The role of amino acids in appetite regulation

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Abstract

There is currently a lack of safe and effective treatment options for obesity. A high protein diet is an effective weight loss and weight maintenance strategy. However, like many diets, high protein diets can be difficult to adhere to. The mechanisms by which protein exerts its weight-reducing effect remain unclear. However, it has been reported that different types of protein exert different effects on appetite. One possible explanation for these differences is the varied amino acid constituents of the protein. Preliminary data from our group investigated the effect of a range of amino acids on food intake in rodents. L-cysteine was identified as the most anorexigenic amino acid. This thesis has investigated the effect of L-cysteine on food intake and explored possible mechanisms by which it mediates this effect.

L-cysteine dose dependently decreased food intake in both rats and mice following oral gavage and intraperitoneal administration. This reduction in food intake did not appear to be secondary to behavioural side effects or feelings of nausea. L-cysteine increased neuronal activation in the area postrema and nucleus tractus solitarius, delayed gastric emptying and suppressed plasma acyl-ghrelin levels. However, the anorectic effect of L-cysteine did not appear to depend on NMDA, GPRC6A or CCK-A receptors, nor on subdiaphragmatic vagal afferent signalling. Repeated administration of L-cysteine also decreased food intake in rats and diet-induced obese mice.

The studies described in this thesis demonstrate the anorectic effects of L-cysteine and identify possible sites of action. It is likely that different amino acids exert different effects on appetite through a number of mechanisms, the combination of which contributes towards the success of high protein diets on body weight and appetite. This thesis provides a framework for future studies to investigate the therapeutic potential of combinations of amino acids that could provide a safe and practical therapeutic treatment for obesity.

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Declaration of contributors

This thesis was composed entirely by the author and all of the work in this thesis was performed by the author with any collaboration and assistance described below:

Chapter 2

Preliminary data presented in the introduction was carried out by Dr Hannah Greenwood. All in-house radioimmunoassays were established and maintained by Professor M. Ghatei. Brainstem c-Fos was counted by Miss Kathryn Miller who was blinded from treatment groups. Cannulation experiments were performed with assistance from Dr James Kinsey-Jones.

Chapter 3

SDA surgeries were performed with assistance from Gina Hyberg (AstraZeneca). The GPRC6A knockout mouse colony was bred and maintained by Dr James Kinsey-Jones.

Abbreviations

3-MST	3-mercaptopyruvate sulfurtransferase
5-HT	5-hydroxytryptamine (serotonin)
5-HT_{2c}	Serotonin 2c receptor
7TM	7 transmembrane
AAV	Adeno-associated virus
AC	Adenylate cyclase
AgRP	Agouti related peptide
AHA	Anterior hypothalamic area
ANOVA	Analysis of variance
AP	Area postrema
APC	Anterior piriform cortex
ARC	Arcuate nucleus
ATP	Adenosine trisphosphate
BBB	Blood brain barrier
BMI	Body mass index
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated transcript
CaSR	Calcium sensing receptor
CAT	Cysteine amino transferase
CB1R	Cannabinoid 1 receptor
CBS	Cystathionine beta synthetase
CCK	Cholecystokinin
CCK-1R	Cholecystokinin 1 receptor
CCK-2R	Cholecystokinin 2 receptor
CDO	Cysteine dioxygenase
cFLI	c-Fos like immunoreactivity
c-Fos	Cellular Fos
CHH	O-carboxymethyl hydroxylamine hemihydrochloride
CL	Cysteine lyase
CNS	Central nervous system
CSD	Cysteine sulfinate decarboxylase
CSE	Cystathionine gamma lyase
CTA	Conditioned taste aversion
DA	Dopamine
DAG	Diacylglycerol
DIO	Diet induced obese
DIT	Diet induced thermogenesis
DMC	Dorsal vagal complex
DMN	Dorsomedial nucleus
DMX	Dorsal motor nucleus of the vagus
eIF2α	Eukaryotic initiation factor 2 α
EMEA	European Medicines Agency
ENS	Enteric nervous system

ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase 1/2
FDA	Food and Drugs Administration
FTO	Fat mass and obesity associated protein
GCGR	Glucagon receptor
GCN2	General control nonrepressed 2
GDP	Guanosine diphosphate
GDW	Glass distilled water
GHRL	Ghrelin gene
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 recetor
GOAT	Ghrelin o-acyltransferase
GPCR	G-protein coupled receptor
GPRC6A	G-protein coupled receptor group C member 6A
GSH	Glutathione
GSSG	Glutathione disulphide
GTP	Guanosine trisphosphate
HFD	High fat diet
HPLC	High pressure liquid chromatography
IAA	Indispensible amino acid
ICV	Intracerebroventricular
IHC	Immunohistochemistry
IP	Intraperitoneal
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor
IQR	Interquartile range
IR	Insulin receptor
KO	Knockout
Lep-R	Leptin receptor
LHA	Lateral hypothalamic area
LTP	Long term potentiation
MBH	Mediobasal hypothalamus
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MCH	Melanin-concentrating hormone
MCHR1	Melanin-concentrating hormone receptor 1
MCHR2	Melanin-concentrating hormone receptor 2
MEF	Mouse embryonic fibroblasts
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NA	Noradrenaline
NAcc	Nucleus Accumbens
NDP-MSH	[Nle4,D-Phe7] alpha-melanocyte stimulating hormone
NMDA	N-methyl-D-aspartate

NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
Ob-R	Leptin receptor
OG	Oral Gavage
OX1R	Orexin receptor 1
OX2R	Orexin receptor 2
OXM	Oxyntomodulin
PBS	Phosphate buffered saline
Pe	Periventricular nucleus
PIP_2	Phosphotidylinositol-4,5 bisphosphate
PLC	Phospholipase C
PLP	Pyridoxal 5' phosphate
POMC	Pro-opiomelanocortin
PPG	Proparagylglycine
PVN	Paraventricular nucleus
PYY	Peptide Tyrosine Tyrosine
RIA	Radio-immunoassay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SC	Subcutaneous
SDA	Subdiaphragmatic vagal deafferentation
SEM	Standard error of the mean
SLC	Solute-linked carrier
SNAC	Sodium N-[8-(2-hydroxybenzoyl) amino caprylate
SNP	Single nucleotide polymorphism
SON	Supraoptic nucleus
T1R1	Taste receptor type 1 member 1
T1R2	Taste receptor type 1 member 2
T1R3	Taste receptor type 1 member 3
T2DM	Type II Diabetes Mellitus
TG	Triglyceride
TRPM5	Transient receptor potential cation channel subfamily M member 5
TSC	Tuberosclerosis complex
VFT	Venus fly trap
VMN	Ventromedial nucleus
VTA	Ventral tegmental area
α-MSH	Alpha-melanocyte stimulating hormone
γGCS	Gamma-glutamyl cysteine synthetase

Table of Contents

List of Figures

List of Tables

CHAPTER 1: GENERAL INTRODUCTION

1.1 The Obesity Epidemic

Humans regulate body weight and appetite through a complex array of neural systems. These systems originated in a period when food supplies were sporadic. This limited availability of food provided an evolutionary drive for neural reward mechanisms to be closely associated with the systems involved in the procurement and consumption of food. This association has been conserved in humans (Ulijaszek, 2002) and, as might be predicted, appears to better defend the lower rather than the upper limits of adiposity (Hill and Peters, 1998). In today's modern industrialized society in which food supply, for most, is plentiful and readily available, this conserved relationship between the neural physiology and psycho-social aspects of eating has resulted in an obesity epidemic.

