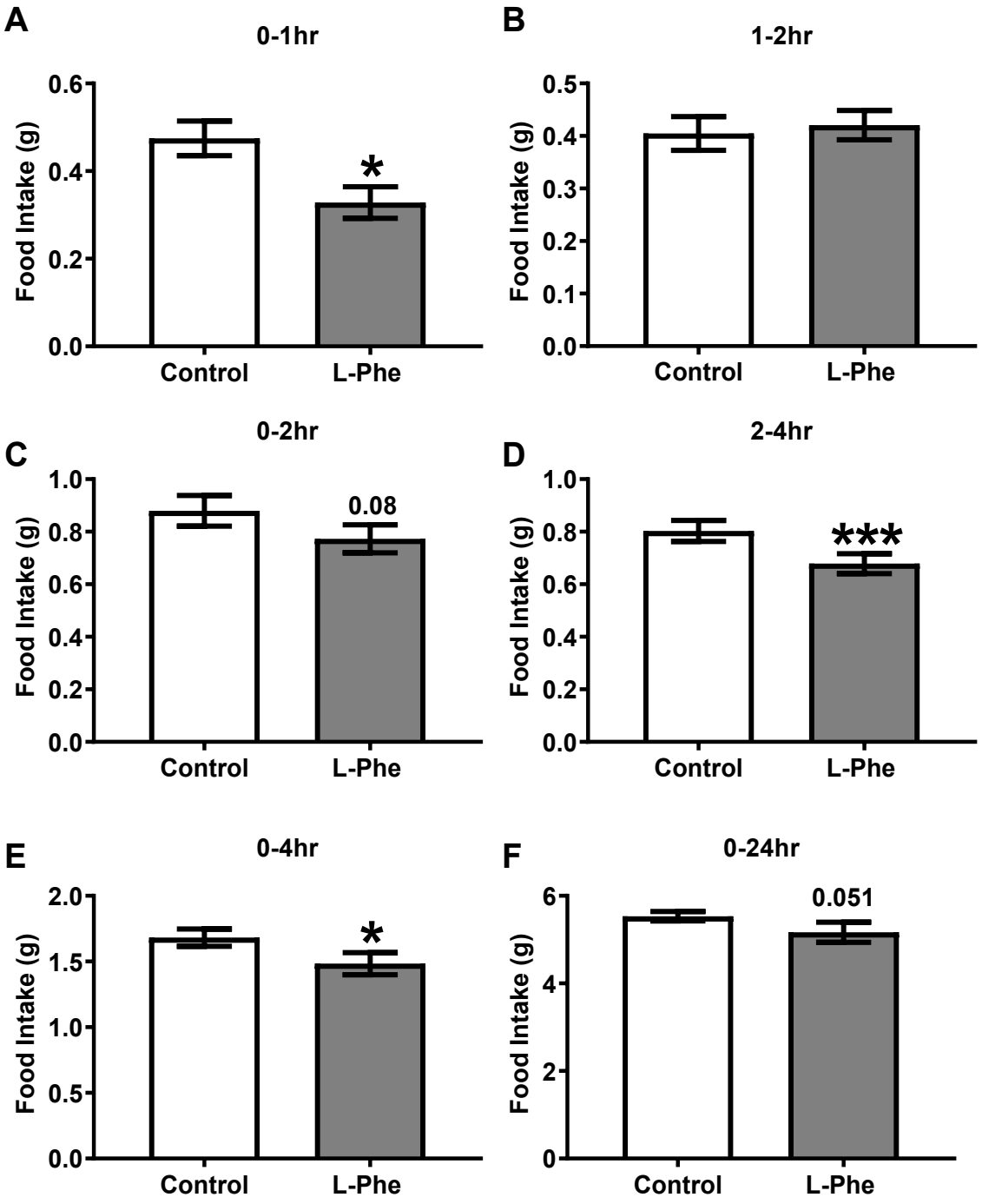


Figure 3.3.9.1 The effect of colonically administered L-Phenylalanine on food intake in *ad libitum* fed mice in the early dark phase.

The effect of colonically administering 0.1ml of 10mM L-Phenylalanine or water (control) on food intake in *ad libitum* fed mice at the onset of the early dark phase was measured at A) 0-1, B) 1-2, C) 0-2, D) 2-4, E) 0-4, and F) 0-24 hours post administration. Data expressed as mean ± SEM (n=16, crossed over). *p<0.05, ***p<0.001 Vs control.

3.3.10. The effect of colonic administration of L-Phenylalanine on c-Fos-like immunoreactivity in mice, quantified manually

Colonic administration of 10mM L-Phenylalanine results in a significant increase in c-Fos like immunoreactivity in the AP (L-Phenylalanine: median: 9, IQR: 7-12; Vs. Vehicle: median: 5, IQR: 3-6.5, $p<0.05$) of the brainstem. There was a trend for increased activation in the NTS and ARC but this did not reach statistical significance.

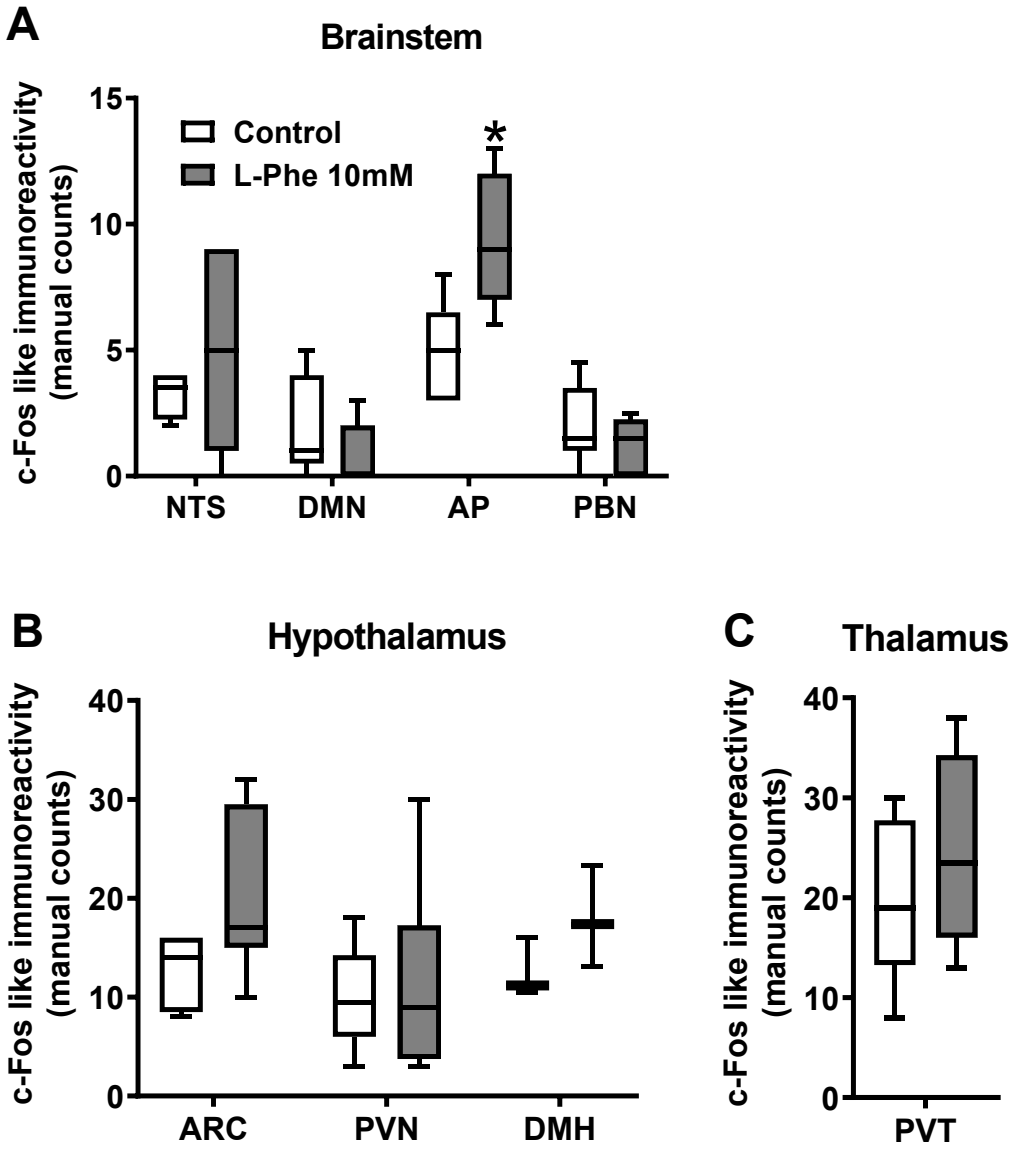


Figure 3.3.10.1 The effects of colonically administered L-Phenylalanine on c-Fos like immunoreactivity in the brain of mice, quantified manually.

C-Fos like immunoreactivity in the A) Brainstem (n=4-7), B) hypothalamus (n=3-8), and C) thalamus (n=6) of mice colonically administered 10mM L-Phenylalanine in the light phase was quantified manually. Data is expressed as median ± interquartile and total range. ARC, arcuate nucleus; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; NTS, nucleus tractus solitarius; DMN, dorsal motor nucleus; AP, area postrema; PVT, paraventricular nucleus of the thalamus. *adjusted p value <0.05.

3.3.11. The effect of colonic administration of L-Phenylalanine on c-Fos-like immunoreactivity in mice, quantified using an automated system

Colonic administration of 10mM L-Phenylalanine results in a non-significant increase in c-Fos like immunoreactivity in the AP and PVN.

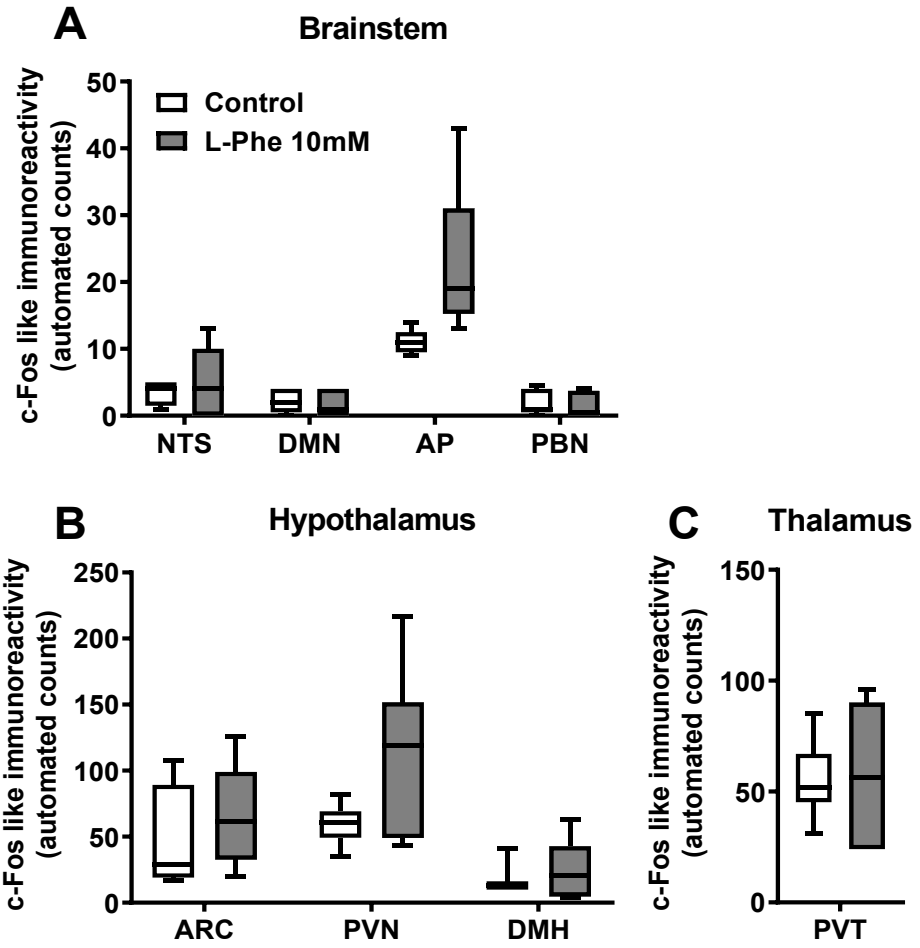


Figure 3.3.11.1 The effects of colonically administered L-Phenylalanine on c-Fos like immunoreactivity in the brain of mice, quantified using an automated system.

C-Fos like immunoreactivity in the A) brainstem (n=5-7), B) hypothalamus (n=3-6), and C) thalamus (n=5-6) of mice colonically administered 10mM L-Phenylalanine in the light phase was quantified using an automated system. Data is expressed as median ± interquartile and total range. ARC, arcuate nucleus; PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; NTS, nucleus tractus solitarius; DMN, dorsal motor nucleus; AP, area postrema; PBN, parabrachial nucleus; PVT, paraventricular nucleus of the thalamus.

3.3.12. The effect of colonic administration of L-Phenylalanine on plasma gut and pancreatic hormone levels in mice in the light phase

Colonic administration of 10mM L-Phenylalanine led to a significant increase in glucagon (Control: 45.04 ± 7.422pg/ml vs. L-Phe: 70.22 ± 5.082pg/ml) and insulin (Control: 71.56 ± 7.458pg/ml vs. L-Phe: 113.9 ± 17.4pg/ml) plasma levels in mice thirty minutes post-administration, compared to water treated controls. There was also a trend towards decreased GIP and increased PYY and PP, but these changes did not reach statistical significance. No difference in plasma levels of acyl ghrelin were detected.

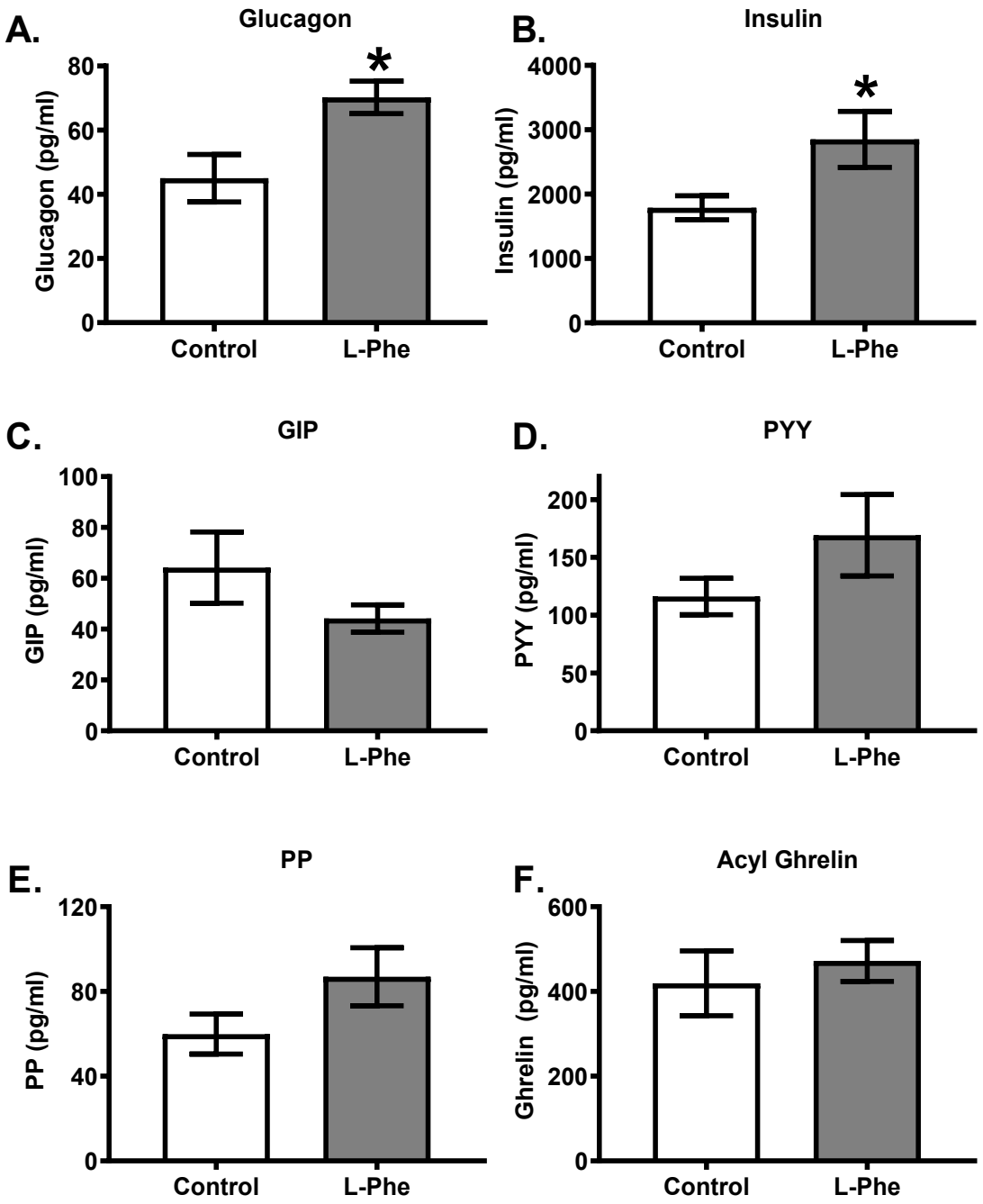


Figure 3.3.12.1 The effect of colonically administered L-Phenylalanine on gut hormone plasma levels.

The effect of administering 0.1ml of 10mM L-Phenylalanine or water control colonically on plasma A) glucagon, B) Insulin, C) GIP, D) PYY, E) PP and F) Ghrelin, thirty minutes post administration in mice, following a four hour fast. Data presented as mean ± SEM (A: n= 6-10; B: n=9-10; C: n= 8-10; D: n= 6-8; E: n=7-8; F: n=9-10). *$p<0.05$ Vs Control.

3.3.13. The effect of colonic administration of L-Phenylalanine on plasma GLP-1 levels

Mice fasted for 4 hours in the light phase were colonically administered 0.1ml of 10mM L-Phenylalanine. L-Phenylalanine had no significant effect on plasma GLP-1 levels 30 minutes post administration, as measured using the in-house RIA. GLP-1 RIA intra-assay variability and limit of detection was 4.2% and 1.2 pmol/L, respectively.

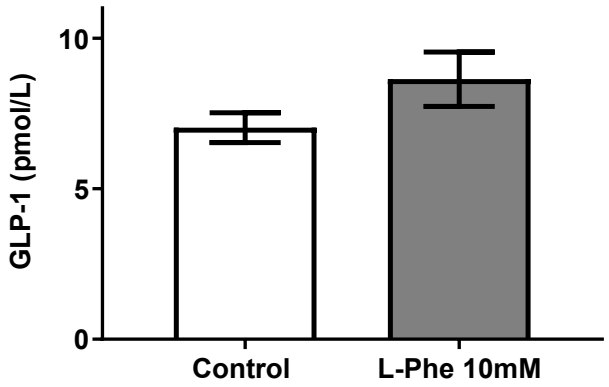


Figure 3.3.13.1 The effects of colonically administered L-Phenylalanine on plasma GLP-1 levels in mice.