In England, 61.7% of the adult population (≥16years) and 30% of children (<16years) are considered overweight or obese (Aresu, 2010). Previously considered a chronic disease associated mainly with Western or other high-income countries, obesity is now approaching the proportions of a global epidemic. In 2008, the World Health Organization (WHO) estimated that over 10% of the world's adult population (≥20years) were obese (WHO, 2008), with global obesity having doubled since 1980. Obesity is now in the top five leading risks for global deaths (WHO, 2009).

Obesity is a significant risk factor for some of the United Kingdom's leading health problems; cardiovascular disease, cancer, type 2 diabetes mellitus, hypertension and osteoporosis. The financial burden of treating these conditions (McPherson, 2007) and their relative high prevalence make obesity a major public health issue. Obesity and the aforementioned co-morbidities are largely preventable by reducing energy intake and/or increasing energy expenditure. However, despite long standing public health advice, the prevalence of obesity is still increasing and a satisfactory treatment has yet to be been found.

1.1.1 Current treatment options

Current strategies for obesity management include lifestyle changes, pharmacological intervention and bariatric surgery. Bariatric surgical procedures most successfully achieve sustained weight loss. However, due to the expensive and highly invasive nature of these procedures, they are generally only available to the morbidly obese. In England,

approximately 2% of the population is morbidly obese (BMI >40kg/m^2), but a further 60% is considered to be overweight or obese (BMI 25-40kg/m^2) (Aresu, 2010). For the majority of the overweight population the only options are therefore lifestyle and/or pharmacological interventions. However, long term compliance with lifestyle interventions is low and the efficacy of currently available drugs is limited, leading to relatively low successful treatment rates (Dansinger et al., 2005).

Orlistat, a gastric and pancreatic lipase inhibitor, is the only prescription medicine for obesity currently licensed in the UK. The European Medicines Agency (EMEA) and the Food and Drugs Administration (FDA) currently recommend that for a new anti-obesity drug to be approved it should result in a statistically significant placebo adjusted weight loss of greater than 5% at the end of a 12 month period. Less than 30% of patients on Orlistat achieve this magnitude of weight loss (Powell et al., 2011).

In 2012, the FDA approved two new pharmacological treatments for obesity, Qsymia® and Belviq®, for use in the United States. Both, however, have limitations. Qsymia® (which was originally named Qnexa® during development) is a combination of the anticonvulsant Topiramate, a weak carbonic anhydrase inhibitor, and the appetite suppressant Phentermine, an amphetamine derivative. Qnexa® demonstrated superior efficacy to currently available treatments; the SEQUEL study reported 79% of Qnexa® treated patients achieved >5% weight loss (VivusInc, 2010). However, its development was burdened with safety concerns: side effects included tachycardia, and it was suggested it might cause teratogenic effects and eye problems. It has therefore been marketed as a treatment for obesity in adults but is contraindicated for women of child bearing age, patients with glaucoma, patients with thyroid problems and for patients taking monamine oxidase inhibitors. Since its marketing approval in 2012, Qsymia® has been met with relatively poor sales, which are likely to be partially related to these safety issues. Belviq® (Lorcaserin), a selective 5-HT_{2C} receptor agonist, is limited more by its relatively low efficacy. Lorcaserin treatment resulted in a 3.6% placebo adjusted weight loss during Phase III clinical trials (Smith et al., 2010), which is below the FDA recommended level of 5%. It is likely this limited efficacy will also negatively impact its future marketing. Neither drug has been approved for use in Europe. It is therefore unlikely either will act as a panacea for the global obesity epidemic and thus new treatment strategies are needed.

1.2 Energy Homeostasis

Maintenance of energy balance is a complex multi-factorial process involving a number of central and peripheral components. Key players include the gustatory system, gastrointestinal (GI) tract, liver, pancreas, skeletal muscle and adipose tissue, which are all in bidirectional communication with the brain via the autonomic nervous system, hormones and metabolites.

1.2.1 Central Regulation of Energy Homeostasis

A number of brain regions are involved in controlling our eating behaviour. The most important are thought to be the hypothalamus, where nutrient, hormonal and neuronal signalling is integrated to regulate feeding, parts of the cortex and limbic system which are affiliated with the emotional and reward aspects of eating, and the caudal brainstem which is involved in the interpretation of peripheral signals from organs including the GI tract.

1.2.1.1 The Hypothalamus and homeostatic neuropeptides

The hypothalamus contains several nuclei including the arcuate nucleus (ARC), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) (Fig. 1.1) that collectively co-ordinate orexigenic and anorexigenic signals from within and from outside the central nervous system (CNS) to control energy homeostasis. The ARC is located at the base of the hypothalamus beside the median eminence, an area devoid of a blood brain barrier, and is thus accessible to circulating nutrients and hormones. The ARC contains two functionally discrete populations of neurons involved in the regulation of energy homeostasis: neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons, which stimulate food intake, and proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) neurons, which inhibit food intake (Fig 1.2). These neurons have extensive connections to higher order neurons in other regions of the hypothalamus and extra-hypothalamic brain regions (Bagnol et al., 1999).

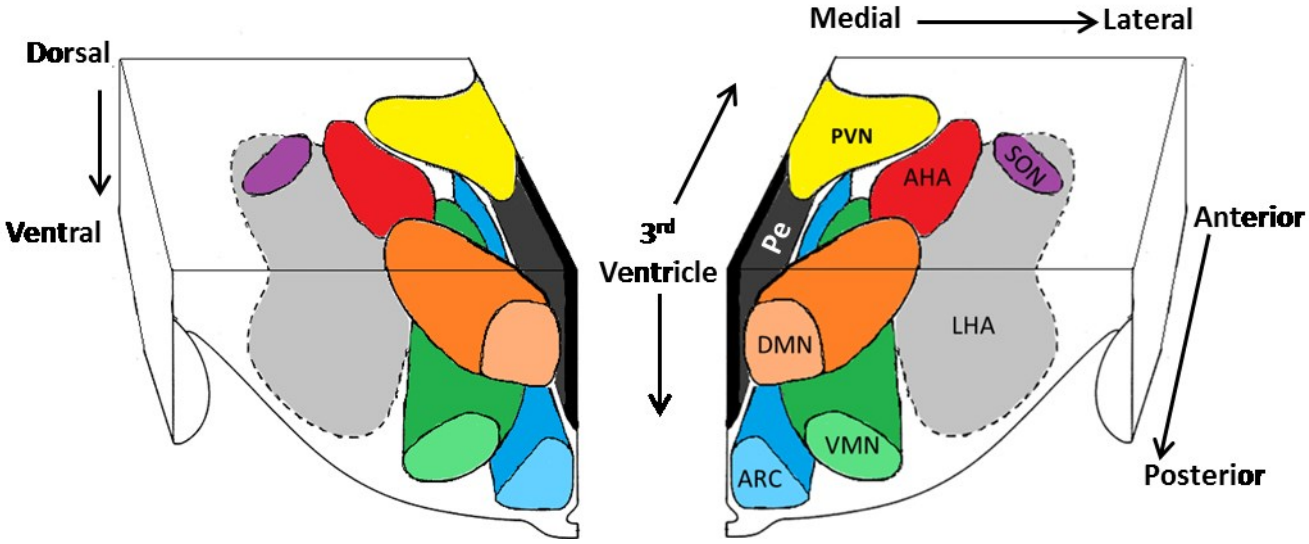


Figure 1.1 –Three-dimensional structure of the rat hypothalamus.