Plasma GLP-1 levels in mice administered 10mM of L-Phenylalanine or water control were measured using the in-house radioimmunoassay. Data expressed as mean ± SEM (n=7).

3.3.14. The role of the calcium sensing receptor in mediating L-Phenylalanine induced GLP-1 and PYY release from murine primary colonic cells

L-Phenylalanine at a dose of 30mM significantly stimulated GLP-1 and PYY release from colonic crypts compared to vehicle control (0.75% DMSO) (Control: 4.98 ± 0.62 % GLP-1 release vs. L-Phe: 14.05 ± 2.10 %GLP-1 release) (Control: 7.55 ± 1.23 % PYY release vs. L-Phe: 17.99 ± 3.98 % PYY release). The effect of L-Phenylalanine on gut hormone release was non-significantly attenuated by 15µm NPS2390, a synthetic antagonist of the CaSR. Treatments with the antagonist alone had no significant effect on the GLP-1 or PYY release.

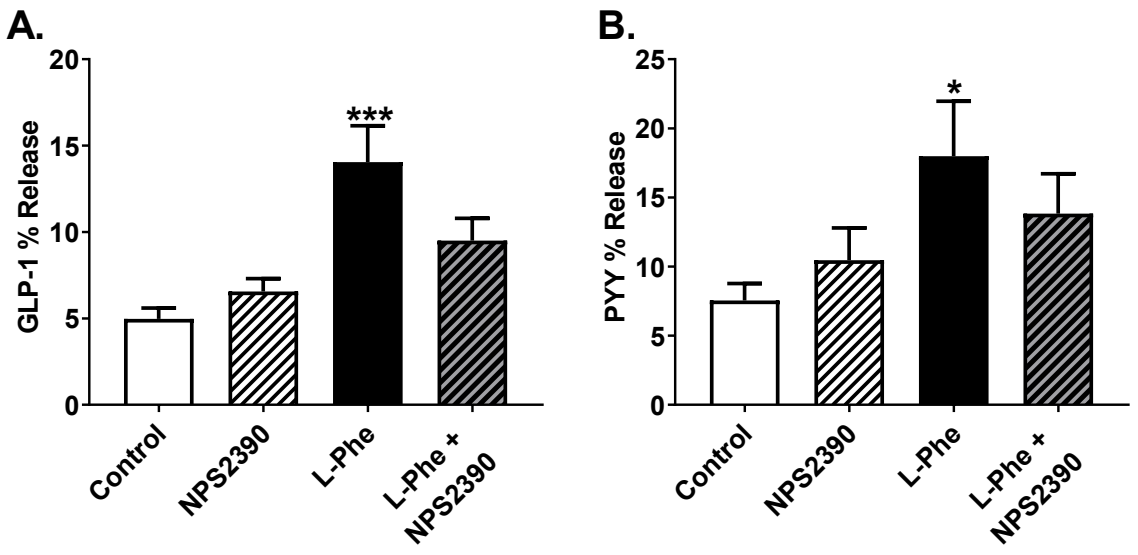


Figure 3.3.14.1 The role of the calcium sensing receptor in mediating L-Phenylalanine induced gut hormone secretion from murine primary colonic crypts.

The effect of 30mM L-Phenylalanine in the presence or absence of 15µM NPS2390 on A) GLP-1 (n=7) and B) PYY (n=5) release from murine primary colonic crypts, following a 2 hour incubation. Data presented as mean ± SEM. *p<0.05, ***p<0.001 Vs control.

3.4. Discussion

The mechanisms underlying L-Phenylalanine's anorectic effects were investigated by examining the effect of L-Phenylalanine on neuronal activation in the central nervous system and gut hormone secretion in the periphery. The role of the CaSR, an amino acid sensor, in mediating the secretion of appetite regulating hormones *in vitro* was also investigated. L-Phenylalanine was found to decrease food intake when given orally and colonically, increase energy expenditure, increase neuronal activation in the brainstem and stimulate the secretion of hormones; an effect mediated in part by the CaSR *in vitro*.

L-Phenylalanine's anorectic effects have been reported in humans (Ballinger and Clark 1994, Rogers and Blundell 1994), rhesus monkeys (Gibbs and Smith 1977) and rodents (Alamshah, Spreckley et al. 2017), though these effects are not consistent between studies (Pohle-Krauza, Navia et al. 2008, Jordi, Herzog et al. 2013). This study found oral administration of 6mmol/kg L-Phenylalanine to acutely supresses food intake and increase energy expenditure in rats, without causing signs of discomfort examined during behavioural studies, data published in (Alamshah, Spreckley et al.

2017). The increase in energy expenditure observed is most likely through L-Phenylalanine's effects on locomotion, which was significantly raised in behavioural studies and non-significantly raised in CLAMS experiments. The literature regarding rodent locomotor response to L-Phenylalanine is limited and inconclusive (Thurmond, Lasley et al. 1977, Mullenix, Tassinari et al. 1991). It is possible that the anorectic effects and the locomotor effects of L-Phenylalanine reflect different mechanisms, for example, reflecting peripheral effects on the gut hormone system, and direct sensing by the brain, respectively.

In vivo this study finds that orally administered L-Phenylalanine, at a dose of 12mmol/kg in *ad libitum* fed mice, is associated with a non-significant trend for increased GLP-1. Administration of 10mM L-Phenylalanine, the dose used by Mace et al. and Alamshah et al., directly into the colon of mice fasted for four hours in the early light phase, also stimulated PYY and GLP-1 secretion non-significantly (Mace, Schindler et al. 2012, Alamshah, Spreckley et al. 2017). The stimulation of GLP-1 and PYY by L-Phenylalanine is supported by Alamshah et al. that found administration of L-Phenylalanine in fasted rats to stimulate the secretion of GLP-1, when administered orally, and PYY, when administered directly into the ileum; and by Steinert et al that found intraduodenal administration of L-Phenylalanine stimulates PYY and, less potently, GLP-1 secretion in humans (Alamshah, Spreckley et al. 2017, Steinert, Ullrich et al. 2017).

L-Phenylalanine has been found to stimulate the secretion of GIP, GLP-1 and PYY from rat small intestinal loops, an effect mediated by the CaSR, an amino acid sensor (Mace, Schindler et al. 2012). This is supported by findings in this chapter where L-Phenylalanine was found to stimulate the secretion of GLP-1 and PYY from colonic crypt primary cell cultures, and this effect was blunted by antagonising the CaSR. Although this finding did not reach significance, a similar pattern with GLP-1 that did reach significance was seen in STC-1 cells and organoid cultures by other members of the group, published in (Alamshah, Spreckley et al. 2017). This suggests the CaSR is able mediate the secretion of anorectic gut hormones in response to L-Phenylalanine in the distal gut.

The CaSR has also been linked to the secretion of anorectic CCK. L-Phenylalanine stimulates the secretion of CCK *in vivo* (Ballinger and Clark 1994, Steinert, Landrock et al. 2015) and *in vitro* (Hira, Nakajima et al. 2008, Wang, Chandra et al. 2010, Liou, Sei et al. 2011). In humans, administration of 10g of L-Phenylalanine via a nasogastric tube stimulated a peak 5 fold increase in CCK 20 minutes post administration (~5.49pmol/L) (Ballinger and Clark 1994). Infusions of 7.28 pmol/L of CCK are sufficient to supress food intake in humans (Ballinger, McLoughlin et al. 1995). However, whether CCK is responsible for L-Phenylalanine's anorectic effects remains to be determined. *In vitro*, the CaSR has been suggested to mediate the secretion of CCK in STC-1 cells (Hira, Nakajima et al. 2008)

and I cells isolated from the mouse duodenum (Wang, Chandra et al. 2010, Liou, Sei et al. 2011). CCK release from STC-1 and mouse proximal small intestine cultures is also partly inhibited by T1R1/T1R3 antagonists (Daly, Al-Rammahi et al. 2013). Therefore, the CaSR seems to play a role in L-Phenylalanine's secretagogue effect in the proximal gut. However, there is redundancy in the system. It would be interesting to examine whether L-Phenylalanine's anorectic effects persist if CCK signalling is blocked.

The CaSR is crucial in calcium homeostasis, and therefore CaSR knockout models result in complex phenotypes that make it difficult to discern its effects on energy homeostasis. Global knock out models result in hyperparathyroidism, hypercalcinaemia, growth retardation and premature death (Chang, Tu et al. 2008); and gut epithelial CaSR knockout results in abnormal crypt structures and epithelial hyperproliferation (Rey, Chang et al. 2012). A gain of function mutation in the CaSR of mice does not affect food intake or body weight. However, it does result in altered islet size, α- to β- cell ratio, α-cell electrophysiology and glucagon response to glucose, as well as impaired insulin secretion and glucose tolerance (Gorvin, Rorsman et al. 2017). This suggests the CaSR also plays a potential role in glucose homeostasis.

In accordance to human studies (Guttler, Kuhl et al. 1978, Nuttall, Schweim et al. 2006, Steinert, Ullrich et al. 2017, Ueda, Sanbongi et al. 2017), this study found both oral and colonic administration of L-Phenylalanine to potently stimulate the secretion of glucagon, though particularly when orally administered. L-Phenylalanine's effects on insulin are modest in the literature in response to oral administration in humans (Guttler, Kuhl et al. 1978, Nuttall, Schweim et al. 2006), rats (Alamshah, Spreckley et al. 2017) and mice (Lin, Efanov et al. 2016). This effect may be mediated in part by GPR142, another aromatic amino acid sensor, in mice (Lin, Efanov et al. 2016). This study found a decrease in insulin secretion, twenty minutes after L-Phenylalanine oral loading in mice. This may be due to differences in the fed state, timing of the cull and dosage of L-Phenylalanine. In this study mice orally gavaged L-Phenylalanine were fed *ad libitum* unlike in Alamshah et al. and Lin et al. (Lin, Efanov et al. 2016, Alamshah, Spreckley et al. 2017). Moreover the dosage used in Lin et al. was approximately 2.5 fold greater (Lin, Efanov et al. 2016). This study did however see an increase in insulin secretion thirty minutes following colonic administration of 10mM L-Phenylalanine. This suggests the fed state and region of the gut targeted by L-Phenylalanine are relevant in determining the response elicited, and that responses may change at different timepoints. L-Phenylalanine administered directly into the colon of mice was also found to non-significantly increase PP levels. Increased PP levels in response to intra-gastric or intra-duodenal L-Phenylalanine has also been observed in humans (Taylor, Byrne et al. 1982) and in dogs (Beglinger, Haecki et al. 1981), respectively.

The ability of proteins and amino acids to stimulate the secretion of glucagon and insulin is well reported in the literature in dogs (Ohneda, Parada et al. 1968, Rocha, Faloona et al. 1972), sheep (Kuhara, Ikeda et al. 1991) and humans (Guttler, Kuhl et al. 1978, Schmid, Schusdziarra et al. 1989, Schmid, Schulte-Frohlinde et al. 1992, Nuttall, Schweim et al. 2006). The secretion of glucagon correlates well with the appearance of amino acids in circulation following a protein load and amino acid/protein induced insulin secretion is potentiated by glucose (Calbet and MacLean 2002). Both glucagon and insulin have anorectic effects, however, amino acids and proteins are thought to stimulate their secretion with the purpose of promoting amino acid utilisation without concurrent hypoglycaemia (Unger, Ohneda et al. 1969).

Amino acids can modulate pancreatic activity indirectly through vagal networks, neuropeptides, central sensing and gut hormones. Alternatively they can directly act on the pancreas, for example, amino acid transport through sodium coupled transporters can depolarise the membrane of β-cells and stimulate insulin release (Newsholme, Brennan et al. 2006). It has been suggested that L-Phenylalanine is able to stimulate the secretion of pancreatic enzymes via neuronal, cholinergic dependent, and hormonal, CCK mediated, pathways (Owyang, Louie et al. 1986, Owyang, May et al. 1986). Moreover, pancreatic polypeptide secretion in response to duodenal administration of L-Phenylalanine in dogs is inhibited by atropine, suggesting the cholinergic system is also involved in mediating pancreatic hormone secretion in response to L-Phenylalanine (Beglinger, Haecki et al. 1981). Whether similar pathways are involved in L-Phenylalanine induced secretion of insulin and glucagon requires further elucidation.

The CaSR is expressed in EECs (Symonds, Peiris et al. 2015), the enteric nervous system (Chattopadhyay, Cheng et al. 1998, Cheng 2012), vagal neurons (Egerod, Petersen et al. 2018) and in the pancreas in islets, acinar and duct cells (Bruce, Yang et al. 1999, Rasschaert and Malaisse 1999, Rácz, Kittel et al. 2002). Whether it plays a role L-Phenylalanine's effects on pancreatic hormone secretion also remains to be investigated.

Overall, the role of the CaSR in mediating L-Phenylalanine's effects should be investigated *in vivo* through tissue specific conditional knock out models.

The central nervous system receives both hormonal and neuronal inputs from across the periphery, including the gastrointestinal tract and adipose tissue; it integrates this information and coordinates an appropriate response to regulate energy homeostasis. It is also able to directly sense amino acids in circulation. To the author's knowledge, this is the first study examining the effect of L-Phenylalanine on the activation of appetite regulating centres of the brain.

In this study neuronal activation was measured using c-Fos like immunoreactivity as a marker. C-Fos is an early gene transcription factor that is transiently expressed in activated neurons. The number of cells positive for the Fos protein was measured either manually, using the cell counting tool on Fiji, or with an automated macro written by Steve Rothery. The macro takes into consideration the size and intensity of stained regions to classify the cells as c-Fos reactive or non-reactive. Both methods showed similar patterns of neuronal activation. However, the automated method detected a greater number of c-Fos reactive cells. This may be because it is more sensitive to lower levels of activation or background noise. The latter is easier to discern when counting manually and may explain the increased significance observed in manually counted nuclei. Nevertheless, with further adjustments, the automated macro may provide a less subjective, more controlled method of c-Fos like immunoreactivity quantification.

Administration of L-Phenylalanine increased neuronal activation in the dorsal vagal complex, particularly the NTS and AP of rats and mice administered L-Phenylalanine orally, and the AP of mice colonically administered L-Phenylalanine. The dorsal vagal complex is formed by a group of nuclei important in appetite regulation: the NTS, AP and DMN, which are described in more detail in chapter 1. Briefly, the NTS receives and integrates gustatory, mechanical and chemical sensory information from the GI tract, as well as inputs from the hypothalamus (Katz, Nicolelis et al. 2000). It projects to the DMN and modulates its activity. The DMN is a major source of vagal efferent neurons that innervate the GI tract, mediating motility and secretion, and the pancreas, regulating pancreatic secretion. It too receives innervation from the periphery, CNS and other nuclei in the brainstem (Mussa and Verberne 2013). L-Phenylalanine has been shown to stimulate the secretion of gut hormones that activate the vagus nerve. Consequently, activation of these nuclei could be downstream of vagal afferent nerve activity in response to gut hormone sensing.

The AP is a circumventricular organ located above the NTS. Its activation could be through direct sensing of circulating L-Phenylalanine, following absorption. However, it is worth noting that the dose of L-Phenylalanine administered colonically is significantly lower and is unlikely to raise circulating levels greatly. Therefore, it is more likely activated indirectly through vagal afferent signalling, NTS signalling or the detection of hormones. The AP is also a chemoreceptor trigger, associated with emesis, raising the possibility that L-Phenylalanine's anorectic effects are due to malaise. However, behavioural studies failed to show any signs of discomfort in response to orally administered L-Phenylalanine, suggesting this is not the case. Moreover, the parabrachial nucleus of the brainstem, thought to be involved in the detection of noxious stimuli (Palmiter 2018), was not activated in mice colonically administered L-Phenylalanine However, administration of L-Phenylalanine to animals that display an emetic response may be more telling.

Greater neuronal activation in the NTS was observed following oral rather than colonic administration of L-Phenylalanine. The vagus innervates the upper GI tract more densely, in comparison to the colon, therefore, agents administered in the upper GI tract may be better at stimulating vagal activity and downstream NTS activation (Berthoud, Kressel et al. 1992, Kressel, Berthoud et al. 1994, Wang and Powley 2000). Moreover, L-Phenylalanine is known to stimulate the secretion of CCK, which activates vagal afferents (Joyner, Smith et al. 1993). CCK expressing EECs are expressed in the upper gut. These are unlikely to be stimulated by colonically administered L-Phenylalanine. On the other hand, colonic L-Phenylalanine was shown to stimulate PYY non-significantly. Whether PYY signals directly to the brain or indirectly via the vagus nerve is still contested. Future work should examine the importance of the vagus nerve and AP in mediating the effects of L-Phenylalanine. Moreover, dual staining to identify the type of neurons activated by L-Phenylalanine administration may help reveal its mechanism of action.

Neuronal activation in the AP and NTS, has also been seen in response to other amino acids including L-Lysine, L-Arginine, L-Glutamic acid (Jordi, Herzog et al. 2013) and L-Cysteine (McGavigan, O'Hara et al. 2015). The appetite supressing effects of L-Arginine and L-Glutamic acid are lost following AP destruction suggesting their appetite regulating effects are centrally mediated. However, whether this is due to direct sensing centrally, peripheral neuronal sensing or downstream of hormonal signalling is not clear. Findings by (Alamshah, McGavigan et al. 2016) suggest that L-Arginine suppresses appetite by directly acting on the brain as its anorectic effects are not mediated by gut hormone secretion nor vagal signalling, and because central administration of L-Arginine decreases food intake. However, whether this is the case with L-Phenylalanine requires further investigation.

In mice, colonic administration of L-Phenylalanine decreased food intake acutely at 0-1 and 2-4 hours post administration. Gut hormone and neuronal activation studies in this chapter investigated the early response, not the delayed 2-4hour effect. The initial suppression of food intake may be mediated by activation of appetite regulating centres such as the AP and the secretion of hormones such as PYY. Even though GLP-1 and PYY secretion in response to L-Phenylalanine did not reach significance in *in vivo* studies, it did *in vitro* in primary colonic crypts. This could be further investigated by, for example, examining the anorectic effects of colonic L-Phenylalanine following PYY and GLP-1 signalling blockade or AP lesions.