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

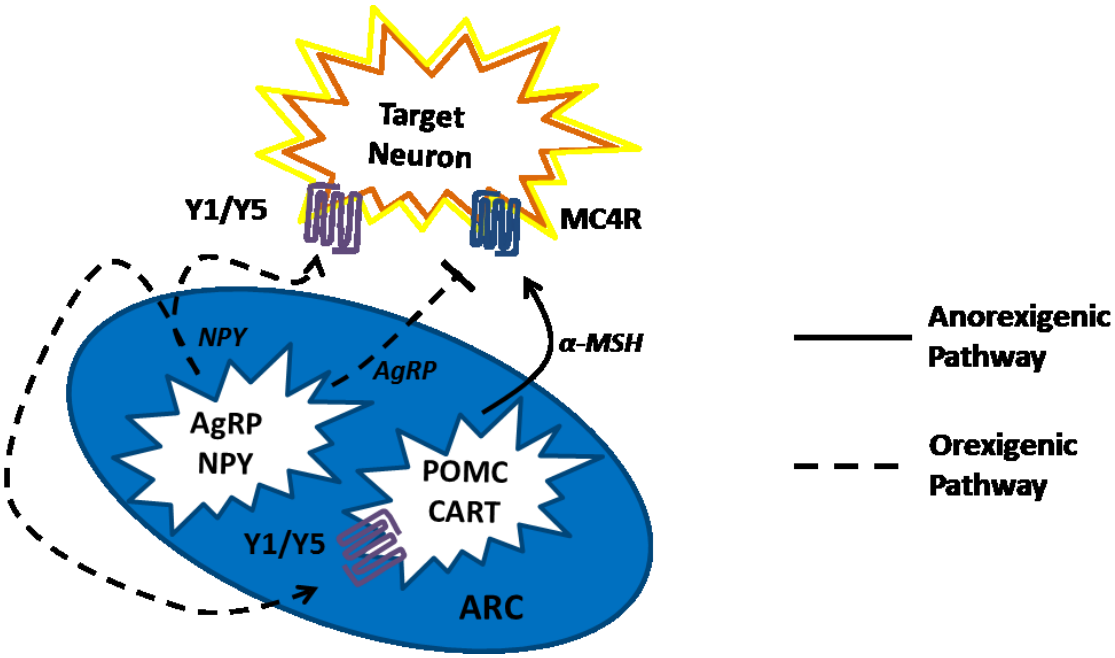


Figure 1.2 – Schematic diagram illustrating the functional roles of the ARC AGRP/NPY and POMC/CART neurons in appetite

α-MSH: alpha-melanocyte stimulating hormone, AgRP: agouti related peptide, ARC: arcuate nucleus, CART: cocaine amphetamine regulated transcript, MC4R: melanocortin 4 receptor, NPY: neuropeptide Y, POMC: proopiomelanocortin

1.2.1.1.1 Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is a neuropeptide widely expressed in the brain and highly expressed in the ARC (Morris, 1989). It is the most potent orexigenic neuropeptide known and acts on Y1 and Y5 receptors to increase food intake (Rudolf et al., 1994, Kanatani et al., 1996, Criscione et al., 1998). The Y1 receptor is widely expressed in the CNS, and within the hypothalamus is expressed specifically in the PVN, ARC and supraoptic nucleus (SON) (Kopp et al., 2002). The Y5 receptor is expressed in the LHA, PVN, SON and ARC (Durkin et al., 2000). ICV administration of NPY, or Y1 or Y5 specific agonists induces feeding (Cabrele et al., 2000, Mullins et al., 2001, Clark et al., 1984). The Y2 receptor, which is the receptor for the anorexigenic gut hormone Peptide YY (see section 1.2.2.3.4), is coexpressed with NPY in the ARC where it is thought to function as an inhibitory autoreceptor to dampen NPY release (Broberger et al., 1997). In addition, arcuate NPY acts as a neuromodulator of neighbouring POMC neurons via the activation of Y1 receptors on these neurons (Fig. 1.2) (Roseberry et al., 2004).

Adeno-associated virus (AAV)- mediated overexpression of NPY in the LHA increases meal size, and in the PVN increases meal frequency (Tiesjema et al., 2007). Chronic central administration of NPY causes obesity (Stanley et al., 1986). Pharmacological evidence therefore suggests NPY is important in appetite regulation. In addition, NPY expression in the ARC is upregulated by fasting (Sahu et al., 1988), and knockdown of ARC NPY expression reduces food intake and body weight in rats (Gardiner et al., 2005). However, germline NPY knock-outs have no appetite or body weight phenotype (Erickson et al., 1996) suggesting developmental compensation.

Specific Y1 and Y5 receptor antagonists have been developed as potential anti-obesity agents (Erondu et al., 2006, Antal-Zimanyi et al., 2008). However, many have failed to progress to clinical trials for reasons including low oral bioavailability, poor brain penetrability and a lack of selectivity, and those that have progressed to clinical trial have shown poor efficacy in long term randomized control trials (Erondu et al., 2006, Antal-Zimanyi et al., 2008).

1.2.1.1.2 Agouti related peptide (AgRP)

Agouti Related Peptide (AgRP) is co-expressed with NPY and γ-aminobutyric acid (GABA) in the arcuate nucleus (Broberger et al., 1998). It is an orexigenic peptide (Rossi et al., 1998) that is an inverse agonist of the melanocortin 4 receptor (MC4R) (Nijenhuis et al., 2001) and an antagonist of the melanocortin 3 receptor (MC3R) (Ollmann et al., 1997). Central administration of AgRP stimulates food intake in rodents (Rossi et al., 1998), and fasting increases AgRP expression in the ARC (Bi et al., 2003). Similar to NPY, germline AgRP knockouts have no appetite or body weight phenotype (Qian et al., 2002) and overexpression leads to obesity (Graham et al., 1997). However, post-natal ablation of NPY/AgRP neurons produces a profound reduction in food intake and body weight (Gropp et al., 2005, Luquet et al., 2005), suggesting there may be a degree of developmental compensation in the knock outs. The effect of post-natal AgRP neuron ablation on food intake and survival can be reversed by administration of a $GABA_A$ receptor agonist into the parabrachial nucleus (PBN), suggesting GABA release from AgRP neurons onto neurons in the PBN is essential for maintenance of food intake and body weight (Wu et al., 2009).

1.2.1.1.3 α-Melanocyte-stimulating hormone (α-MSH)

Alpha-Melanocyte-stimulating hormone (α-MSH) is an anorexigenic neuropeptide that is a post-transcriptional cleavage product of POMC. POMC is expressed in the ARC and also in the nucleus tractus solitarius (NTS) of the brainstem. However, α-MSH can be found in the PVN, DMN, ARC and NTS amongst other areas (Jacobowitz and O'Donohue, 1978). Fasting increases POMC expression in the rodent ARC (Mizuno et al., 1998). Alpha-MSH is an agonist of the MC3 and MC4 receptors and central administration in rodents inhibits food intake (Tsujii and Bray, 1989).

MC3R has relatively limited expression, but is found in the ARC, DMN, thalamus, ventral tegmental area (VTA), raphe nucleus and hippocampus (Roselli-Rehfuss et al., 1993). MC3R knock-out mice have a mild phenotype with a small increase in body weight and fat mass, but without hyperphagia (Chen et al., 2000).

MC4R is widely expressed, being found in the amygdala, thalamus, cortex, striatum, hippocampus and brainstem, and within the hypothalamus in the ARC, PVN and DMN (Gantz et al., 1993, Mountjoy et al., 1994). MC4R knock-outs are obese and hyperphagic

(Huszar et al., 1997) and selective re-expression of MC4R in the PVN and central amygdala attenuates approximately 60% of this obese phenotype through reduced overeating, suggesting these regions are major targets of MC4R appetite signalling (Balthasar et al., 2005). Human MC4R mutations are the most common known cause of monogenic obesity (Farooqi et al., 2000, Vaisse et al., 2000, Vaisse et al., 1998). These findings suggest that the MC4R mediates an anorectic tone that when disrupted results in obesity.

A number of MC4R agonists have been developed as potential anti-obesity agents (Fehm et al., 2001, Hallschmid et al., 2006, Krishna et al., 2009). However, their development has been hampered by poor efficacy (Hallschmid et al., 2006, Krishna et al., 2009) and adverse side effects such as pressor activity (Kuo et al., 2004, Skibicka and Grill, 2009).

1.2.1.1.4 Cocaine and amphetamine-regulated transcript (CART)

Cocaine and amphetamine-regulated transcript (CART) has a less clear role in energy homeostasis. CART is expressed in a number of hypothalamic nuclei, including the ARC, PVN, LHA, DMN, and SON (Koylu et al., 1997). It is colocalised with POMC in the ARC, oxytocin and vasopressin in the PVN and melanin concentrating hormone (MCH) in the LHA (Vrang et al., 1999). There is no known receptor for CART and thus the mechanisms by which it affects energy homeostasis are poorly understood. ICV administration of CART peptide reduces food intake in rodents and ICV administration of CART antiserum increases food intake (Lambert et al., 1998). In contrast, direct administration of CART peptide into specific hypothalamic nuclei has an orexigenic effect, suggesting the anorectic effects of CART are not mediated within the hypothalamus (Abbott et al., 2001). It has been suggested that the anorectic actions of CART peptide may be a brainstem mediated effect as blockage of the cerebral aqueduct attenuates the anorectic effect of 3rd ventricle ICV CART peptide (Aja et al., 2001).

1.2.1.1.5 Melanin-concentrating hormone (MCH)

Melanin-concentrating hormone is an orexigenic hormone that is highly expressed in the LHA and zona incerta (Bittencourt et al., 1992). It acts on the receptor MCH-R1 (Chambers et al., 1999) and in humans also on MCH-R2 (Sailer et al., 2001, Hill et al., 2001). Central administration of MCH increases food intake in rodents (Della-Zuana et al., 2002). MCH knockout results in hypophagia, leanness and increased basal metabolic rate (Shimada et al.,

1998), whereas MCH overexpression causes hyperphagia and increased susceptibility to obesity (Ludwig et al., 2001).

1.2.1.1.6 Orexins

Orexin A and B are putative orexigenic neuropeptides also expressed in the LHA (Sakurai et al., 1998). There are two orexin receptors, OX1R and OX2R. Orexin A binds to both with high affinity, whereas orexin B binds preferentially to OX2R. OX1R is most highly expressed in the VMN and OX2R most highly in the PVN (Trivedi et al., 1998). Although orexin knockout mice are hypophagic, their predominant phenotype is narcolepsy. Central injection of either orexin A or B stimulates food intake in rats (Sakurai et al., 1998) but it is unclear whether this is a primary effect, or a secondary effect as a result of increased arousal.

An overview of the expression of the discussed hypothalamic neuropeptides and their receptors is summarized in Fig. 1.3.

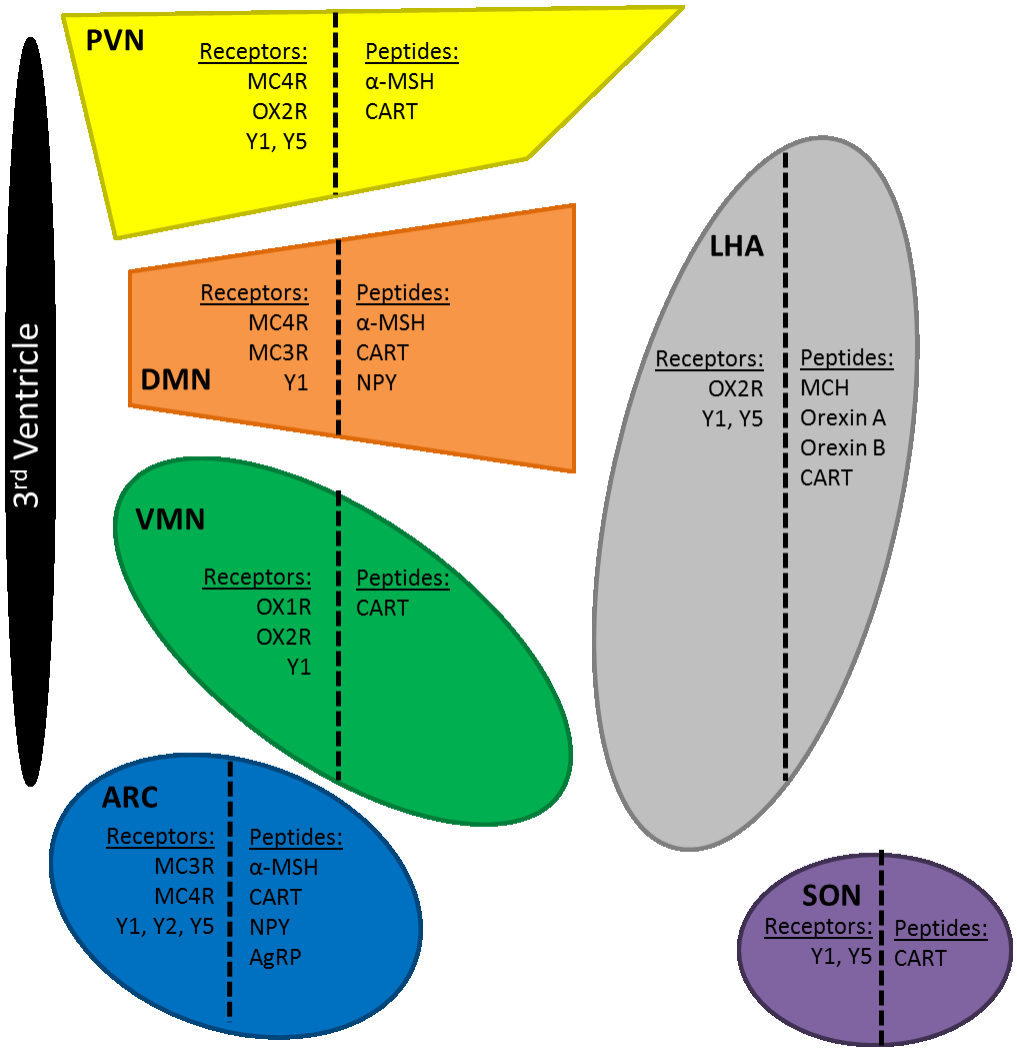


Figure 1.3 – Overview of the hypothalamic expression of neuropeptide and neuropeptide receptors involved in energy homeostasis.