The delayed response at 2-4hours may be due to the absorption and detection of L-Phenylalanine and/or its metabolites by neurons found in the gut (vagal terminals, submucosal plexus and myenteric plexus) or in the central nervous system. The latter would be surprising considering the

small dose used. It would have been interesting to measure c-Fos immunoreactivity three and a half hours after colonic administration of L-Phenylalanine to examine the differences in neuronal activation between the early and delayed food intake responses observed. Additionally, it would have been interesting to track L-Phenylalanine absorption, metabolism and distribution following colonic administration, via radio-labelled L-Phenylalanine, to determine its site of action. Moreover, future studies should utilise D-Phenylalanine as a control. This was not possible at the doses given orally due to solubility problems but should be tested for doses given rectally.

In conclusion, the data in this chapter supports a role for L-Phenylalanine in appetite suppression when administered into the upper and lower GI tract of rodents. This anorectic effect does not appear to be due to discomfort, however, this is difficult to demonstrate conclusively in rodents. Furthermore, this work shows that L-Phenylalanine stimulates the secretion of pancreatic and gut hormones, and that the secretion of appetite regulating gut hormones may be mediated by the CaSR. Additionally, I show that administration of L-Phenylalanine increases neuronal activation in the brainstem. Whether this is direct, downstream of vagal sensing or downstream of gut hormone secretion is not clear from the studies described in this chapter.

Chapter 4: The Role of the CaSR in Vagal Afferents in Appetite Regulation and Glucagon Secretion

4.1. Introduction

4.1.1. Vagus nerve

The vagus nerve is the 10th out of 12 cranial nerves and is a major source of extrinsic GI tract innervation. It consists of a pair of parasympathetic nerves, compromised of both afferent sensory and efferent motor pathways. The proportion of vagal afferent and efferent neurons varies across the vagal branches. However, vagal afferents consistently outnumber vagal efferents in rats (~70% vs ~30%) (Prechtl and Powley 1990). Sensory pathways relay mechanical, chemical, thermal and osmotic sensory information, from peripheral organs, including the heart, lungs, liver, pancreas and GI tract, to the NTS and, infrequently to the AP (Norgren and Smith 1988, Berthoud and Neuhuber 2000, de Lartigue 2016). Efferent neurons originate in the DMN and nucleus ambiguus (Norgren and Smith 1988).

Vagal afferent neurons are pseudo bipolar and have the majority of their cell bodies in the nodose ganglia, which are located bilaterally at the cervical level, below the jugular foramen (Norgren and Smith 1988). Unlike in humans, the nodose ganglia in mice are fused with the jugular ganglia. For sake of simplicity, this jugular/nodose ganglia complex will be referred to as 'NG' throughout (Kaelberer and Jordt 2016).

The NG is heterogeneous in its cellular composition. Most neurons are type C fibres; these neurons have small diameter axons that are unmyelinated and propagate slow rise but sustained electrical potentials. A type fibres are significantly less frequent and are myelinated (Schild, Clark et al. 1994). The NG also contains Schwann cells, microglia, endothelial cells and satellite glial cells (Waise, Dranse et al. 2018).

The cervical branches of the vagus nerve are important in regulating the cardio-respiratory system. They innervate various tissues including the pharynx, larynx, acoustic meatus, bronchi of the lungs, aortic arch, heart and oesophagus (see figure 4.1.1.1) (Berthoud and Neuhuber 2000, de Lartigue 2016). The two vagal trunks (dorsal and ventral) enter the abdomen where they divide into the sub-diaphragmatic branches, which are important in metabolism. The different sub-diaphragmatic branches are illustrated in figure 4.1.1.1. In brief, they include the ventral and dorsal gastric branch which innervate the stomach and proximal duodenum; the celiac and accessory celiac branches that innervate the distal duodenum and the remaining levels of the gut, except the rectum; the ventral common hepatic branch innervating the liver, portal vein and biliary tracts; and the gastro-duodenal and pyloric branches that innervate the lower parts of the stomach, pyloric sphincter, proximal duodenum and pancreas (Altschuler, Escardo et al. 1993, Berthoud and Neuhuber 2000). In addition,

the accessory celiac branch arising from the left cervical vagus/ventral trunk also innervates the liver weakly (Berthoud and Neuhuber 2000).

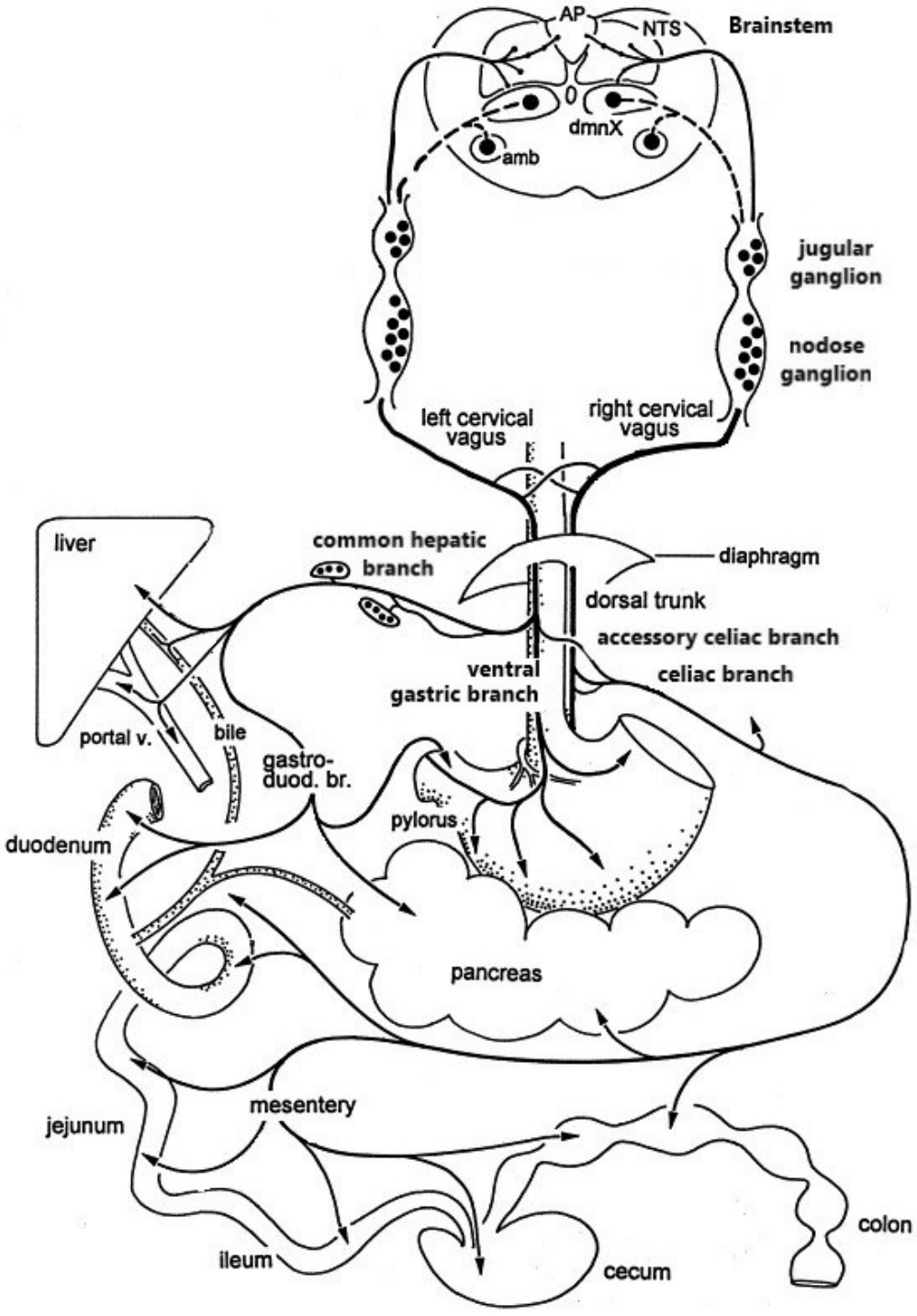


Figure 4.1.1.1 Sub-diaphragmatic branching of the vagus nerve in rats. Adapted from (Berthoud and Neuhuber 2000).

The vagus nerve has both mechanoreceptors and chemoreceptors providing information on the volume and composition of food, respectively. Vagal afferent mechanoreceptors in the stomach are activated by gastric distension which results in increased NTS activity and stimulation of meal termination, irrespective of the macronutrient type (Mathis, Moran et al. 1998, de Lartigue 2016). This results in a volumetric dependent, vagally mediated, negative feedback on food intake. On the other hand, chemosensing by vagal afferents in the duodenum are thought to be involved in a nutrient composition-mediated negative feedback (Powley and Phillips 2004).

Vagal mucosal terminals in the intestine are predominant in the submucosa and can be close to EECs and the lamina propia and are thus well situated to detect absorbed nutrients and secreted hormones. This contrasts with gastric vagal afferents that primarily innervate the myenterix plexus and intramuscular arrays. Gastric mucosal endings are less abundant. Nevertheless, the existence of vagal afferents in the gastric mucosa, and the secretion of hormones by the stomach that bind to receptors expressed in the NG (eg leptin and ghrelin), suggests gastric vagal afferents may also act as chemosensors, not just mechanosensors as classically thought (Powley and Phillips 2004, Berthoud 2008).

Direct infusion of nutrients into the duodenum increases vagal firing. Different nutrients give rise to different patterns of activation, with peptones increasing vagal spike activity more than glucose, suggesting vagal afferents are able to detect food composition (Schwartz and Moran 1998). These effects can be due to direct sensing of absorbed nutrients or secondary to the detection of gut hormones (Lal, Kirkup et al. 2001).

4.1.1.1. Hormone receptors in the vagus

The vagus nerve expresses a variety of hormone receptors including CCK-A, GLP-1R, GHSR, Y_2, leptin receptor, OX-1R (orexin receptor), 5-HT_3 (serotonin receptor), and MC4R (Gautron, Lee et al. 2010, Egerod, Petersen et al. 2018, Waise, Dranse et al. 2018)

CCK signalling in the vagus has been extensively explored, as discussed in the introduction. Briefly, CCK stimulates activity of vagal afferents and increases c-Fos activation in the NTS; its anorectic effects are lost following vagotomy (Joyner, Smith et al. 1993). Antagonism of CCK-A, expressed in the vagus and not centrally, increases food intake (Moran, Ameglio et al. 1993, Beglinger, Degen et al. 2001), and mice lacking CCK-A are resistant to CCK's anorectic effects and eat larger meals, without altering overall food intake (Kopin, Mathes et al. 1999).

CCK and leptin act synergistically in vagal afferent neurons. Leptin receptor signalling modulates vagal afferent CCK sensitivity. Without leptin signalling, CCK's anorectic effects are lost at lower concentrations (de Lartigue, Barbier de la Serre et al. 2012, de Lartigue, Ronveaux et al. 2014).

The leptin receptor in the vagus is also thought to regulate mechanosensitivity of gastric vagal afferents, depending on the fed status of the mouse (Kentish, O'Donnell et al. 2013). Moreover, mice lacking the leptin receptor in the NG display increased fat mass, weight gain, meal duration and meal size, suggesting leptin signalling in vagal afferents may play a role in meal termination (de Lartigue, Ronveaux et al. 2014).

4.1.1.2. Nutrient sensing receptors in the vagus

In addition to hormone receptors, the vagus nerve expresses a limited number of nutrient sensing GPCRs, suggesting the vagus may be able to directly detect absorbed nutrients. The expression of four metabolite sensors have been reported: the CaSR, the aromatic amino acid metabolite sensor GPR35, the short chain fatty acid receptor FFAR 3, and the lipid metabolite sensor GPR119 (Egerod, Petersen et al. 2018). Expression of the long-mid length fatty acid receptor GPR 40, not detected by Egerod et al., most likely due to its low expression, has also been observed in 7% of NG neurons (Darling, Zhao et al. 2014, Egerod, Petersen et al. 2018). *In vitro*, the NG is activated by GPR40 endogenous agonists (Darling, Zhao et al. 2014), supporting a potential role for vagal afferents in direct nutrient sensing. However, whether vagal activity is modulated *in vivo* by direct GPR40 sensing and signalling remains a question.

Ablation of Nav1.8 neurons results in a decreased CaSR expression in the nodose, suggesting the CaSR is expressed in Nav1.8 positive neurons (Egerod, Petersen et al. 2018). Nav 1.8 is expressed in >75% of sensory vagal neurons in the NG (Gautron, Sakata et al. 2011). Moreover, the CaSR has also been identified in rats where it modulates the sensitivity of TRPV-1 channels *in vitro* in bronchopulmonary vagal neurons (Gu, Vysotskaya et al. 2013). Therefore, it may be possible for the CaSR to modulate the activity/sensitivity of other branches of the vagus nerve.

In addition to the receptors mentioned above, there may be other mechanisms for sensing nutrients. For example, glucose-excited and glucose-inhibited neurons have been identified in the NG. These neurons can sense glucose, through its downstream metabolism which modulates membrane potential (Grabauskas, Song et al. 2010). Interestingly, glucose sensing by vagal neurons has been show to modulate the density of serotonin receptors in vagal afferents innervating the GI tract (Babic, Troy et al. 2012).

4.1.1.3. Vagal plasticity

The nutritional status of rodents induces characteristic changes in the expression of peptides and receptors in the NG, highlighting its plasticity. Prolonged fasting decreases the expression of the anorectic receptor Y_2 and peptide CART and increases the expression of orexigenic peptide MCH and

cannabinoid receptor (CB1) in the NG of rodents. This phenotypic switch is thought to be mediated by CCK, and is potentiated by leptin and inhibited by ghrelin signalling (Burdyga, Lal et al. 2004, Burdyga, Varro et al. 2006, de Lartigue, Dimaline et al. 2007, Burdyga, de Lartigue et al. 2008, de Lartigue, Barbier de la Serre et al. 2012, de Lartigue, Ronveaux et al. 2014). Consequently, rodents with leptin receptor deficiency do not undergo this phenotypic switch when fasted (de Lartigue, Barbier de la Serre et al. 2012, de Lartigue, Ronveaux et al. 2014).

The macronutrient composition of diets is also able to modify receptor expression in the NG. For example, prolonged HPDs in mice decrease the expression of the orexigenic receptor OX-1R (Journel, Chaumontet et al. 2012). On the other hand, HFD in mice increases the expression of OX-R1/2 and decreases the expression of CCK-A (Nefti, Chaumontet et al. 2009). The ability of the vagus to change chemical and mechanical sensitivity in response to nutrient availability and content provides another level of fine tuning and complexity to the vagal regulation of food intake. Moreover, vagal neurons have also been found to form synapses with EEC/neuropod cells, allowing for a more rapid transmission of nutrient availability to the CNS (Kaelberer, Buchanan et al. 2018).

4.1.1.4. Proteins and vagal signalling

Prolonged high protein intake increases c-Fos expression in the NTS, the main site of central vagal afferent innervation (Darcel, Fromentin et al. 2005, Faipoux, Tomé et al. 2008). This suggests the vagus may play a role in the effects of HPDs. However, sub-diaphragmatic vagotomy (severing the vagus) failed to inhibit the anorectic effects of a HPD in rats; on the contrary, they ate even less (Rampin, Larue-Achagiotis et al. 2003). However, this could be due to loss of efferent signalling and redundancy in the system, and does not conclusively rule out vagal sensing in mediating the effect of a HPD on food intake and energy homeostasis.

The effects of amino acids on vagal activation have also been investigated. Tanaka et al. infused the portal vein of rats with Alanine and Lysine, at physiological concentrations reflecting the absorption of amino acids into circulation, without the confounding effects of gut hormones. Both Lysine and Alanine were found to stimulate the activation of vagal neurons in the hepatic branch (Tanaka, Inoue et al. 1990), suggesting a role for the vagus in amino acid sensing. Later studies examining the effects of other amino acids on hepatic vagal activity found that amino acids could be either excitatory or inhibitory. The mechanism underlying these effects were unclear, but the latency period (10-20 minutes) suggested that they may be indirect in rats (Niijima and Meguid 1995).

On the other hand, intra-duodenal amino acid infusions in cats stimulated vagal activity with a latency of 9s, hinting at direct vagal afferent stimulation through chemosensing or neuronal signals from neuropods (Jeanningros 1982). Some of the responses were amino acid and neuron specific,

suggesting the possible existence of a heterogeneous population with the ability to discern amino acids.

Moreover, Lysine's anorectic effects in rats, but not Arginine's, are lost following capsaicin induced vagal lesions, which are more selective for afferent neurons (Jordi, Herzog et al. 2013). Therefore, these amino acid sensing neurons, may be important in appetite regulation. However, whether this is through direct vagal nutrient sensing, or indirectly via other signals such as gut hormones, is unclear. Nevertheless, as mentioned previously, the vagus does express the machinery to directly detect aromatic amino acids and their metabolites (Egerod, Petersen et al. 2018). Therefore, investigating the role of these receptors in appetite and glucose homeostasis would be interesting.

It is worth noting that capsaicin induced vagal lesions can also damage neurons in the CNS, which may play a role in Lysine's appetite regulating effects. Additionally, capsaicin fails to target all vagal afferents and damages some vagal efferent fibres (Browning, Babic et al. 2013, Udit and Gautron 2013).

4.1.1.5. Vagal regulation of pancreatic secretion and the entero-pancreatic reflex

Parasympathetic vagal efferent neurons are thought to be involved in the regulation of endocrine and exocrine pancreatic secretions. They originate in the DMN which is heavily regulated by the NTS, which receives sensory vagal information from peripheral organs including the gut; the DMN is also innervated by the AP and hypothalamic nuclei (Loewy, Franklin et al. 1994, Streefland, Maes et al. 1998, Buijs, Chun et al. 2001). This pathway, in which sensory signals from the gut can mediate changes in pancreatic secretion via vagal efferents, is referred to as the vago-vagal entero-pancreatic reflex. Its existence was alluded to over a century ago by Pavlov (Pavlov 1903). However, it wasn't until several decades later that it was investigated further.

The role of this pathway in the secretion of the exocrine pancreas has been extensively studied. Amino acids, particularly the aromatic amino acids L-Phenylalanine and L-Tryptophan are potent stimulants of pancreatic exocrine secretion (Meyer, Kelly et al. 1976). Studies examining the effect of intraduodenally administered L-Tryptophan suggest the pancreatic response to low nutrient loads is primarily regulated by the entero-pancreatic vagal reflex, and the response to high nutrient loads is hormonally mediated by CCK, for example (Vazquez-Echarri, Baumgartner et al. 1986, Singer, Niebel et al. 1989, Niebergall-Roth, Teyssen et al. 1997). L-Phenylalanine is also thought to stimulate the secretion of pancreatic enzymes via neuronal, cholinergic dependent, and hormonal, CCK-mediated, pathways (Owyang, Louie et al. 1986, Owyang, May et al. 1986).

The vagus is also important in mediating pancreatic endocrine secretion. In humans, truncal vagotomy results in a blunted glucagon response to insulin-induced hypoglycaemia, and a blunted insulin response to oral glucose, compared to selective vagotomy that preserves the hepatic and celiac branches of the vagus (Russell, Thomson et al. 1974). Impaired insulin response following truncal vagotomy is also reported in other studies, which also found vagotomised patients to hyper-secrete glucagon in response to oral glucose (Sudo, Ishiyama et al. 1982).

Electrical stimulation of rat vagal efferents, with cell bodies in the DMV, stimulates the secretion of insulin and glucagon, via the hepatic and gastric branch in rats (Berthoud, Fox et al. 1990). Moreover, stimulation of the DMN increases the secretion of insulin and glucagon, an effect blocked by atropine or vagotomy (Jeanrenaud, Ionescu et al. 1983, Laughton and Powley 1987, Mussa, Sartor et al. 2011). Likewise, stimulation of the NTS, which regulates DMN activity, also promotes insulin secretion (Jeanrenaud, Ionescu et al. 1983). Interestingly, even a small number of parasympathetic preganglionic neurons can mount a significant insulin and glucagon response (Berthoud, Fox et al. 1990).

The vagus has phenotypically and functionally distinct subpopulations. Pancreas-projecting vagal efferent neurons from the DMV can respond to either GLP-1 or PP, but not both (Wan, Coleman et al. 2007). Microinjections of GLP-1 into the DMV stimulate insulin secretion, whereas PP microinjections decrease pancreatic exocrine secretion, without altering basal insulin secretion (Krowicki and Hornby 1995, Okumura, Pappas et al. 1995, Babic, Browning et al. 2012). However, PP microinjections do potentiate glucose-stimulated insulin secretion in rats (Krowicki and Hornby 1995). The existence of functionally distinct subpopulations is supported by the existence of specific vagal branches that mediate the secretion of pancreatic hormones (Berthoud, Fox et al. 1990), at specific frequencies (Berthoud and Powley 1987).

Proteins and amino acids stimulate the secretion of glucagon and insulin in dogs (Ohneda, Parada et al. 1968, Rocha, Faloona et al. 1972), sheep (Kuhara, Ikeda et al. 1991) and humans (Guttler, Kuhl et al. 1978, Schmid, Schusdziarra et al. 1989, Schmid, Schusdziarra et al. 1989, Schmid, Schulte-Frohlinde et al. 1992, Nuttall, Schweim et al. 2006). L-Phenylalanine in isolation also stimulates the secretion of glucagon (Rocha, Faloona et al. 1972, Guttler, Kuhl et al. 1978, Nuttall, Schweim et al. 2006, Steinert, Ullrich et al. 2017, Ueda, Sanbongi et al. 2017). Whether L-Phenylalanine's effects on glucagon secretion are vagally mediated, like its effects on the exocrine pancreas, is unclear. A study examining the effect of celiac or hepatic branch vagotomy on the ability of amino acids to stimulate insulin and glucagon secretion was inconclusive with regards to L-Phenylalanine as they failed to show a pancreatic hormone response to L-Phenylalanine in sham vagotomised rats (Tanaka, Inoue et

al. 1993). However, the study did find altered glucagon and insulin responses to alanine and leucine, respectively, in vagotomised (hepatic branch) rats (Tanaka, Inoue et al. 1990, Tanaka, Inoue et al. 1993). Further studies are required to establish the role of vagal afferent protein/amino acid sensing on pancreatic endocrine secretion.

4.1.2. Hypothesis and aims

The vagus nerve is known to modulate food intake and pancreatic secretion. It expresses a limited number of nutrient sensors, including the CaSR, whose function in the vagus is unknown. The CaSR is modulated by L-Phenylalanine. Findings from the previous chapters found orally administered L-Phenylalanine to activate neurons in the NTS, a site of vagal afferent innervation, and DMN, where vagal efferents arise. Therefore, this study was interested in investigating whether vagally-expressed CaSR could modulate any of L-Phenylalanine's pancreatic secretagogue or appetite-suppressing effects.

Hypothesis

Vagally expressed CaSR mediates the anorectic and pancreatic secretagogue effects of L-Phenylalanine in mice.

Aims

To investigate:

1. The ability of adeno-associated viruses expressing cre recombinase to knockdown the CaSR in the NG of CaSR floxed mice
2. The effect of NG CaSR knockdown on L-Phenylalanine's anorectic effects and ability to stimulate the secretion of pancreatic hormones.

4.2. Methods

4.2.1. Animals

Mice were housed in individually ventilated cages under temperature controlled (21-25ºC) and light controlled (12 hours light; 12 hours dark) conditions. Mice were given *ad libitum* access to RM3 diet (Special Diet Services Ltd, Witham, Essex, UK) during breeding and RM1 diet (Special Diet Services Ltd, Witham, Essex, UK) thereon after, unless otherwise stated.

All animal procedures were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, the project license number 70/8068 and with the approval of the Central Biomedical Services unit at Imperial College London.

4.2.1.1. Generation of CaSR floxed mice

CaSR floxed mice were kindly donated by Wenhan Chang. Details regarding the generation of these mice can be found in (Chang, Tu et al. 2008). Mice were bred from CaSR homozygous floxed parents.

4.2.1.2. Genotyping of CaSR floxed mice

Ear tissue biopsies (<2mm^2), obtained using a sterile ear puncher, were digested using 50µl of proteinase K digestion mix (appendix 2). Samples were then heated in a thermal cycler (Veriti™ 96-Well Thermal Cycler, Applied Biosystems) for 40 minutes at 60ºC to increase the activity of the enzyme and 10 minutes at 95ºC to inactivate the enzyme. Samples were stored at -20ºC until further use.

CaSR floxed mice were discriminated from wild type mice using the primers in table 4.2.1.1.

	Sequence
P3U	5'-GTG ACG GAA AAC ATA CTG C-3'
P3L	5'-CGA GTA CAG GCT TTG ATG C-3'

Table 4.2.1.1 CaSR genotyping primer sequences. (Chang, Tu et al. 2008)

Three µl of DNA, obtained from the proteinase digestion, were added to 22µl of the mastermix (appendix 2). Using a thermocycler, the DNA was initially denatured at 94ºC for 3 minutes. The samples were subsequently cycled 40 times at 94ºC (1 minute), 58ºC (1 minute) and 72ºC (1 minute) before being incubated at 4ºC until removal.

Three µl of 6 x DNA loading buffer (Appendix 2) were added to the amplified DNA that was run on a 2% agarose Type-II A gel. The agarose gel was made in 1 x TAE buffer (appendix 2) containing

0.5µg/ml of ethidium bromide. The gel was run in 1 x TAE at 130mV for 50 minutes. A 1kb plus ladder (Invitrogen) was used to determine the approximate size of the DNA bands and therefore genotype of the mouse. A 167bp band signified the presence of the floxed cassette and a 133bp band corresponded to a WT allele; no floxed cassette.

4.2.2. CaSR immunoreactivity in the NG

NG extracted from mice were fixed in 4% PFA for 2.30hrs at 4ºC, transferred to 70% ethanol, and sent for paraffin embedding and sectioning by Marokh Nohadani. Slides were deparaffinised and rehydrated by undergoing washes in xylene followed by decreasing concentrations of ethanol, respectively. Heat and enzymatic antigen retrieval were used to unmask the epitope. First, samples were boiled in 10mM citrate solution pH 6 (appendix 2) for 20minutes using a rice cooker, then, after being rinsed in GDW followed by 1x tris-buffered saline (TBS) + 0.1% Triton X-100 (TTBS) (appendix 2), they were delineated using a wax pen. Next, samples underwent enzymatic antigen retrieval. Briefly, the samples were pre-warmed to 37ºC in 2M HCl for 20 minutes before being rinsed and incubated in 0.05% working trypsin solution (appendix 2) for 15 minutes at 37ºC.

Slides were then washed three times for 5 minutes in TTBS and blocked for 2 hours in blocking solution (TTBS containing 4% goat serum, 1% BSA, 0.05% tween20 and 0.1% gelatine) to minimise non-specific binding. Slides were incubated in a humidifying chamber at 4ºC overnight in a 1:200 dilution of rabbit IgG anti-CaSR antibody (NSJ Bioreagents, Suffolk) made in blocking solution.

The following day slides were washed and incubated with a goat anti-rabbit IgG Alexa Fluor 488 secondary antibody at a 1:1000 concentration, diluted in TBS 1% BSA, for 1 hour at room temperature. Following staining slides were washed in PB no saline (appendix 2) before being stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, Poole, UK) and cover-slipped. Slides were stored at 4ºC in the dark until visualisation using a Zeiss AxioObserver widefield microscope from the Imperial College FILM Facility.

4.2.3. Quantification of CaSR expression in the NG using RT-qPCR

The NG of CaSR homozygous floxed male mice were bilaterally extracted, frozen using liquid nitrogen and stored at -80ºC until further use.

RNA from individual NG was extracted using TRIsure (Bioline, Germany). Briefly, NG were homogenised in 500 µl of TRIsure for 2 minutes using the TissueLyser II (Qiagen, Hildon, Germany). One hundred microliters of chloroform, to aid phase separation, were added and the samples were separated by centrifugation (15 minutes; 13,130g; 4ºC). The aqueous phase containing RNA was then cleaned by alcohol precipitation. RNA was visualised throughout using 1µl/ml of GlycoBlue Blue

co-precipitant (Invitrogen) to avoid loss. The resulting RNA was re-suspended in 10µl of RNase free water and quantified using a nanodrop Lite spectrophotometer (Thermofisher Scientific, Madison, USA).

Contaminant genomic DNA was removed using RQ1 RNase-Free DNase (Promega, Southampton, UK), and 0.3µg, 0.18µg or 0.13µg of RNA were reverse transcribed described using high capacity cDNA reverse transcription kit (Life Technologies, Paisley, UK). CaSR expression was quantified by RT-qPCR using maxima probe master mix (Life Technologies, Paisley, UK) and validated taqman CaSR probes (Mm00443375), targeting exon boundary 4-5, and β-actin probes (Mm02619580) (Life Technologies, Paisley, UK).

4.2.4. Quantification of CaSR expression in the NG of fasted mice

Group housed three-month-old glucagon receptor floxed mice, which are not expected to show a specific phenotype in the absence of cre recombinase, were kindly donated by Victoria Salem. Mice were fed *ad libitum* or fasted for 24 hours prior to schedule 1 via pentobarbital overdose and NG extraction. RNA was isolated and CaSR mRNA levels were quantified as per above.

4.2.5. Bilateral injection of adeno-associated virus (AAV) viral constructs into the NG

NG injections were kindly performed by Dr Simon Cork. Mouse anaesthesia induction was performed in an induction chamber using 4% isofluorane, 2L/min oxygen flow rate. Once the mouse lost its pedal reflex, anaesthesia was maintained with 2% isofluorane, 1L/min oxygen. All fur was removed from the chin to the base of the rib cage using clippers and mice were treated with 2µg/kg of atropine, a muscarinic acetylcholine receptor antagonist, to prevent the heart from stopping due to overstimulation of the vagus, which also has parasympathetic control of the heart. Mice were also treated with 4mg/kg of carprofen, an analgesic.

Following area sterilisation with 10% iodine, a midline vertical incision at the neck (~1cm in length) was made, the salivary glands and overlaying muscle was blunt dissected to expose the vagus nerve that runs alongside the internal carotid artery. The vagus nerve was followed to the posterior lacerated foramen at the base of the skull, using the glossopharyngeal nerve as a landmark. Towards the entry, a swelling of the vagus nerve is observed, this is the NG. The vagus nerve was isolated from surrounding connective tissue and blood vessels making the NG accessible. Then, using a pulled glass capillary tube attached to a micromanipulator and pneumatic picopump (PV820, world instruments, Hertfordshire, UK) the viral vectors were injected bilaterally into the NG. Following

surgery mice were injected with 0.12mg/kg buprenorphine and given carprofen in their water (2.72mg/100ml) as needed. Mice were also fed wet diet.

The AAV vectors used in this chapter were purchased from (Penn Vector Core, Pennsylvania) and are listed in table 4.2.5.1.

Short name	Full name	Viral titre
AAV9-Control	AAV9.hSyn.eGFP.WPRE.bGH	1.32e14 GC/ml
AAV9-Cre	AAV9.hSyn.HI.eGFP-Cre.WPRE.SV40	5.506e13 GC/ml
AAV5-Control	AAV5.hSyn.eGFP.WPRE.bGH	2.224e13 GC/ml
AAV5-Cre	AAV5.hSyn.Cre.hGH	2.484 e13 GC/ml

Table 4.2.5.1 Adeno-associated viral vectors used in this study with corresponding viral titre.

AAV vectors expressing cre recombinase are expected to excise CaSR exon 7, which is flanked by LoxP sits, in the CaSR floxed mice. Exon 7 encodes the 7TMD and intracellular loops of the CaSR, necessary for the receptor to function (Chang, Tu et al. 2008).

4.2.6. Verification of CaSR knockdown in the NG of mice bilaterally injected with AAV using RT-qPCR

CaSR knockdown in the NG was examined using RT-qPCR as described in section 4.2.3 with a probe targeting the exon boundary 6-7 (Mm00443377_m1, Life Technologies, Paisley, UK), as the Lox P sites in CaSR floxed mice are located at either side of exon 7. CaSR expression relative to β-actin was normalised to mice injected with a control virus that does not express cre recombinase.

4.2.7. The effect of CaSR knockdown in the NG on the anorectic effects of CCK

Vagal function was verified by examining the response of mice to CCK-8, the effects of which are mediated via the CCK-A receptor in the vagus, four weeks after NG injections with AAV. Mice were i.p injected with 100µl of 5µg/kg of CCK octapeptide sulfated (Tocris) made in saline, at the onset of the early dark phase. Food intake in *ad libitum* fed mice was monitored 1 hour post administration.

4.2.8. The effect of CaSR knockdown in the NG on body composition

Five weeks following NG injection of AAV9-Control or AAV9-Cre, body composition was assessed using an Echo MRI-100 body composition analyser (Echo medical systems, Houston, Texas) as described in chapter 2.

4.2.9. The effect of CaSR knockdown in the NG on energy expenditure and food intake in mice orally administered L-Phenylalanine

AAV9-Control or AAV9-Cre NG injected mice were individually housed in over-head feeder CLAMS cages (Columbus instruments). Following a 24hour acclimatisation period, *ad libitum* fed mice were orally gavaged with 0.3ml of 12mmol/kg L-Phenylalanine or vehicle control (10% Tween20, water) during the light phase. Following a 48 hour wash out period, the mice were crossed over and the study was repeated.

Metabolic parameters, oxygen consumption (VO_2) and carbon dioxide production (VCO_2), were measured by indirect calorimetry, normalised for body weight and used to calculate the respiratory exchange ratio (RER), an indirect indicator of the main substrate used during metabolism. Activity was determined from the number of consecutive beam breaks. Measurements of these parameters were taken every 19 minutes.

4.2.10. The effect of CaSR knockdown in the NG on glucagon and glucose secretion in mice

Individually housed mice were bilaterally injected with either AAV9-Control or AAV9-Cre to knockdown the CaSR in the NG. Mice were fed *ad libitum*. At the end of the light phase their food was removed and they were orally gavaged with 0.3ml of 12mmol/kg L-Phenylalanine or vehicle (10% Tweeen20 in GDW).

Alternatively, individually housed mice were bilaterally administered either AAV5-Control or AAV5-Cre directly into the NG. Mice were fed *ad libitum*. At the end of the light phase their food was removed and they were orally gavaged with 0.3ml of 12mmol/kg L-Phenylalanine vs vehicle or 60% (w/v) whey protein (Bulk Powders, pure whey isolate 90, UK) vs water.

Forty microliters of blood were collected via tail venesection into K2 EDTA tubes, containing 500 KIU/ml of blood of Aprotinin, at 0 and 30 minutes post administration. The blood was centrifuged for 15mins at 3300g. The plasma was then separated, aliquoted and stored at -80C until further use.

Glucagon levels were quantified using mouse glucagon sandwich ELISA bioassays (Cisbio Bioassays, Codolet, France), according to manufacturer's instructions. Glucose levels were quantified using colorimetric enzymatic glucose assay (Randox, UK, GL364), as described in chapter 3 section 3.2.6.3.

4.2.11. Statistical analysis

Data is expressed as mean ± standard error of the mean. Knockdown, fed vs fasted, and body composition studies were analysed using an unpaired t-test. CCK food intake studies were analysed

using paired t-tests. Energy expenditure studies were analysed by calculating the area under the curve and then, like all the remaining studies, analysed by performing a one-way ANOVA with Tukey's post hoc multiple comparisons test, except for the RER which was analysed using a repeated measures two-way ANOVA with Tukey's post hoc multiple comparisons test. All analysis was performed using GraphPad Prism software (Prism 7.02 or 8.02, GraphPad Software Inc., CA, USA). P values <0.05 were considered statistically significant.

4.3. Results

4.3.1. CaSR immunoreactivity in the NG

CaSR immunoreactivity, in green, was identified on the surface (white arrow) and nucleus (yellow arrow) of neuronal cell bodies; and on satellite cells (red arrow). This pattern of staining was not observed in the no primary antibody control. Nuclear staining, in blue, was performed using DAPI.

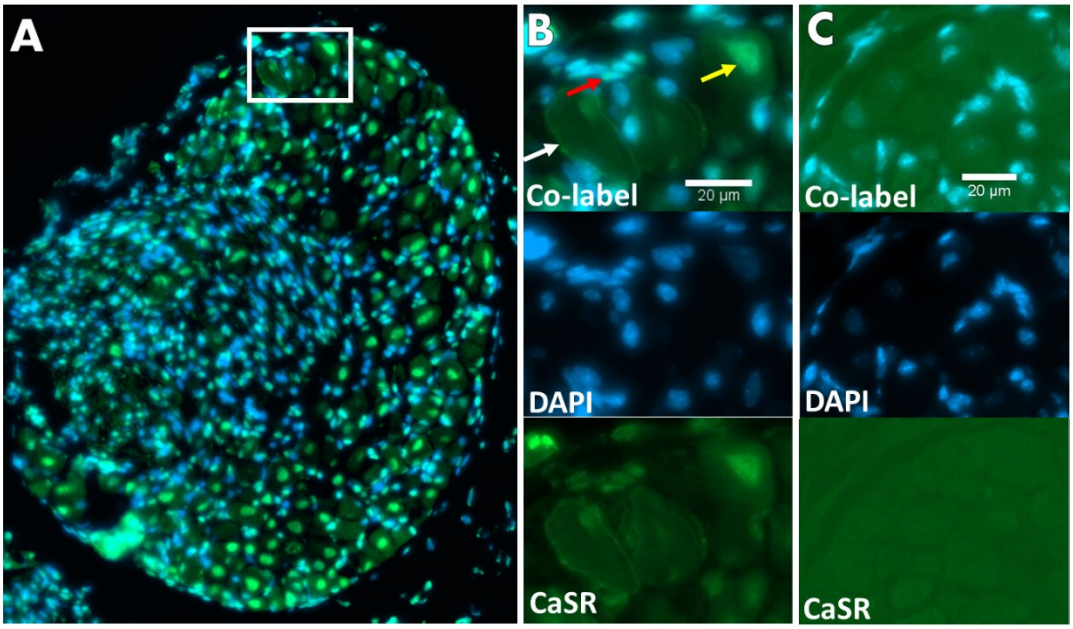


Figure 4.3.1.1: CaSR immunoreactivity in the NG of mice.