α-MSH: alpha-melanocyte stimulating hormone, AgRP: agouti related peptide, AHA: anterior hypothalamic area, ARC: arcuate nucleus, CART: cocaine and amphetamine regulated transcript, DMN: dorsomedial nucleus, LHA: lateral hypothalamic area, MC3R: melanocortin 3 receptor, MC4R: melanocortin 4 receptor, MCH: Melanin-concentrating hormone, NPY: neuropeptide Y, OX1R: Orexin 1 receptor, OX2R: Orexin 2 receptor, Pe: periventricular nucleus, PVN: paraventricular nucleus, SON: supraoptic nucleus, VMN: ventromedial nucleus

1.2.1.2 Central non-peptidergic systems and energy homeostasis

Although hypothalamic neuropeptide systems play a crucial role in energy homeostasis, it is the non-peptidergic systems which have been the predominant target in pharmacological treatment of obesity to date. These systems include the monoamines and the cannabinoids.

1.2.1.2.1 Monoamines

The monoaminergic system was one of the earliest targets in the fight against obesity. This system includes the neurotransmitters dopamine (DA), noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT), all of which are involved in a number of physiological processes including emotion, cognition and feeding.

Centrally acting sympathomimetics, such as the amphetamine derivatives phentermine, diethylpropion and phendimetrazine, were some of the earliest pharmacological agents used for weight loss (Rodgers et al., 2012). The general mechanism of action of these agents included competitive inhibition of monoamine re-uptake by the monoamine transporters and subsequent release of stored neurotransmitters, collectively increasing the synaptic levels of monoamines (Nelson and Gehlert, 2006). However, their use as anti-obesity agents was limited due to their stimulant properties, particularly their actions on the sympathetic nervous system which can result in adverse cardiovascular effects and the abuse potential of these compounds. Nonetheless, these three amphetamine derivatives are still licensed by the FDA for the treatment of obesity, albeit only as a short term (less than 12 weeks) treatment. Phentermine is the most commonly used and is also found in the combination drug Qsymia®, mentioned previously (see section 1.1.1). These early amphetamine derivatives, although still available, were superseded in the 1970-80s by the 5-HT releasing agents fenfluramine and dexfenfluramine. In the 1990's a combination therapy of fenfluramine and phentermine known as fen-phen showed superior efficacy and was widely prescribed off-label in the USA. This combination, and both fenfluramine and dexfenfluramine were withdrawn from the market in 1997 due to side effects including pulmonary hypertension and cardiac valvulopathy (Boughner, 1997, Wadden et al., 1998, Tomita and Zhao, 2002). Sibutramine, a dual monoamine (NA and 5-HT) re-uptake inhibitor was introduced into the market in 1997 as a treatment for obesity. However, it was withdrawn in 2010 following evidence that it increased cardiovascular events in post-

marketing trials (James et al., 2010). Despite the relatively unsuccessful history regarding monoamine targeted pharmacotherapy for obesity, the 5-HT_{2c} agonist Lorcaserin (Belviq®) was recently approved by the FDA as a long term treatment for obesity in the United States (safety and efficacy discussed previously in Section 1.1.1). The 5-HT_{2c} receptor is predominantly expressed on POMC neurones, and Lorcaserin is believed to mediate its anorectic effect by stimulating α-MSH release (Redman and Ravussin, 2010).

1.2.1.2.2 Cannabinoids

The ability of endocannabinoids, a group of endogenous neuromodulatory lipids, and phytocannabinoids, such as those found in cannabis plants, to stimulate appetite in animals and humans has been well documented (Williams and Kirkham, 1999, Kirkham and Williams, 2001, Di Marzo et al., 2001, Pagotto et al., 2006). They mediate their orexigenic effects via the CB1 receptor (Colombo et al., 1998, Williams and Kirkham, 1999), which is expressed widely within the central and peripheral tissues (Herkenham et al., 1991, Pagotto et al., 2006, Herkenham et al., 1990, Engeli et al., 2005). The utility of the CB1 receptor as a pharmacological target for anti-obesity drugs has thus been widely investigated. A CB1 receptor inverse agonist, Rimonobant, was approved as an anti-obesity agent in Europe in 2006. However, it was withdrawn from the market in 2008 due to reports of serious psychiatric problems, such as anxiety, depression and suicidal ideation (Christensen et al., 2007). Despite these side effects, this receptor is still under investigation as a potential target for anti-obesity agents. Neutral CB1 antagonists and CB1 antagonists that do not cross the blood brain barrier and thus potentially attenuate these side effects are being developed. Several pre-clinical studies have demonstrated that peripherally acting CB1 antagonists can reduce feeding (LoVerme et al., 2009, Pavon et al., 2008, Tam et al., 2010)

1.2.1.3 The Brainstem

The brainstem is another key player in the regulation of energy homeostasis. The caudal brainstem, in particular, integrates neural gustatory and gut vagal afferent signals, relaying them to higher brain centres to modulate food intake (Schwartz, 2006). The dorsal vagal complex (DVC), which comprises the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMX) and the area postrema (AP), is the main region of the caudal brainstem involved in the control of energy homeostasis (Schwartz, 2006).

The NTS receives input from the glossopharyngeal nerve, which relays taste information from the gustatory system, and from vagal afferents which relay information from the gastrointestinal tract, including mechano- and chemo-sensory information as well as endocrine signals (discussed in more detail in Section 1.2.2.2). The NTS has projections to hypothalamic and reward centre nuclei (Ito and Seki, 1998).

The DMX provides parasympathetic motor innervation to peripheral regions including the upper GI tract. The DMX receives input from the NTS and is the location from which vagal efferents stem, thus the NTS and DMX form the centre of the vagovagal reflex.

The AP is a circumventricular organ, devoid of a blood brain barrier making it accessible to circulating nutrients and peptide hormones, and an important site for the integration of circulating peripheral signals. It is also important for the detection of circulating toxins and is involved in the emetic response. The AP has projections to the NTS, DMX and parabrachial nucleus (Ito and Seki, 1998).

1.2.1.4 Cortex and Limbic system

The homeostatic control of eating is more sensitive to under rather than over-nutrition. Key drivers of this asymmetric regulatory system are the hedonic aspects of eating, including reward based pathways which are activated by highly palatable foods. Activation of these pathways increases the motivation to eat, and appear to override the physiological signals which promote in the cessation of eating. Major areas of the brain involved in hedonic eating include the VTA and nucleus accumbens (NAcc) (Berridge et al., 2010).

1.2.1.5 Central obesity targets

The anti-obesity pharmaceutical program has been largely unsuccessful. With the exception of Orlistat, the only available prescription treatment for obesity available in Europe, the industry has focused on central targets. These have been largely met with adverse side effects and poor efficacy, the latter of which may be secondary to poor oral bioavailability or low penetrability of the blood brain barrier. Nonetheless, a greater understanding of the complexity of these systems and better pre-marketing analysis of potential side-effects will be needed for the development of successful and safe centrally acting anti-obesity agents.

1.2.2 Peripheral Regulation of Energy Homeostasis

Homeostatic signals arising from the periphery are classically divided into either short-term episodic signals that are rhythmically released in response to eating, such as gastrointestinal peptide hormones, or long term tonic signals, such as insulin and leptin that are released in proportion to fat stores. These signals inform central homeostatic pathways of the body's current metabolic status.

1.2.2.1 Adiposity signals

To effectively regulate energy homeostasis the brain must be aware of energy availability in the periphery.