A) A cross section of the whole NG was imaged at x40 magnification using a Zeiss AxioObserver widefield microscope. B) Post-hoc zoomed in images of the region delineated by the white box were generated on Fiji. Staining was observed on the surface (white arrow) and nucleus (yellow arrow) of neuronal cell bodies; and on satellite cells (red arrow). C) No primary antibody control images. Cell nuclei were stained blue with DAPI and the CaSR was stained green.

4.3.2. Quantification of CaSR expression in the NG using RT-qPCR

CaSR mRNA expression relative to β-actin was quantified in the NG of *ad libitum* fed mice. CaSR expression in the kidney was used as a positive control and levels in the liver as a negative control. CaSR expression in the NG was ~22 fold lower, compared to the kidney (kidney: 1.000 ± 0.087 AU; NG: 0.045 ± 0.002 AU, p<0.001). CaSR expression in the liver was negligible in comparison.

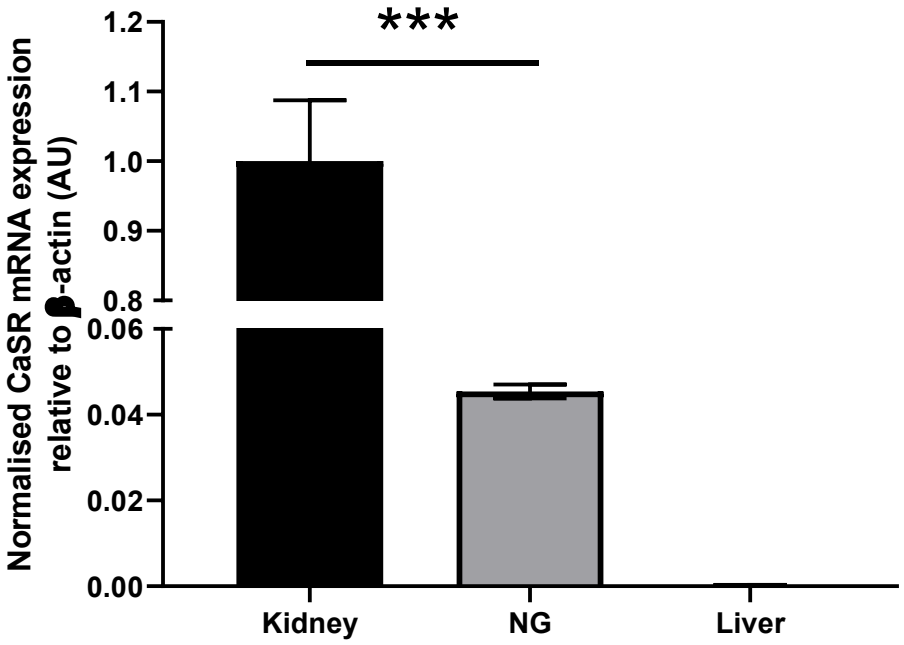


Figure 4.3.2.1: CaSR expression relative to β-actin in the NG, kidney and liver of *ad libitum* fed mice.

CaSR mRNA expression relative to β-actin was quantified in the kidney, NG and liver of *ad libitum* fed mice. Data expressed as mean ± SEM (n=4-5). ***p<0.001.

4.3.3. The effect of fasting on CaSR mRNA expression in the NG

Mice fasted for 24 hours had ~45% more CaSR mRNA expression in the NG than mice fed *ad libitum* (fasted: 1 ± 0.14 AU vs fed: 0.55 ± 0.10 AU, *p<0.05).

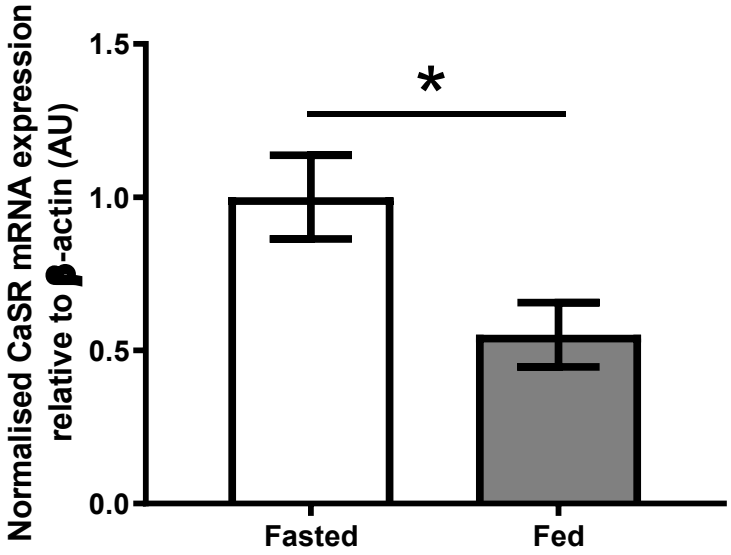


Figure 4.3.3.1: The effect of fasting on CaSR mRNA expression relative to β-actin in the NG

CaSR mRNA expression, relative to β-actin, in mice fed *ad libitum* or fasted for 24 hours. Data was normalised to the fasted group. Data expressed as mean ± SEM (n= 5-7). *p<0.05.

4.3.4. Quantification of CaSR knockdown following NG injection of AAV9-Control or AAV9-Cre using RT-qPCR

CaSR expression in the NG of CaSR floxed mice bilaterally injected with AVV9-Control or AAV9-Cre was measured using RT-qPCR to quantify cre recombinase induced knockdown.

Mice in A) were injected bilaterally, on the same day, 3 months prior to mice in cohort B) that were injected bilaterally with a week between each injection.

In A) left and right NG were processed in separate batches with different amounts of template RNA (0.13µg left NG vs 0.18µg right NG). Therefore, CaSR expression in the cre animals was normalised to the corresponding right or left control NG. CaSR knockdown in the left NG was ~88% and in the right NG it was ~98%. Due to the low n numbers, no statistical analysis was performed.

Samples from cohort B) were processed together and normalised to control left NG. From cohort B it appears there are no differences in the expression of the CaSR between the left and right NG. Knockdown in the left NG was ~46% (Control left NG: 1.000 ± 0.142 AU vs. Cre left NG 0.539 ± 0.029 AU, $p<0.05$) whereas in the right NG it was ~85% (Control right NG: 0.916 ± 0.098 AU vs Cre right NG: 0.136 ± 0.067 AU, $P<0.001$).

Mice lacking RT-qPCR verification information or with a knockdown <30% in either ganglion were excluded from the study.

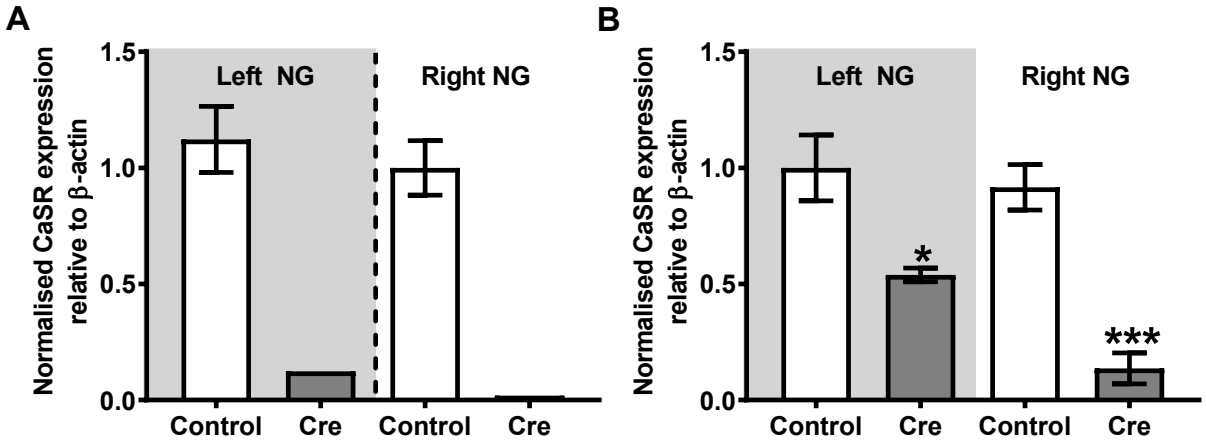


Figure 4.3.4.1: CaSR expression relative to β-actin in the NG of mice injected with control or cre expressing virus.

CaSR mRNA expression relative to β-actin was quantified in the individual NG of mice injected with either AAV9-Control (Control) or AAV9-Cre (Cre). A) CaSR expression in the first cohort of mice was normalised to the side specific (left vs right NG) control group, as indicated by the dotted line separating the left and right NG. B) CaSR expression in the second cohort of mice, injected with a week in between each injection, was normalised to control left NG. Data expressed as mean ± SEM. *$p<0.05$, ***$P<0.001$. A) n=1-2, B) n=4-6.

4.3.5. The effect of CaSR knockdown in the NG on the anorectic effects of CCK

Intraperitoneal administration of 0.1ml of 5μg/kg CCK or saline control in *ad libitum* fed mice at the onset of the dark phase decreased food intake at 0-1 hours post injection, regardless of AAV9-Cre mediated CaSR knockdown in the NG (AAV9-Control saline: 0.38 ± 0.09g vs. AAV9-Control CCK: 0.15 ± 0.03g, $p<0.05$) and (AAV9-Cre saline: 0.38 ± 0.10g vs. AAV9-Cre CCK: 0.16 ± 0.01g, $p= 0.11$).

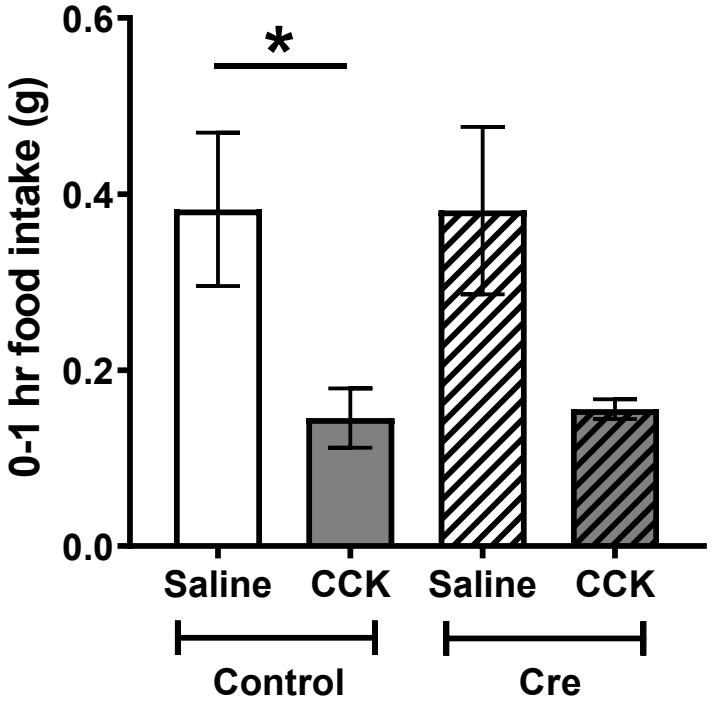


Figure 4.3.5.1: The effect of CaSR knockdown in the NG on CCK's anorectic effects.

Ad libitum fed CaSR floxed mice, previously bilaterally injected with either AAV9-Control or AAV9-Cre into the NG, were administered 0.1ml of saline or 5μg/kg of CCK-8 intra-peritoneally at the onset of the early dark phase. Food intake was monitored 1-hour post administration. Data expressed as mean ± SEM (n=4-6, crossed over). *$p<0.05$.

4.3.6. The effect of CaSR knockdown in the NG on the body weight and body composition of mice

Five weeks post-surgery, AAV9-control mice and CaSR knockdown mice (AAV9-Cre) displayed no differences in body weight, fat mass or lean mass.

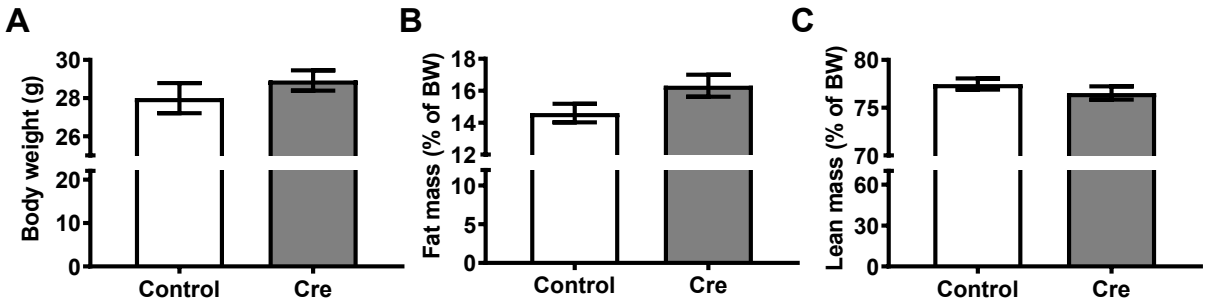


Figure 4.3.6.1: The effect of CaSR knockdown in the NG on body weight and body composition, 5 weeks post-surgery.

The A) body weight B) fat mass and C) lean mass (calculated as a percentage of body weight) of CaSR floxed mice injected with AAV9-Control (Control) or AAV9-Cre (Cre) into the NG. Data expressed as mean ± SEM (n=5-9).

4.3.7. The effect of orally administered L-Phenylalanine on food intake in mice that have the CaSR knocked down in the NG

CaSR floxed mice bilaterally injected with AAV9-Control or AAV9-Cre, to knockdown the CaSR, were habituated for 24 hours to CLAMS cages before being orally gavaged with 12mmol/kg L-Phenylalanine or a vehicle control (10% Tween 20, water) during the light phase.

L-Phenylalanine non-significantly decreased food intake in AAV9-Control mice, compared to AAV9-Control mice gavaged with vehicle solution, for approximately 7.30hrs. The food intake of CaSR knockdown mice (AAV9-Cre) gavaged with vehicle solution was overall lower than control mice gavaged with vehicle solution.

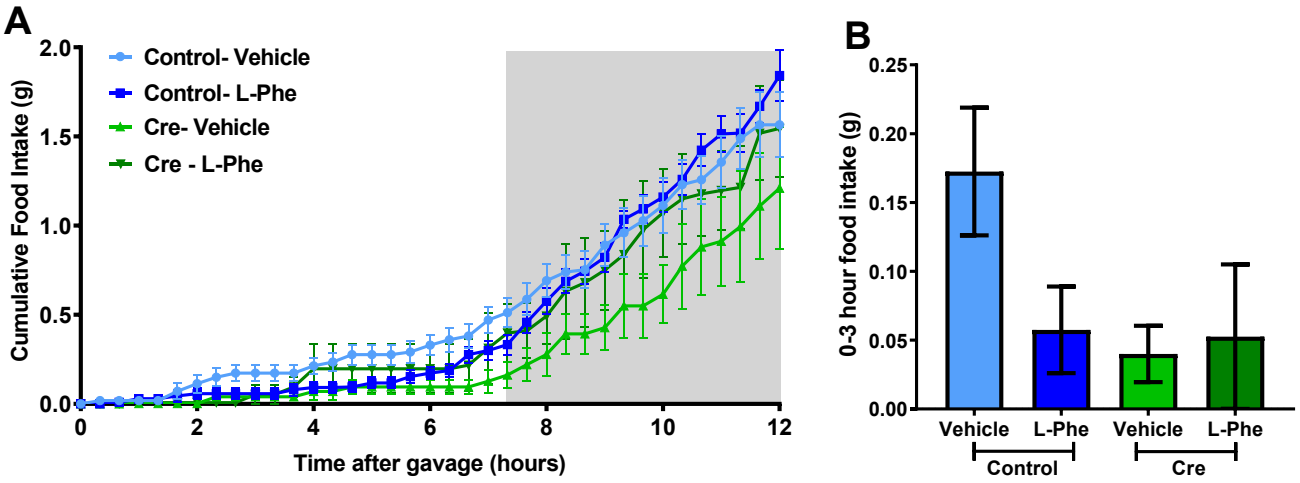


Figure 4.3.7.1: The effect of orally administered L-Phenylalanine on food intake in mice that have the CaSR knocked down in the NG

The effect of 12mmol/kg L-Phenylalanine on cumulative food intake in CaSR floxed mice bilaterally injected with AAV9-Control or AAV9-Cre over A) 12 hours and B) 3 hours. Data expressed as mean ± SEM (n= 4-8, crossed over).

4.3.8. The effect of CaSR knock down in the NG on the energy expenditure of mice orally gavaged with L-Phenylalanine

Ad libitum fed mice bilaterally injected with AAV9-Control or AAV9-Cre were orally gavaged with 12mmol/kg L-Phenylalanine or vehicle during the early light phase. Energy expenditure was examined using the CLAMS metabolic cages.

There was a trend for increased oxygen consumption, carbon dioxide production, respiratory exchange ratio and total activity in control mice administered L-Phenylalanine. No difference was observed between NG CaSR knockdown mice administered L-Phenylalanine or vehicle.

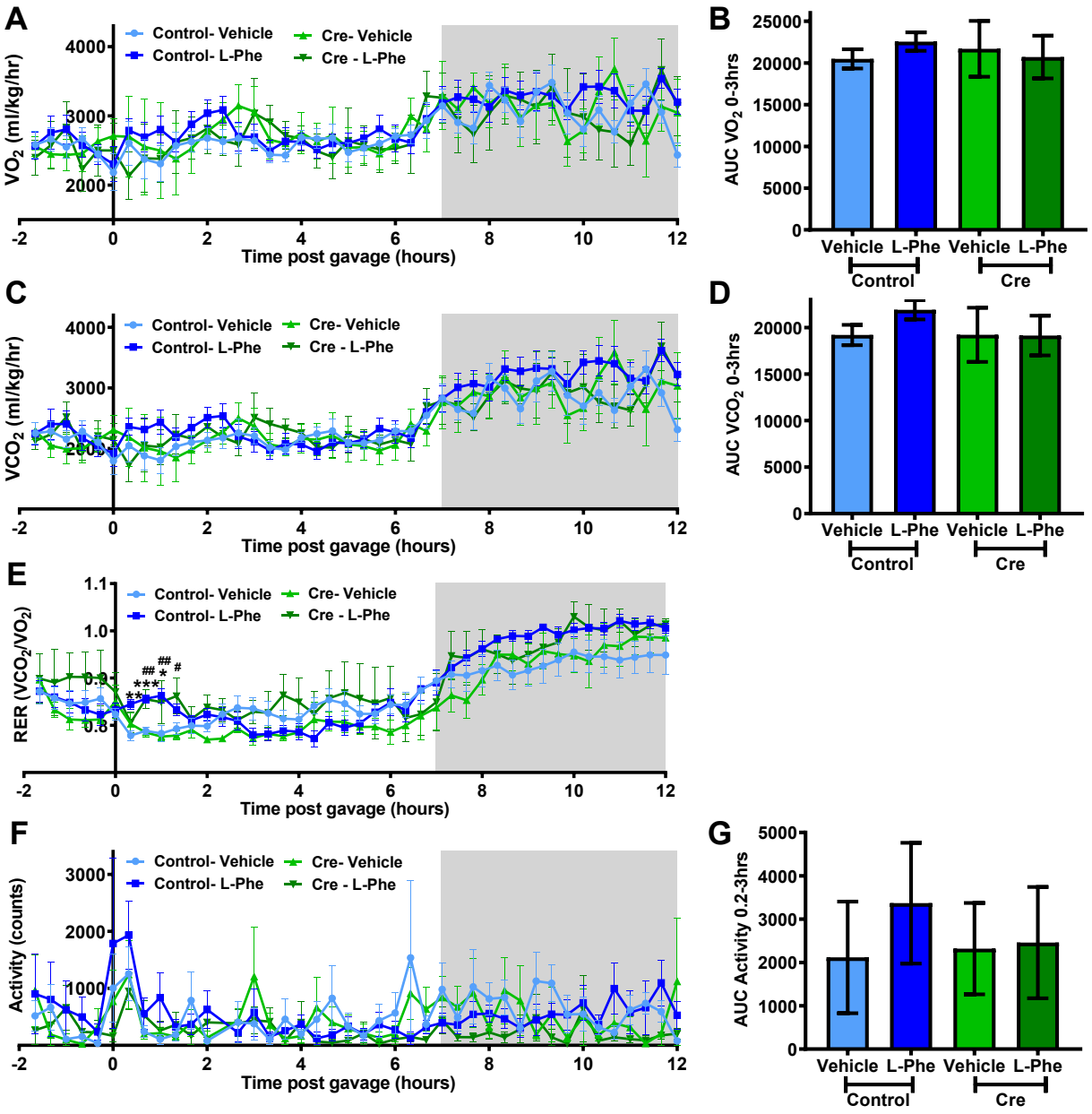


Figure 4.3.8.1: The effect of orally administered L-Phenylalanine on energy expenditure in mice that have the CaSR knocked down in the NG

The effect of orally administered 12mmol/kg L-Phenylalanine in CaSR floxed mice bilaterally injected with AAV9-Control or AAV9-Cre on A) oxygen consumption (VO_2) 0-12 hours post treatment and B) 0-3 hours post treatment (AUC); C) carbon dioxide production (VCO_2) 0-12 hours post treatment and D) 0-3 hours post treatment (AUC); E) respiratory exchange ratio (RER) 0-12 hours; F) activity 0-12 hours post treatment and G) 0.20-3 hours post treatment (AUC). Data expressed as mean ± SEM (n= 4-8, crossed over), *p<0.05 Control-Vehicle vs Control L-Phe. #p<0.05 Control-L-Phe vs Cre-Vehicle.

4.3.9. The effect of CaSR knockdown using AAV9-Cre in the NG on plasma glucagon and glucose levels in response to L-Phenylalanine

CaSR floxed mice bilaterally injected with either AAV9-control or AAV9-Cre virus, which expresses cre recombinase to knockdown the CaSR in the NG, were orally gavaged with 12mmol/kg L-Phenylalanine or vehicle solution (10% Tween20, GDW).

Administration of L-Phenylalanine significantly increased glucagon secretion in both AAV9-Control and AAV9-Cre injected mice 30 minutes post administration, compared to AAV9-Control vehicle treated mice prior to treatment (AAV9-Control vehicle t0: 42.38 ± 4.44 pg/ml Vs AAV9-Control L-Phe t30: 82.66 ± 5.22 pg/ml, $p<0.05$) (AAV9-Control vehicle t0: 42.38 ± 4.44 pg/ml Vs AAV9-Cre L-Phe t30: 87.24 ± 15.17 pg/ml, $p<0.01$).

Glucose levels were increased in AAV9-Cre mice 30 minutes after L-Phenylalanine oral administration in comparison to AAV9-Cre mice 30 minutes after Vehicle administration (AAV9-cre Vehicle t30: 5.5 ± 0.5 mmol/L vs AAV9-Cre L-Phe t30: 8.4 ± 1.4 mmol/L). Levels were also raised in comparison to AAV9-Control mice 0 and 30 minutes post vehicle administration, and AVV9-Cre mice 0 minutes post administration.

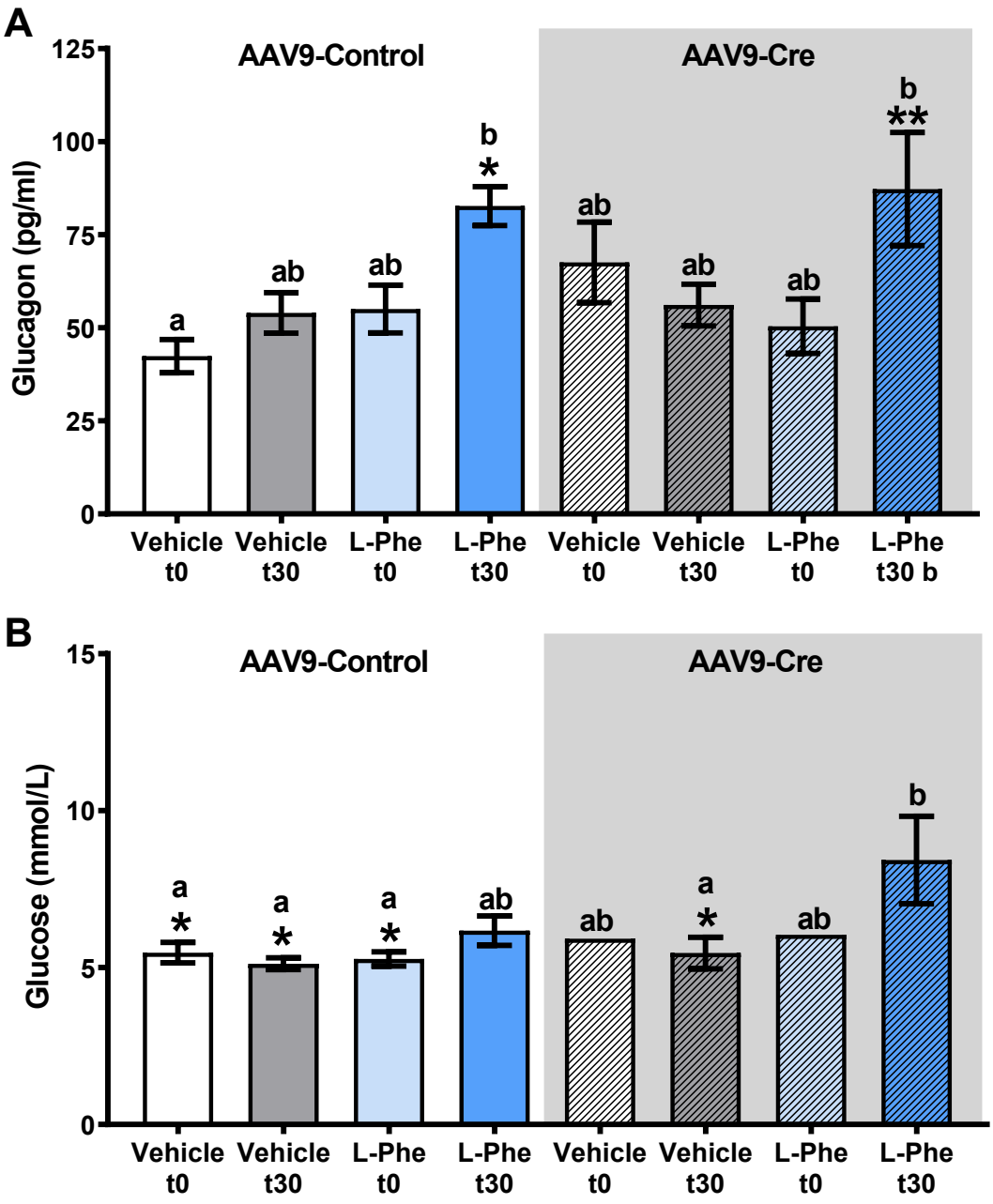


Figure 4.3.9.1: The effect of CaSR knockdown in the NG on glucagon and glucose secretion in response to L-Phenylalanine

CaSR floxed mice bilaterally injected with AAV9-Control or AAV9-Cre were orally administered 0.3ml of 12mmol/kg L-Phenylalanine or vehicle solution. Circulating levels of A) glucagon (n= 4-5, crossed over) and B) glucose (n= 1-4, crossed over) were measured 0 (t0) and 30 (t30) minutes post administration. Data expressed as mean ± SEM. Different letters indicate statistically significant differences. A) Level of significance compared to AAV9-Control vehicle t0 is denoted with *p<0.05, **p<0.001. B) Level of significance compared to AAV9-Cre L-Phe t30 is denoted with *p<0.05.

4.3.10. Quantification of CaSR knockdown following NG injection of AAV5-Cre using RT-qPCR

CaSR knockdown in the NG of mice bilaterally injected with AAV5-Control or AAV5-Cre was quantified using RT-qPCR. Samples were normalised to AAV5-Control left NG.

No difference in the expression of the CaSR between the left and right NG was detected. Knockdown in the left NG was ~44% (AAV5-Control left NG: 1.000 ± 0.136 AU vs. AAV5-Cre left NG 0.664 ± 0.028 AU, p=0.09) whereas in the right NG it was ~63% (AAV5-Control right NG: 0.996 ± 0.102 AU vs AAV5-Cre right NG: 0.377 ± 0.143 AU, P<0.05). Mice lacking RT-qPCR verification information from either NG were excluded from the study.

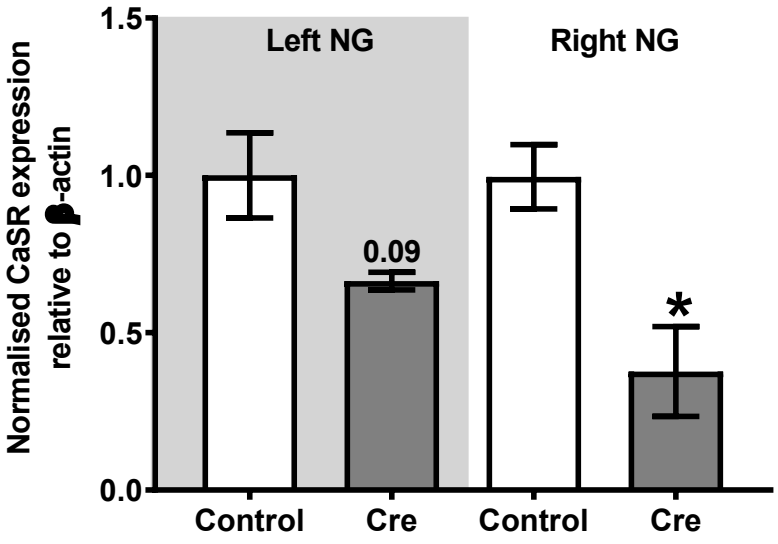


Figure 4.3.10.1: CaSR expression relative to β-actin in the NG of mice injected with AAV5-Cre.

CaSR mRNA expression relative to β-actin was quantified in the individual NG of mice injected with either AAV5-Control (control) or AAV5-Cre (Cre). CaSR expression was normalised to AAV5-control left NG. Data expressed as mean ± SEM. *p<0.05 (n=3-4).

4.3.11. The effect of AAV5- Cre mediated CaSR knockdown in the NG of mice on the anorectic effects of CCK

Intraperitoneal administration of 0.1ml of 5µg/kg CCK or saline control in *ad libitum* fed mice at the onset of the dark phase non-significantly decreased food intake at 0-1 hours post injection, independently of AAV5-Cre mediated CaSR knockdown in the NG (AAV5-control saline: 0.27 ± 0.06g vs. AAV5-control CCK: 0.17 ± 0.07g, p=0.47) and (AAV5-Cre saline: 0.27 ± 0.11g vs. AAV5-Cre CCK: 0.12 ± 0.07g, p= 0.49).

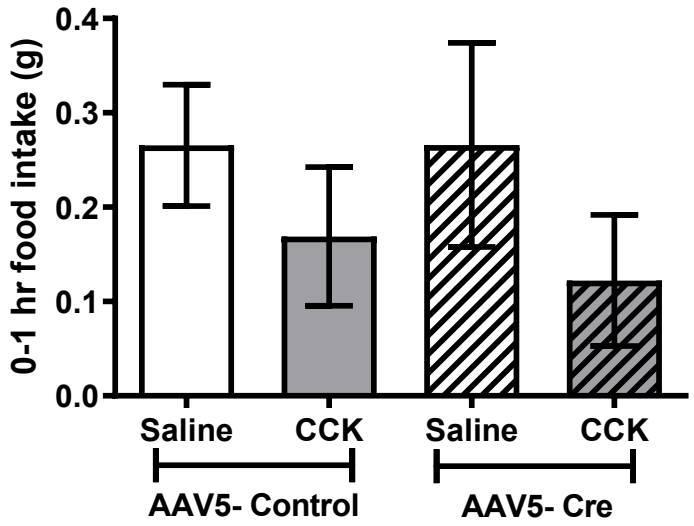


Figure 4.3.11.1: The effect of AAV5-Cre mediated CaSR knockdown in the NG of mice on CCK's anorectic effects.

Ad libitum fed CaSR floxed mice, previously bilaterally injected with either AAV5-Control or AAV5-Cre into the NG, were administered 0.1ml of saline or 5µg/kg of CCK-8 intra-peritoneally at the onset of the early dark phase. Food intake was monitored 1 hour post administration. Data expressed as mean ± SEM (n=3-4, crossed over).

4.3.12. The effect of CaSR knockdown via AAV5-Cre in the NG on glucagon secretion in response to L-Phenylalanine

CaSR floxed mice bilaterally injected with either AAV5-control or AAV5-Cre, which expresses cre recombinase to knockdown the CaSR in the NG, were orally gavaged with 12mmol/kg L-Phenylalanine or vehicle solution.

Administration of L-Phenylalanine increased glucagon secretion in both AAV5-control and AAV5-Cre injected mice 30 minutes post administration of L-Phenylalanine, compared to 0 minutes post administration. This increase was statistically significant in AAV5-Cre mice (AAV5-Cre L-Phenylalanine t0: 38.7 ± 2.9 pg/ml vs AAV5-Cre L-Phenylalanine t30: 157.4 ± 45.8 pg/ml, $p<0.05$) (AAV5-Control L-Phenylalanine t0: 47.9 ± 7.3 pg/ml vs AAV5-Cre L-Phenylalanine t30: 157.4 ± 45.8 pg/ml, $p<0.05$).

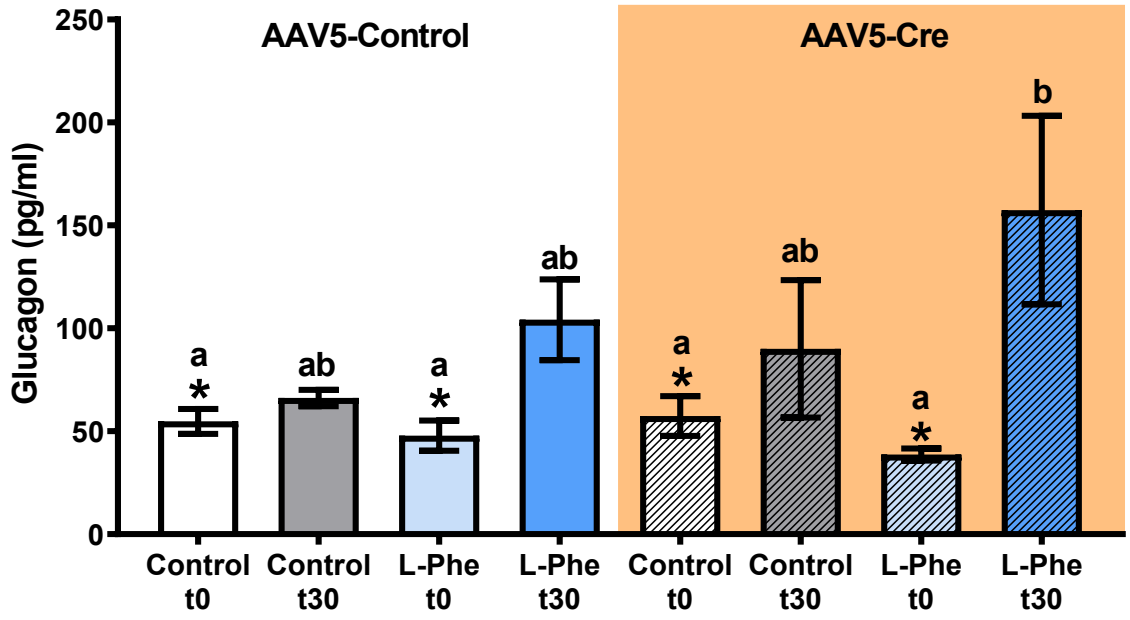


Figure 4.3.12.1: The effect of CaSR knockdown in the NG on glucagon secretion in response to L-Phenylalanine

Mice bilaterally injected with a control virus (AAV5-Control) or a virus expressing cre recombinase (AAV5-Cre) to knockdown the CaSR were orally administered 0.3ml of 12mmol/kg L-Phenylalanine or vehicle solution. Circulating levels of glucagon were examined. Data expressed as mean ± SEM (n= 3-4, crossed over). Different letters indicate statistically significant differences. Level of significance compared to AAV5-Cre L-Phe t30 denoted with *$p<0.05$.

4.3.13. The effect of CaSR knockdown via AAV5-Cre in the NG on plasma glucagon and glucose levels in response to whey protein

Mice bilaterally injected with either AAV5-Control or AAV5-Cre virus, which expresses cre recombinase to knockdown the CaSR in the NG, were orally gavaged with 0.3ml of 60% (w/v) whey protein or water.