1.2.2.1.1 Leptin

Leptin is the protein hormone product of the ob gene, and its discovery in 1994 (Zhang et al., 1994) transformed the field of obesity research. It is an adipocyte-derived hormone (adipokine) with multiple roles within the body, but predominantly acting as a long term signal informing the brain of adipose energy reserves. Plasma leptin levels correlate with body adiposity (Schwartz et al., 1996, Maffei et al., 1995). Circulating leptin is transported across the blood brain barrier (BBB) in proportion to peripheral levels, informing the central appetite centres of peripheral energy availability (Schwartz et al., 1996). Leptin binds to the leptin receptor (Ob-R or LEP-R) to exert its effects. Increased leptin signalling decreases food intake and increases energy expenditure (Weigle et al., 1995, Pelleymounter et al., 1995, Halaas et al., 1995). Alternatively, a decrease in leptin signalling, either through low levels of adiposity or through gene/receptor defects has a profound effect on many regulatory systems, including reproductive and energy homeostatic pathways (Boden et al., 1996, Keim et al., 1998, Chehab et al., 1996).

Following its discovery, exogenous leptin therapy was proposed as a treatment for obesity. However, it was later discovered that obesity is associated with leptin resistance, meaning that this therapeutic strategy was largely unsuccessful. Nevertheless, the therapeutic potential of leptin has not been entirely ruled out. One of the most difficult aspects of curbing the obesity epidemic is weight maintenance after initial weight loss. Weight loss is associated with a decrease in energy expenditure that is more than that expected from the decrease in the fat and lean mass. Thus a previously obese individual has a lower energy

expenditure than a person with the same body composition who has not previously been obese (Leibel et al., 1995, Kissileff et al., 2012). This reduction in energy expenditure is counter intuitively coupled with higher hunger and delayed satiation. Collectively these characteristics are reminiscent of reduced leptin signalling. Leptin replacement after moderate weight loss has been shown to correct these deviations and to assist weight maintenance in the post-obese state (Rosenbaum et al., 2002). Leptin may therefore be a useful therapeutic strategy for weight maintenance.

1.2.2.1.2 Insulin

Insulin, produced by pancreatic β-cells, is vital for regulating the storage of absorbed nutrients and also acts as an adiposity signal to the brain in the regulation of energy balance. Insulin secretion increases rapidly post prandially, acting on tissues such as the liver, skeletal muscle and adipose tissue to stimulate the uptake of excess glucose from the blood. Plasma insulin correlates with body mass index (Polonsky et al., 1988) and with adiposity (Bagdade et al., 1967) suggesting a role in the communication of peripheral energy availability. Plasma insulin levels also reflect more acute changes in energy status, increasing during meals and situations of positive energy balance in direct proportion to the degree of adiposity, and decreasing during fasting and negative energy balance.

Peripheral insulin enters the CNS, where it is proposed to act as an anorexigenic signal, in proportion to its circulating levels, crossing the BBB via a saturable transport mediated mechanism (Woods et al., 2003). Insulin signals through the insulin receptor (IR), which is widely expressed throughout the periphery and CNS, including regions involved in the regulation of food intake and energy homeostasis. Insulin appears to regulate feeding at least in part through the modulation of hypothalamic neuropeptide expression. Third ventricle administration of insulin decreases preproNPY expression in the ARC of rats and reduces fasting-induced NPY release in the PVN (Schwartz et al., 1992), and also increases hypothalamic POMC expression. Repeated administration of insulin in small doses, or as a continuous infusion, decreases food intake and increases energy expenditure (Woods et al., 1996). Peripheral administration of insulin, at levels that do not cause hypoglycaemia, also reduces food intake in rats (VanderWeele et al., 1982, McGowan et al., 1990). Additionally, neuron-specific disruption of the insulin receptor leads to diet induced obesity and sex-

specific increases in food intake (Bruning et al., 2000). These data collectively demonstrate a role for insulin in appetite regulation.

1.2.2.2 The enteric nervous system and vagus nerve

The enteric nervous system (ENS) contains two major nerve plexuses, the submucosal plexus and myenteric plexus, which lie entirely within the digestive tract wall (intrinsic nerves) and can operate autonomously of the CNS (Furness, 2012). The ENS is implicated in every aspect of gut function, and has a major influence on gastric and pancreatic exocrine secretion, motility, blood supply and the secretion of gut hormones. The primary role of the ENS is to co-ordinate local activity within the digestive tract.

The vagus nerve is one of the major extrinsic nerves involved in gut function, and is a predominant pathway in the gut-brain axis. Vagal afferents originating in the mucosa or submucosa have mechano- and chemo- sensory roles, and relay sensory information to the NTS. Vagal afferents are also sensitive to a number of gastrointestinal hormones, such as CCK, GLP-1, PYY and Ghrelin (these gastrointestinal hormones are discussed in more detail in section 1.2.2.3) (Koda et al., 2005, MacLean, 1985, Imeryuz et al., 1997, Date et al., 2002). Vagal efferent fibres originating from the DMX influence motility, gastric and pancreatic exocrine secretion and the secretion of gut hormones, with the postganglionic efferent fibres considered as part of the ENS.

1.2.2.3 Gut hormones

The gastrointestinal tract contains a number of different enteroendocrine cells, secreting a range of peptide hormones. These hormones have a number targets, including gastrointestinal exocrine glands, smooth muscle, afferent terminals and the brain. Enteroendocrine cells are distributed throughout the length of the gastrointestinal tract. They are polarized cells with their apical membrane at the gut lumen facilitating direct sensing of the nutritional milieu within the gut. The release of gut hormones can be stimulated by gastric distension, nutrient detection and by neuronal signals (Cummings and Overduin, 2007).

1.2.2.3.1 Ghrelin

Ghrelin, the only known orexigenic gut-derived hormone (Wren et al., 2001), is predominantly secreted from X/A-like endocrine cells in the stomach and proximal small intestine (Date et al., 2000, Kojima et al., 1999). In addition to stimulating food intake, ghrelin helps maintain fasting blood glucose (Scott et al., 2012) and promotes gastric motility (Levin et al., 2006) and adipogenesis (Thompson et al., 2004). Ghrelin was discovered as the endogenous ligand of the growth hormone secretagogue receptor (GHSR). Its gene (GHRL) encodes a 117 amino acid (aa) pre-proghrelin, which is subsequently cleaved and processed to the 28aa mature ghrelin. There are two major forms of ghrelin: acyl and des-acyl ghrelin. Acyl ghrelin has an n-octanoyl group attached to the serine at position 3 by a post-translational modification catalysed by ghrelin O-acyltransferase (GOAT) (Yang et al., 2008). Acyl-ghrelin is believed to be the biologically active form of ghrelin, with the physiological role of des-acyl ghrelin, the more abundant form, being unclear. It is thought that the arcuate NPY/AgRP neurons are involved in mediating acyl-ghrelin's orexigenic effects (Chen et al., 2004) and that in hindbrain an AP-NTS-DMX-vagal efferent pathway mediates the effect of ghrelin on pancreatic secretion (Li et al., 2006, Scott et al., 2012).

Obesity is associated with disrupted ghrelin regulation: obese individuals fail to significantly suppress ghrelin levels after a meal (le Roux et al., 2005). Strategies to reduce ghrelin signalling are being explored as a treatment for obesity. For example GHSR antagonists, ghrelin antibodies and RNA spiegelmers have been investigated (Shearman et al., 2006, Kobelt et al., 2006, Vizcarra et al., 2007, Asakawa et al., 2003). However these have thus far not yielded any promising clinical data. An additional strategy is targeting the enzymes involved in the regulation of acyl ghrelin levels. GOAT antagonists seem to be a promising target, reducing food intake, body weight and fat mass in high fat fed mice (Barnett et al., 2010).