Administration of whey significantly increased glucagon secretion in AAV5-Control mice, 30 minutes post administration, compared to AAV5-Control mice administered water (AAV5-Control water t30: 37.17 ± 7.28pg/ml Vs AAV5-Control Whey t30: 118.20 ± 19.97 pg/ml, $p<0.01$). This effect was not observed in NG CaSR knockdown mice (AAV5-Cre). No significant differences in glucose levels were observed across any of the groups.

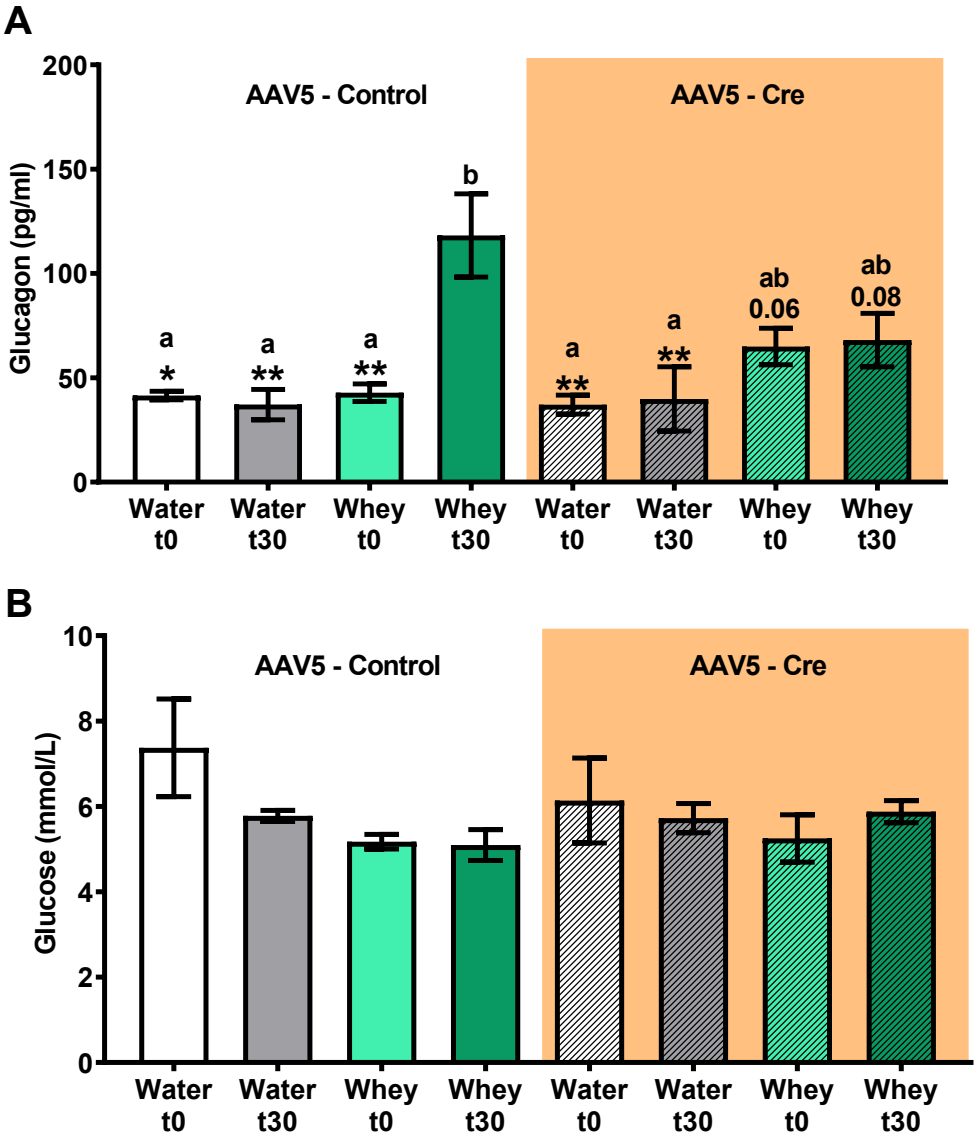


Figure 4.3.13.1: The effect of CaSR knockdown via AAV5-Cre in the NG on glucagon and glucose secretion in response to whey protein

CaSR floxed mice bilaterally injected with AAV5-Control or AAV5-Cre to knockdown the CaSR were orally administered 0.3ml of 60% (w/v) whey protein or water. Circulating levels of A) glucagon and B) glucose were measured 0 (t0) and 30 (t30) minutes post administration. Data expressed as mean ± SEM (n=2-4, crossed over). Different letters indicate statistically significant differences. Columns with 'ab' are not significantly different to any of the groups. Level of significance compared to AAV5-Control whey t30 is denoted with *p<0.05, **p<0.001.

4.4. Discussion

Studies in the previous chapter found L-Phenylalanine to supress appetite and stimulate the secretion of hormones, including pancreatic hormones. Moreover L-Phenylalanine was found to activate nuclei in the brainstem including the NTS, the site of sensory vagal innervation, and the DMN, where vagal efferents originate. Vagal neurons express a limited number of nutrient sensors including the CaSR, which is modulated by L-Phenylalanine. Consequently, this chapter aimed to determine whether the CaSR in vagal afferents plays a role in appetite regulation or the secretion of pancreatic hormones in response to L-Phenylalanine.

Studies in this chapter found administration of AAV vectors expressing cre recombinase into the NG to be effective at knocking down the CaSR. The effect of CaSR knockdown in the NG on L-Phenylalanine's anorectic effects were inconclusive. However, findings from this chapter suggest vagally expressed CaSR plays a role in mediating protein-induced glucagon secretion.

CaSR expression was identified in the NG by immunohistochemical staining and RT-qPCR, as reported in the literature (Gu, Vysotskaya et al. 2013, Egerod, Petersen et al. 2018). CaSR immunoreactivity was identified on vagal afferents and satellite cells of the NG. In accordance with Egerod et al., expression levels were low (Egerod, Petersen et al. 2018). In this chapter they were ~22 fold lower than in the positive control, the kidney, where the CaSR is widely expressed and crucial in mineral ion transport (Riccardi and Brown 2010). Nevertheless, small populations of vagal neurons can surmount significant physiological responses (Berthoud, Fox et al. 1990).

Within the vagus there are phenotypically and functionally distinct vagal subpopulations which show plasticity. Low levels of CaSR are probably due to its expression being limited to a subpopulation of neurons; the phenotype and function of which would be interesting to investigate further. Fluorescence-activated cell sorting and RNA sequencing of NG CaSR positive neurons would be useful for such studies.

To the author's knowledge, the role of the CaSR in the vagus remains largely unexplored. Papers investigating this solely focused on the role of the CaSR on transient receptor potential vanilloid receptor type 1 currents in the broncho-pulmonary branch of the vagus in rats (Gu, Vysotskaya et al. 2013).

The vagus nerve displays plasticity and responds to the nutritional status of rodents by altering the expression of appetite regulating peptides and sensors. This allows the small population of neurons found in the NG to relay complex signals from the periphery to the CNS. Prolonged fasting decreases the expression of anorectic receptors and peptides, and increases the expression of orexigenic

peptides and receptors in the NG of rodents (Burdyga, Lal et al. 2004, Burdyga, Varro et al. 2006, de Lartigue, Dimaline et al. 2007, Burdyga, de Lartigue et al. 2008, de Lartigue, Barbier de la Serre et al. 2012, de Lartigue, Ronveaux et al. 2014). To the author's knowledge, previous studies have not examined the effect of fasting on vagal expression of nutrient sensors. This study found the CaSR to be upregulated following a 24 hour fast. This contrasts with what would be expected from an anorectic mediator and may reflect the importance of the CaSR in other systems e.g. calcium homeostasis and neuronal excitability. The expression of GPR35 and GPR119 did not appear to be altered (unpublished findings in the group).

Long term viral transduction of vagal sensory neurons can be achieved through the injection of AAV vectors into the nodose ganglion, without any apparent outward effect on the NG or health of the animal (Kollarik, Carr et al. 2010). This allows for post-developmental knockdown of genes in the NG.

To examine the role of the CaSR in the NG, CaSR floxed mice, kindly donated by Wenhan Chang (Chang, Tu et al. 2008), were injected with control or cre recombinase expressing AAV5 and AAV9 vectors. Different AAV serotypes display different tropisms, AAV9 has been shown to transduce vagal sensory neurons successfully. Chang et al. reported infection of ~50% of sensory neurons by AAV9 vectors (Chang, Strochlic et al. 2015). This low infection rate was probably caused by the low viral titres used, due to the mixing of two different viruses in a 1:1. However, higher infection rates, have been reported using higher viral titres, with most vagal sensory neurons being successfully transduced 4-5 weeks post injection, and with limited efferent vagal infection (Kollarik, Carr et al. 2010).

In this study, AAV9-Cre stimulated ~85% CaSR knockdown in the NG, quantified 5 months after injection. Efficacy of knockdown was greater in the right NG compared to the left NG. This is likely due to the increased leakage reported during the surgery of left NG injections (qualitative observations). No differences in CaSR expression were identified between the left and right NG.

AAV5 is reported to have the highest transduction rate in dorsal root ganglion neurons (versus AAV1-8) (Mason, Ehlert et al. 2010). However, AAV5-Cre injected mice showed less effective CaSR knockdown (~63%, right NG), compared to AAV9-Cre injected mice. This could be due to the slightly lower viral titre (AAV5-Cre: 2.484 e13 GC/ml vs AAV9-Cre; 5.506e13 GC/ml), due to tropism differences, or due to differences in the vector expression cassettes. The AAV9-Cre vector, unlike the AAV5-Cre vector, contained the expression cassette WPRE (woodchuck hepatitis post-transcriptional regulatory element) which improves the expression of transgenes in AAV (Choi, Yu et al. 2014). Moreover, AAV9-Cre and AAV5-Cre vectors had different polyadenylation signal sequences which also affects transgene expression (Choi, Yu et al. 2014).

Most vagal sensory neurons are successfully transduced 4-5 weeks post injection (Kollarik, Carr et al. 2010). Therefore, prior to this, no experiments were performed.

CCK is a hormone that signals via CCK-A receptors in the vagus. To examine whether vagal afferents were still able to respond to satiety signals, the anorectic response of bilaterally injected mice to CCK was examined. This study found no changes in the anorectic response to CCK in control or CaSR knockdown mice, suggesting vagal satiety signals, at least those mediated by CCK, were still functional. Moreover, CCK responsiveness is modulated by other receptors in the vagus, for example leptin receptor signalling (de Lartigue, Barbier de la Serre et al. 2012, de Lartigue, Ronveaux et al. 2014). It would appear this is not the case with the CaSR.

CaSR knockdown in the NG did not appear to affect the body composition of mice 5 weeks post-surgery. This suggests that vagally expressed CaSR does not play a role in adiposity, unlike vagally expressed Leptin receptor (de Lartigue, Ronveaux et al. 2014). No differences in body weight were observed at later dates, however whether body composition was altered in the long run was not examined.

To examine the effect of CaSR knockdown on L-Phenylalanine's anorectic response, mice were placed into CLAMS metabolic cages and, following a 24 hour acclimatisation period, orally gavaged with L-Phenylalanine at a dose previously shown to supress food intake (Alamshah, Spreckley et al. 2017). Mice with <30% knockdown in either of the NG, or who we were missing knockdown information, were excluded post-hoc from CLAMS data. During this post-hoc analysis it was found that NG CaSR knockdown mice took longer to acclimatise to the CLAMS cages. This was evident from their increased weight loss during the CLAMS study and their supressed food intake during the light and dark phase of the first three days. By the final night, the food intake of CaSR NG knockdown mice was comparable to that of control mice, suggesting CaSR NG knockdown mice are not normally hypophagic. This is supported by the lack of food intake and body weight effect when the mice are in their accustomed cages (data not shown). However, this may suggest that NG CaSR knockdown makes mice more susceptible to stress or makes it harder for them to adapt to new environments.

Due to the hypophagic behaviour of NG CaSR knockdown mice during the CLAMS studies, this study was unable to determine whether L-Phenylalanine's anorectic effects persist in NG CaSR knockdown mice. Nevertheless, control mice in the CLAMS cages did acutely decrease their food intake and have a trend for increased oxygen consumption, potentially due to increased activity, in response to orally administered L-Phenylalanine. This is in alignment with findings in the previous chapter in rats.

Futures studies should allow for a longer acclimatisation period in the CLAMS cages to determine the effects of L-Phenylalanine on NG CaSR knockdown mice. Moreover, the cause underlying the

prolonged hypophagic behaviour of NG CaSR knockdown mice during acclimatisation should be further investigated.

L-Phenylalanine can modulate pancreatic exocrine secretion, in part, via an entero-pancreatic vagal pathway in which vagal afferents send sensory information to the NTS which modulates DMN vagal efferents that innervate the pancreas to regulate exocrine secretion (Owyang, May et al. 1986). Additionally, stimulation of the DMN, NTS and vagus have all been shown to stimulate the endocrine pancreas (Jeanrenaud, Ionescu et al. 1983, Laughton and Powley 1987, Berthoud, Fox et al. 1990, Mussa, Sartor et al. 2011).

Studies in the previous chapter showed L-Phenylalanine stimulates the secretion of glucagon and activates brainstem nuclei involved in the vagal entero-pancreatic reflex. Additionally, the vagus expresses machinery to detect absorbed aromatic amino acids and their metabolites, which may be able to modulate vagal activity, and potentially downstream pancreatic secretions. One of these receptors is the CaSR, which is modulated by L-Phenylalanine as discussed in the previous chapter. Therefore, whether vagal sensing of L-Phenylalanine via the CaSR could mediate glucagon secretion was investigated.

NG CaSR knockdown did not alter baseline glucagon secretion, nor secretion of glucagon in response to L-Phenylalanine, 30 minutes post administration, regardless of the AAV-Cre vector used. Interestingly, however, glucagon release in response to whey protein, though similar in magnitude to L-Phenylalanine, was lost in CaSR NG knockdown mice, in comparison to control mice. This suggests nutrient sensing via vagal afferent neurons may mediate endocrine secretion. However, this data should be interpreted with caution considering the low n numbers and low level of knockdown in AAV5-Cre mice, even though a small population of neurons can have a significant physiological effect.

The lack of NG CaSR knockdown glucagon suppression in L-Phenylalanine treated mice may be due to the pharmacological dose used. This would likely increase circulating levels of L-Phenylalanine, leading to systematic effects that may mask any vagally mediated pathway. On the other hand, administration of a protein load, instead of the amino acid itself, would result in a more physiological appearance of amino acids with regards to concentration, rate of appearance, absorption and detection, even though the dose used was pharmacological. It would also stimulate the secretion of other factors which may be involved in priming the vagus.

Future studies should verify findings in a larger cohort and examine the circuit using lower doses directly administered into the gut at different levels. The ability of L-Phenylalanine and protein loads to stimulate glucagon secretion, following blockade of the cholinergic system, should also be investigated to establish whether the vagus modulates glucagon secretion. Moreover, the effect of CaSR modulation on vagal activity, the phenotype of CaSR expressing vagal fibres and the location of CaSR positive vagal terminals in the gut should be examined.

Overall this study found NG injections of AAV expressing cre recombinase vectors an effective tool for knocking down the CaSR in vagal afferents. The viral serotype, titre, and construct are all factors that should be carefully considered. The effect of NG CaSR knockdown on L-Phenylalanine's anorectic effects are inconclusive, however, data in this chapter suggests vagally expressed CaSR may play a role in mediating the secretion of glucagon in response to an oral protein load. Whether this is mediated via an entero-pancreatic vagal reflex requires further investigation. The effect of CaSR vagal knockdown on other systems regulated by the vagus nerve were not covered in this chapter.

Chapter 5: General Discussion

Obesity is a growing concern. It was estimated that in 2016, across the world 39% of adults were overweight and 13% were obese (WHO 2018). This is a problem because obesity is associated to several comorbidities including cancer, diabetes and cardiovascular disease (Wang, McPherson et al. 2011).

Current anti-obesity therapies are either ineffective, or highly invasive and difficult to provide on a global scale. Therefore, there is a growing need for new treatments. Lifestyle interventions are the first line of treatment. These include increasing energy expenditure, through exercise, and decreasing energy intake, through dieting, to restore energy balance (Inelmen, Toffanello et al. 2004, Moroshko, Brennan et al. 2011). High protein diets are effective at promoting weight loss, but long-term adherence is low (Batterham, Heffron et al. 2006, Leidy, Clifton et al. 2015). By understanding the mechanisms by which these diets promote satiety and weight loss, we can potentially exploit the system with new therapeutic agents. Therefore, the first part of this thesis aimed to understand the mechanisms underlying the appetite supressing effects of elevated protein intake.

Acute studies have shown protein loads to increase DIT and stimulate the secretion of anorectic hormones which are likely to promote satiety and decrease food intake (Westerterp 2004, Batterham, Heffron et al. 2006, Blom, Lluch et al. 2006, Lejeune, Westerterp et al. 2006, Veldhorst, Nieuwenhuizen et al. 2009, Belza, Ritz et al. 2013, van der Klaauw, Keogh et al. 2013). Consequently, it is reasonable to consider that prolonged protein intake may modulate the expression and/or secretion of anorectic gut hormones. However, studies to date regarding the effects of chronic HPD on gut hormones have demonstrated conflicting results.

In this thesis, mice fed a HPD for 50 days did not show significant changes in their food intake or body weight gain, compared to mice on a NPD. However, HPD fed mice did show a trend for improved body composition, supported by a corresponding decrease in circulating leptin. Considering changes in gut hormone can take place independently of food intake effects (Zhou, Keenan et al. 2011), the secretion and synthesis, of different hormones was still examined in response to prolonged protein intake. A non-significant increase in PYY expression was identified in the jejunum of HPD-fed mice. Though small, especially in comparison to PYY levels in the colon, this increase in PYY may have effects during the early stages of digestion. It is unclear where the PYY released into the circulation shortly after food ingestion comes from, and it is possible that the small increase in mRNA observed may be translated to a significant difference in circulating PYY levels, given the right stimulus. Additionally, the jejunum is normally the principal site of protein digesta

absorption (H J Freeman and Kim 1978, Chung, Kim et al. 1979). This may further suggest the importance of protein breakdown products, such as amino acids, in mediating the effects of HPDs.

The timing of protein-induced satiety has been found to correlate with the appearance and concentration of amino acids in circulation (Mellinkoff, Frankland et al. 1956, Veldhorst, Nieuwenhuizen et al. 2009). This suggests amino acid sensing may be important in mediating the effects of protein-induced satiety (Mellinkoff, Frankland et al. 1956, Veldhorst, Nieuwenhuizen et al. 2009). However, it may be that the amino acids are sensed in the gut, as levels there would also correlate with levels in circulation once they were starting to be absorbed.

Work previously carried out by our laboratory has identified various appetite supressing amino acids including L-Cysteine (McGavigan, O'Hara et al. 2015), L-Arginine (Alamshah, McGavigan et al. 2016) and L-Phenylalanine (Alamshah, Spreckley et al. 2017). The mechanism underlying L-Phenylalanine's anorectic effects were further investigated in this thesis.

L-Phenylalanine was found to decrease food intake when given orally and colonically, to increase neuronal activation in the DVC and stimulate the secretion of anorectic hormones; an effect mediated in part by the CaSR *in vitro*. Additionally, orally administered L-Phenylalanine increased energy expenditure in rats and mice, most likely due to increased locomotor activity.

The oral dose used was, when linearly scaled up, approximately 79 times greater than the daily recommended consumption of L-Phenylalanine in humans and therefore pharmacological. The concentration administered colonically was within physiological range according to (Tanaka, Inoue et al. 1990) but five times greater than concentrations measured in the jejunal content of men 3 hours after a high protein meal (Adibi and Mercer 1973).

The CaSR has been reported to modulate the secretion of various gut hormones, including GLP-1, PYY and CCK, in response to L-Phenylalanine, which acts as a positive allosteric modulator of this receptor (Liou, Sei et al. 2011, Wang, Chandra et al. 2011, Mace, Schindler et al. 2012, Alamshah, Spreckley et al. 2017). Considering combinatorial gut hormone therapies are more effective at supressing food intake, than treatment with individual anorectic hormones (Field, Wren et al. 2010, Cegla, Troke et al. 2014), the CaSR may be an interesting anti-obesity target. The CaSR has been reported to undergo agonist-driven insertional signalling, making it highly resistant to functional desensitisation and thus a promising pharmacological target (Grant, Stepanchick et al. 2011). In accord with this, I found the CaSR was not downregulated in the gut in response to a chronic HPD. However, its broad expression, importance in calcium homeostasis and other roles in the GI tract could lead to several unwanted side effects if targeted pharmacologically. Nonetheless, CaSR agonist

that are largely retained within the gut have been designed, and might prove useful to drive more selective effects (Sparks, Spearing et al. 2017).

Neuronal activation in the DVC, particularly in the NTS and AP, may indicate involvement of the vagus nerve in L-Phenylalanine's effects. The NTS receives and integrates gustatory, mechanical and chemical sensory information from the GI tract, as well as inputs from the hypothalamus (Katz, Nicolelis et al. 2000). It also projects to and regulates the DMN. The DMN is a major source of vagal efferent neurons that innervate the GI tract, mediating motility and secretion, and the pancreas, regulating pancreatic secretion. The DMN is also innervated by the AP, a circumventricular organ and chemoreceptor trigger zone (Loewy, Franklin et al. 1994, Streefland, Maes et al. 1998, Buijs, Chun et al. 2001, Travagli, Hermann et al. 2006). Interestingly, prolonged HPDs also increase c-Fos staining in the NTS (Darcel, Fromentin et al. 2005, Faipoux, Tomé et al. 2008).

L-Phenylalanine stimulates the secretion of gut hormones that can activate the vagus nerve, for example CCK. Additionally, the vagus expresses a limited number of nutrient sensors, including the CaSR, which is modulated by L-Phenylalanine. Consequently, activation of nuclei in the DVC could be downstream of vagal afferent nerve activity in response to gut hormone and/or nutrient sensing. Neuronal activation in the brainstem could also be downstream of CNS signalling, or due to the direct sensing of hormones or nutrients by brainstem nuclei, particularly the AP. The AP expresses a range of receptors for satiety factors including CCK, GLP-1 and PYY, which are secreted in response to L-Phenylalanine (Hill, Campbell et al. 1987, Luckman, Hamamura et al. 1993, Blevins, Chelikani et al. 2008, Baumgartner, Pacheco-López et al. 2010, Richards, Parker et al. 2014). More work is thus required to determine the pathways by which L-Phenylalanine mediates its effects on appetite.

L-Phenylalanine also stimulates the secretion of pancreatic hormones, particularly glucagon, a well reported effect supported by findings in this thesis (Guttler, Kuhl et al. 1978, Nuttall, Schweim et al. 2006, Steinert, Ullrich et al. 2017, Ueda, Sanbongi et al. 2017). Amino acids can modulate pancreatic activity indirectly through vagal networks, neuropeptides, central sensing and gut hormones. Alternatively they can directly act on the pancreas, for example, amino acid transport through sodium coupled transporters can depolarise the membrane of β-cells and stimulate insulin release (Newsholme, Brennan et al. 2006). The dose administered colonically in this study is unlikely to reach organs such as the pancreas at sufficiently high concentrations to cause an effect. Consequently, glucagon secretion in response to colonically administered L-Phenylalanine is most likely mediated by local sensors.

It has been suggested that L-Phenylalanine is able to stimulate the secretion of pancreatic enzymes via neuronal, cholinergic dependent, and hormonal, CCK mediated, pathways (Owyang, Louie et al.

1986, Owyang, May et al. 1986). Moreover, PP secretion in response to duodenal administration of L-Phenylalanine in dogs is inhibited by atropine, suggesting the cholinergic system is also involved in mediating pancreatic hormone secretion in response to L-Phenylalanine (Beglinger, Haecki et al. 1981). Whether similar pathways are involved in L-Phenylalanine induced secretion of glucagon requires further elucidation.

Considering entero-pancreatic vagal reflexes have been associated with L-Phenylalanine's effects on the pancreas, and that vagal afferents express the CaSR which is modulated by L-Phenylalanine, this thesis aimed to investigate the role of the CaSR in vagal afferents in glucagon secretion. To do so, CaSR knockdown was successfully achieved by injecting AAV vectors expressing cre recombinase directly in the NG of CaSR floxed mice. Vagal afferent CaSR knockdown had no effect on baseline or L-Phenylalanine induced glucagon secretion. However, it did block protein induced glucagon secretion. This suggests nutrient sensing via the CaSR in vagal afferent neurons may mediate endocrine secretion.

The lack of NG CaSR knockdown glucagon suppression in L-Phenylalanine treated mice may be due to the pharmacological dose used. This would likely increase circulating levels of L-Phenylalanine, leading to systematic effects that may mask any vagally mediated pathways. On the other hand, administration of a protein load, instead of the amino acid itself, would result in a more physiological levels of pertinent amino acids. Whether vagal CaSR mediated nutrient sensing takes place in the gastric mucosa or other sites such as the hepatic portal vein requires investigation.

The vagus nerve also plays an important role in appetite regulation. It expresses mechanoreceptors and chemoreceptors providing information on the volume and composition of food respectively. Chemosensing of, for example, nutrients and hormones by vagal afferents in the duodenum is thought to be involved in the nutrient composition-mediated negative feedback control of appetite (Powley and Phillips 2004). Consequently, the effect of CaSR NG knockdown on the anorectic effects of L-Phenylalanine were also examined. However, results were inconclusive due to the impaired acclimatisation of CaSR NG knockdown mice to the metabolic cages. This phenomenon suggests vagally expressed CaSR may also play a role in stress or the ability of mice to adapt to new environments.

Overall this thesis was interested in identifying the mechanisms underlying the beneficial effects of HPDs, an effect that is potentially mediated by amino acids. Protein-induced satiety is likely to be mediated by various overlapping pathways. However, by looking at individual amino acids and their effects on energy homeostasis it may be possible to tease these pathways apart to identify new anti-obesity agents. This work focused on the effects of L-Phenylalanine, which was found to modulate

both peripheral and central systems involved in appetite regulation. Moreover, some of its effects were mediated by the CaSR, which may play a role in mediating glucagon secretion through vagal afferent nutrient sensing. Further work is required to determine the importance of vagal nutrient sensing in energy and glucose homeostasis, and whether chronic administration of L-Phenylalanine can reduce long-term food intake and promote weight loss in humans. Furthermore, there are other amino acids that have been shown to supress food intake, and it is possible that combination approaches may be more successful.

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Appendix 1: High protein diet and normal protein diet composition

Product #	NPD		HPD	
%	gm	kcal	gm	kcal
Protein	17.8	15	47.0	40
Carbohydrate	47.3	40	18.1	15
Fat	23.6	45	23.6	45
Total	88.7	100	88.7	100
kcal/gm	4.73		4.73	
Ingredient	gm	kcal	gm	kcal
Casein	150	600	400	1600
L-Cystine	3	12	3	12
Corn Starch	72.8	291	45	180
Maltodextrin 10	100	400	50	200
Sucrose	223	892	50	200
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Lard	177.5	1598	177.5	1598
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0	0	0	0
FD&C Red Dye #40	0.05	0	0	0
FD&C Blue Dye #1	0	0	0.05	0
Total	858.35	4058	857.55	4055

Appendix 2: List of solutions

General

4% formaldehyde solution (PFA): 100ml of 36% formaldehyde, 800ml of 0.01M PBS. Store at 4°C.

40% sucrose: Add 400g of sucrose to 500ml of glass distilled water and stir to dissolve. Adjust volume to 1 litre.

Antifreeze: 30% (v/v) ethylene glycol and 20% (v/v) glycerol in 0.01M PBS

Hepranised PBS for transcardial perfusions: 1:20 dilution of 0.2M PBS containing 1:100 v/v 5000IU/ml heparin solution, cooled to 4°C.

C-Fos immunohistochemistry

Phosphate buffer saline (PBS) (0.2M): 174g Sodium chloride (NaCl), 5.44g potassium dihydrogen phosphate (KH_2PO_4), 28.2g di-sodium hydrogen phosphate dihydrate (Na_2HPO_4. $2H_2O$) in 1 litre of glass distilled water.

Phosphate buffer saline (0.01M): 1:20 dilution of 0.2M phosphate buffer saline

50mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution: 1g of 3,3'-diaminobenzidine tetrahydrochloride, 15ml GDW, 5ml 10x TBS. Stir in the dark on tube rotator for 30 minutes. Filter through 11µm pore diameter filter paper. Store at -20°C.

1% DAB solution: 1ml of 50mg/ml DAB solution, 33µl of 30% H_2O_2, 99ml of 0.01M PBS. Make on the day.

Blocking solution for c-Fos immunohistochemistry: 3% (v/v) normal goat serum, 0.25% (v/v) Triton-X 100 in 0.01M PBS

Secondary antibody diluent for c-Fos immunohistochemistry: 0.3% (w/v) bovine serum albumin, 0.25% (v/v) Triton-X 100 in 0.01M PBS.

ABC solution for c-Fos immunohistochemistry: 1:200 dilution of avidin (A) and biotin (B) in 0.05M TrisHCl.

Tris/HCl solution (1M) (pH 7.6) for c-Fos immunohistochemistry: Add 121.14g Trizma base to 800ml of GDW, pH to 7.6 with HCl, adjust volume to 1 litre.

Tris/HCl solution (0.05M) (pH 7.6) for c-Fos immunohistochemistry: A 1:20 dilution of 1M Tris/HCl

Murine colonic crypt isolation

Digestion media: 0.375mg/ml collagenase from clostridium histolyticum (sigma) made in complete DMEM (10% Foetal bovine serum, 100U/ml penicillin, and 0.1mg/ml streptomycin).

Secretion Buffer:

Salt	**Final concentration** *mM*	**Amount added to 1L GDW** *g*
KCl	4.5	0.335
NaCl	138	8.065
$NaHCO_3$	4.2	0.353
$NaH_2PO_4.2H_2O$	1.2	0.187
$CaCl_2$	2.6	2.6ml of 1M stock solution
$MgCl_2$ (*OR* $MgCl_2.6H_2O$)	1.2	1.2ml of 1M stock solution (*0.244*)
HEPES	10	2.383
pH to 7.4 using NaOH		

On the day, add 0.1% *96% essentially FFA free* BSA and sterile filter (0.2microns)

Lysis buffer

Reagent	**Half strength**
GDW	40ml
Na deoxycholic acid (monohydrate)	0.125g
Igepal	0.25ml
NaCl (5M)	0.75ml Or 0.44 g
Tris HCL (1M, pH 8)	1.25ml

Keep this in a water bath for 15 min then add 1 tablet of complete EDTA-free protease inhibitor cocktail (Roche) and from then on keep on ice.

CaSR immunohistochemistry

10x TBS: 24.2g Trizma base ($C_4H_{11}NO_3$)**,** 80g sodium chloride (NaCl)**, m**ix in 800ml ultra-pure water and adjust pH to 7.6 with pure HCl. Top up with ultra-pure water to 1 litre

1x TBS: 100 ml of 10X TBS, 900 ml GDW

10mM Sodium Citrate, 0.05% Tween 20, pH 6.0: Add 2.94 g of Tri-sodium citrate dehydrate ($C_6H_5Na_3O_7.2H_20$) to 800 ml of GDW, pH to 6.0 with 2M HCl, adjust volume to 1L and add0.5 ml of Tween 20.

Trypsin Stock Solution (0.5% in Distilled Water): Add 50mg of trypsin to 10ml of GDW and mix well. Store at -20 ºC.

Calcium Chloride Stock Solution (1%): Add 0.1g of calcium chloride to 10ml of GDW and mix well. Store at 4 ºC.

Trypsin Working Solution (0.05%): Add 1 ml of trypsin stock solution (0.5%) and 1ml of calcium chloride stock solution 1% to 8mls of 1x TBS. Adjust pH to 7.8 with 1N N NaOH. Store at 4 ºC for one month or -20 ºC for long term storage.

PB no saline: Dissolve 35.61g of $Na_2HPO_4 \bullet 2H_2O$ in 1L of GDW. Dissolve 27.6 g of $NaH_2PO_4 \bullet H_2O$ in 1L of water. Add 770 mL of the $Na_2HPO_4 \bullet 2H_2O$ solution to 230 mL of the $NaH_2PO_4 \bullet H_2O$ solution to make 0.2M PB no saline. Dilute stock 1 in 20 using GDW to make 0.01M PB no saline.

CaSR genotyping

6 x loading buffer: 2.5g Ficoll 400 (Pharmacia Biotech, Sweden), 400µl 0.5M EDTA solution and 18µl 10% (w/v) $CH_3(CH_2)_{11}OSO_3Na$ (sodium dodecyl sulphate, SDS) (Sigma-Aldrich, UK) and bring volume to 10ml using ultra-pure water. Add $C_{16}H_{10}N_2Na_2O_7S_2$ (Orange G) (Merck Milllipore, USA) to desired colour intensity and mix by inverting.

50X Tris-acetate-EDTA (TAE): Add 242g $NH_2C(CH_2OH)_3$ (Trizma base) (Sigma-Aldrich, UK), 57.1ml of glacial acetic acid, 100ml of 0.5M $C_{10}H_{14}H_2ONa_2.2H_2O$ (EDTA) to 600ml of H_2O. Adjust pH to 8.0 and bring solution to a final volume of 1 litre.

Composition of proteinase K digestion mix.

Reagent	Volume (µl)
20mg/ml Proteinase K	1.25
10 X Bioline PCR buffer	5
Water	44
	50/ear notch

Composition of mastermix for CaSR genotyping

Reagent	Volume (µl)
Water	17.5
Bioline 10X PCR buffer	2.5
Bioline 50mM MgCl2	0.875
25mM dNTPs	0.2
Primer pair mix 50 uM	0.25
DMSO	1.25
Bioline Taq polymerase	0.1
DNA Digest	3
	25

Solutions for in house radioimmunoassay

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

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 4ºC.

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

Appendix 3: Macro used for c-Fos automated counting

analysiscfos.ijm

File Edit Language Templates Run Tools Tabs

analysiscfos.ijm

```
fn=getTitle();
run("Duplicate...", "duplicate");
nn=getString("Rename image", fn);

rename("temp");

roiManager("Reset");
run("RGB Color");
run("Colour Deconvolution", "vectors=[H DAB]");

selectWindow("temp (RGB)-(Colour_2)");
rename(nn+" Ananlysis");
run("Smooth");
setAutoThreshold("Yen");
run("Threshold...");
title = "Get info";
msg = "set threshold \n and select OK to continue";
waitForUser(title, msg);

run("Analyze Particles...", "size=20-Infinity summarize add");

selectWindow("temp (RGB)-(Colour_1)");
close();
selectWindow(nn+" Ananlysis");
close();
selectWindow("temp (RGB)-(Colour_3)");
close();
selectWindow("Colour Deconvolution");
close();

selectWindow("temp (RGB)");
roiManager("Show All without labels");
run("Flatten");
rename(nn+" Overlay");

selectWindow("temp (RGB)");
close();
selectWindow("temp");
close();

```

This macro was kindly written by Steven Rothery based at the Imperial College Film facility.

Appendix 4: Assay cross reactivity and sensitivity

Cisbio- Glucagon

ASSAY PERFORMANCES

Assay range	15.6 to 2,000 pg/mL
Limit of detection (LoD*) = Std 0 Mean + 2 SD	12 pg/mL
incubation time	4h to overnight at RT

*The analytical sensitivity was calculated from data obtained with the PHERAstar Plus reader (flash lamp excitation) after overnight incubation. This may vary from one HTRF compatible reader to another.

SPECIFICITY:

The different peptides listed below were tested up to 10 µM

	Specificity (% of recognition)
Glucagon	100%
Glucagon fragment 1-18	<1.81%
Glucagon fragment 19-29	<0.03%
Oxyntomodulin	<0.07%
Glicentin	<0.07%
GLP-1 (7-36 amide)	<0.06%
GLP-1 (7-37)	<0.11%
GLP-2	<0.3%
GRPP (Glicentin-Related Pancreatic Peptide)	<0.01%

Cisbio- Insulin

ASSAY PERFORMANCES AND CALIBRATION

Calibration	NIBSC International standard 66/304 1 ng Insulin HTRF ⟷ 1 ng Insulin NIBSC 66/304 (i.e.: 2.3 x 10-05 IU)
Assay range	0.24 to 8 ng/mL
Limit of detection (LoD*) = Std 0 Mean + 2 SD	0.064 ng/mL
incubation time	Overnight at RT

ASSAY CHARACTERISTICS

Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies for an analyte and any of the other analytes in this panel.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions (mean minDC + 2 standard deviations, N=8 assays).

Analyte	MinDC	MinDC+2SD
Amylin	19	30
C-Peptide 2	20	39
Active Ghrelin	3	4
GIP	1	1
GLP-1	23	41
Glucagon	20	41
IL-6	8	15
Insulin	14	30
Leptin	19	40
MCP-1	13	26
PP	2	6
PYY	5	11
Resistin	23	43
TNFα	8	16

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Expected presentation date	Jul 2019
Portions	Figure 1

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