1.2.2.3.2 Cholecystokinin

Cholecystokinin (CCK) is an anorexigenic gut hormone released from mucosal enteroendocrine I-cells in the proximal intestine (Buchan et al., 1978). CCK is mainly secreted in response to fats and proteins. Two CCK receptors have been identified: CCK- 1R

(or CCK_A) and CCK-2R (or CCK_B). The anorexigenic actions of CCK are primarily mediated through the CCK-1R located on vagal afferent neurons (Moran et al., 1990, MacLean, 1985). CCK-1R is also expressed in the pancreas, gallbladder and pylorus and in specific brain regions, and in addition to its action on vagal afferents CCK also acts via the CCK-1R to stimulate pancreatic secretion, gallbladder contraction and bile release, and slow gastric emptying (Moran and Kinzig, 2004).

Infusion of CCK suppresses food intake in humans (Muurahainen et al., 1988). However, in rodent models it appears that CCK is more important in satiation than in satiety, with acute CCK administration causing a reduction in calorie intake during meals, but a concomitant increase in meal number (West et al., 1984). Additionally, chronic administration of CCK does not decrease food intake (West et al., 1987). Thus the therapeutic potential of CCK1-R agonism as a stand-alone treatment is limited. However, it seems CCK can synergistically enhance the response of other peripheral anorexigenic hormones such as leptin and amylin (Matson et al., 2000, Bhavsar et al., 1998) and thus a combination therapy involving the CCK system may be a more viable option for the treatment of obesity.

1.2.2.3.3 Glucagon-like Peptide 1

Glucagon-Like Peptide-1 (GLP-1) is an anorexigenic peptide produced by post-translational enzymatic cleavage of the pre-proglucagon gene product, and released from enteroendocrine L-cells in response to nutrient ingestion. Its biological activities include well characterised incretin effects, and the inhibition of gastric emptying and food intake (Holst, 1999). There are two bioactive forms of GLP-1: $GLP\text{-}1_{7\text{-}37}$ and $GLP\text{-}1_{7\text{-}36\ amide}$, the latter is thought to be the most important physiologically. The effects of GLP-1 are mediated by the GLP-1 receptor (GLP-1R), which is expressed in several hypothalamic nuclei including the ARC and PVN, and also in the brainstem and peripheral nervous system, pancreatic islets, heart, kidney and GI tract (Bullock et al., 1996, Campos et al., 1994). Peripheral administration of GLP-1 increases c-Fos expression in the PVN and brainstem (Baggio et al., 2004).

GLP-1 has a half-life of 1-2minutes and is rapidly degraded by dipeptidyl-peptidase IV (DPP-4), limiting the therapeutic potential of the endogenous hormone. Nonetheless, the incretin effects of GLP-1 have formed the basis of a number of anti-diabetic drugs. Two long acting

GLP-1 analogues, Exenatide and Liraglutide, are widely used for the treatment of type II diabetes. In clinical trials both also induced weight loss to a similar extent. However Liraglutide appears to be better tolerated by patients and thus may be a more viable treatment option for weight management (Buse et al., 2009). Liraglutide, developed by Novo Nordisk, is an acylated analogue of human GLP-1, with a considerably extended half-life *in vivo*. It was approved for clinical use in Europe in 2009 and in the USA in 2010 as a treatment for type II diabetes. Liraglutide is currently undergoing phase III clinical trials as an anti-obesity therapy.

However, safety concerns have arisen from post marketing surveillance of GLP-1 analogues which may impede their development as anti-obesity therapies. These include an apparent increased incidence of acute pancreatitis in patients treated with Exenatide or Liraglutide compared to other treatment strategies for type II diabetes (Anderson and Trujillo, 2010, Lee et al., 2011). An additional concern is the development of treatment specific-antibodies. Liraglutide is associated with a reduced frequency and lower levels of treatment-associated antibodies compared to Exenatide (Buse et al., 2011), which is predicted to make it a safer and more efficacious option for development as an anti-obesity drug. However, the increased incidence of acute pancreatitis is still a concern.

1.2.2.3.4 Peptide YY

Peptide Tyrosine Tyrosine (PYY), a member of the PP-fold family of peptides, is, like GLP-1, secreted from enteroendocrine L-cells in response to food ingestion. Two major forms are found in circulation; PYY_{1-36} and a truncated form, PYY_{3-36} (Eberlein et al., 1989), produced by enzymatic cleavage of PYY_{1-36} by DPP-IV (Mentlein et al., 1993). PYY_{1-36} has agonist activity at Y_1, Y_2 and Y_5 receptors, whereas PYY_{3-36} is a selective Y_2 receptor agonist. There is little evidence to suggest PYY_{1-36} has a role in energy intake (Sloth et al., 2007), but PYY_{3-36} is widely accepted as an anorexigenic hormone that can reduce food intake in lean and obese animals and humans (Challis et al., 2003, Chelikani et al., 2005, Degen et al., 2005, Batterham et al., 2003, Batterham et al., 2002, Chelikani et al., 2007, Reidelberger et al., 2008). PYY_{3-36} may act on the Y2R in the ARC, where this receptor acts as a presynaptic inhibitory receptor on arcuate NPY/AgRP neurons, though there is also evidence that it may work via the vagus (Blevins et al., 2008, Koda et al., 2005).

The utility of exogenous PYY_{3-36} as a treatment for obesity is limited by its rapid metabolism (Lluis et al., 1989). Furthermore, the supraphysiological doses likely required for periodic administration of PYY_{3-36} to reduce food intake are associated with nausea (le Roux et al., 2008). As with GLP-1, long-acting PYY_{3-36} analogues may be more useful than the endogenous molecule.

1.2.2.3.5 Oxyntomodulin

Oxyntomodulin (OXM), a 37-amino acid peptide secreted from L-cells, is another pre-proglucagon product demonstrated to reduce food intake in animal models and in humans (Dakin et al., 2001, Liu et al., 2010, Wynne et al., 2005, Baggio et al., 2004, Cohen et al., 2003, Wynne et al., 2006). OXM is thought to have a lower incidence of treatment-associated nausea than other exogenously administered gut hormone peptides (Parkinson et al., 2009, Cohen et al., 2003). No OXM specific receptor has been identified to date. However, OXM has weak affinity for the glucagon receptor (GCGR) and also binds to the GLP-1R, though at a lower affinity than GLP-1. In mice, the anorectic effect of OXM is blocked by the GLP-1R antagonist $Exendin_{9-39}$, and is absent in GLP-1R knockout models but not in GCGR knockout models (Baggio et al., 2004). The anorectic effects of OXM are thus thought to be mediated primarily through the GLP-1R (Baggio et al., 2004). Despite its relatively weak affinity for GLP-1R, OXM has a more potent anorectic effect in acute food intake studies compared to GLP-1 at similar doses (Dakin et al., 2004) suggesting it may also mediate its effect though additional mechanisms. It has been suggested that OXM also stimulates energy expenditure, likely via the GCGR, (Wynne et al., 2006) and is thus a good target for obesity therapeutics.

Much like GLP-1, OXM has a short circulating half-life due to DPP-IV degradation which thereby limits its utility as an anti-obesity agent. However, the generation of DPP-IV resistant or long acting analogues of OXM and utilisation of the mechanisms by which it exerts its effects on appetite and energy expenditure may be a promising approach for the development of novel anti-obesity drugs.

1.2.2.4 Peripheral obesity targets

1.2.2.4.1 Gut hormone therapies

Targeting the gut brain-axis seems a safer option than targeting central pathways directly. However, to achieve efficacy without adverse events, it is likely a multi-target approach will be needed. Simultaneous administration of multiple gut hormones or gut hormone analogues may provide a safe and efficacious strategy.

A combined infusion of OXM and PYY_{3-36}, at doses not associated with nausea, has an additive anorectic effect in humans (Field et al., 2010), highlighting the fact that these hormones work through different mechanisms that can be simultaneously exploited. Perhaps the most promising combination of gut hormones is that of GLP-1_{7-36} and PYY_{3-36}. This combination has been shown to have an additive anorectic effect in both mice and humans (Neary et al., 2005). Most interestingly, a GLP-1_{7-36} and PYY_{3-36} oral combination therapy utilising sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC) delivery technology to mimic endogenous secretion of the peptides also has an additive anorectic effect in humans (Steinert et al., 2010). Oral administration is considered the most convenient and economical method of drug delivery and tends to encourage a higher rate of compliance than other administration routes (Gomez-Orellana, 2005). Therefore the development of anti-obesity drugs that can be delivered orally and are as efficacious as injectables would be of great clinical significance.

1.2.2.4.2 Targeting the endogenous production of gut hormones and nutrient sensing systems

An emerging field in anti-obesity research is the study of nutrient sensing receptors. Many nutrient sensing receptors, including those for glucose, fatty acids and amino acids, are present in the GI tract where they have been localized to the enteroendocrine cells. Stimulation of specific nutrient receptors in immortalized and primary enteroendocrine cell cultures has been shown to stimulate gut hormone release (Jang et al., 2007, Steinert et al., 2011, Oya et al., 2013, Tolhurst et al., 2012). However, there is currently very little *in vivo* data on these systems, and thus the physiological relevance of these effects requires further investigation. Theoretically, directly targeting these nutrient sensing receptors to stimulate endogenous gut hormone release might provide a relatively physiological means of suppressing appetite and reducing energy intake. The oral administration of nutrient

sensing receptor agonists which stimulate the endogenous release of gut hormones may be an effective long term treatment for obesity.

1.3 Macronutrient Diets

Control of food intake is one of the most important factors involved in maintaining energy homeostasis. For decades, nutritional intervention studies have focussed on reducing energy content to promote weight loss. This recommendation, although seemingly successful in the short term, is often not sustainable for long periods of time. Most overweight or obese individuals are unable to sustain dietary induced weight loss because of increased hunger levels and energy expenditure adaptations. Therefore, more recently, the focus has shifted to intervention programs utilising diets which not only aid weight loss but also help sustain weight loss and improve obesity-associated metabolic disturbances (Abete et al., 2010).

Current evidence indicates that diets with a low glycemic index (GI) have a number of beneficial effects. Low GI diets facilitate rapid weight loss, improve management of glucose and insulin levels and help reduce blood pressure and triglyceride (TG) levels (Westman et al., 2008, Goff et al., 2013). Additionally, diets rich in omega-3 fatty acids improve cardiovascular health by having beneficial effects on blood pressure, TG levels, and insulin and leptin sensitivity (Due et al., 2008, Ramel et al., 2008). However, perhaps the most promising diet studied for its effects on weight loss and weight maintenance is the high protein diet.

1.4 High Protein Diets

The macronutrient composition of a normal diet typically consists of approximately 55% energy from carbohydrates, 30% from fats and 15% from proteins. Evidence from both animal and human studies suggests that increasing protein to 25-30% of dietary energy can increase satiety and facilitate weight loss (Layman et al., 2009, Soenen et al., 2013). Protein as a macronutrient induces the strongest feeling of satiety per calorie and thus satisfies hunger more easily (Weigle et al., 2005). Subjects on high protein *ad libitum* diets consistently consume fewer calories than when on an *ad libitum* isocaloric normal protein diet, yet report a similar degree of fullness (Weigle et al., 2005). The higher satiating power and reduced calorie intake associated with a high protein diet suggests that such a diet

would make an effective weight loss and subsequent weight management strategy (Larsen et al., 2010). Additional benefits from high protein diets include enhanced fat mass loss and lean mass retention, which helps to curb weight loss-associated decreases in energy expenditure (Soenen et al., 2013).

Several mechanisms have been proposed to mediate the beneficial effects of high protein diets. These include the thermic effect of protein, the stimulation of gluconeogenesis, and altered secretion of appetite-regulating gut hormones (Blom et al., 2006).

1.4.1 Thermic effect of protein

The energy required for digestion, absorption and disposal of ingested nutrients is termed dietary induced thermogenesis (DIT). The thermic effect of protein is greater than that of carbohydrate and fats. DIT for protein is approximately 20-30% of energy consumed whereas for carbohydrate it is 5-10%, and for fat, 0-3% (Westerterp et al., 1999, Tappy, 1996). Unlike carbohydrate and fat, there is no storage capacity for protein, therefore ingested protein is metabolically processed more rapidly. Following digestion, protein can be 'disposed' of in a variety of ways dependent on the metabolic needs of an individual. This can include protein synthesis, urea production and gluconeogenesis, all of which are high energy requiring pathways. Numerous studies have demonstrated that increasing the protein content of the diet increases dietary induced energy expenditure (Nair et al., 1983, Luscombe et al., 2003, Luscombe et al., 2002). Interestingly, the degree of protein-induced thermogenesis can be influenced by the protein source. Animal proteins, which often contain all the essential amino acids and are thus considered 'complete' proteins, produce a higher thermogenic response than vegetable proteins such as soy (Mikkelsen et al., 2000). Increasing energy expenditure without concomitantly increasing energy intake would result in negative energy balance, which could in the long run promote weight loss. Certain studies have attempted to establish whether the increase in DIT seen following an increase in dietary protein is sufficient to affect body weight (Crovetti et al., 1998, Eisenstein et al., 2002). Despite significant increases in DIT these changes are quantitatively small and as such could not account for the magnitude of weight loss achieved on such diets, although they may contribute to it (Eisenstein et al., 2002).

1.4.2 Gluconeogenesis

High protein diets increase gluconeogenesis. Increased gluconeogenesis has been suggested to contribute towards the beneficial effects of high protein diets; adding to the thermic effect of protein and also contributing towards protein-induced satiety via the modulation of glucose homeostasis (Veldhorst et al., 2009f). Additionally, intestinal gluconeogenesis has been implicated in mediating the effects of high protein diets on satiety (Mithieux et al., 2005, Duraffourd et al., 2012). However, not all amino acids are preferentially used for gluconeogenesis, and therefore different proteins may stimulate gluconeogenesis to varying degrees.

1.4.3 Protein induced satiety

Protein is the most satiating macronutrient. Protein induced satiety has been demonstrated acutely by comparing macronutrient preloads (Johnson and Vickers, 1993, Poppitt et al., 1998, Stubbs et al., 1999), comparing high and low protein pre-loads (Veldhorst et al., 2009c, Veldhorst et al., 2009e, Porrini et al., 1995, Veldhorst et al., 2009d) and comparing different types of protein (Veldhorst et al., 2009a, Veldhorst et al., 2009b, Vandewater and Vickers, 1996). A number of these studies have shown, at least in part, that the satiating power of the protein load synchronizes with the timing of amino acid profiles following intake (Veldhorst et al., 2009d, Luhovyy et al., 2007, Veldhorst et al., 2007), suggesting specific amino acids may be responsible for inducing satiety.

The effect of protein preloads and high protein meals on gut hormone mediated satiety has also been widely studied. A high protein meal has been shown to elicit a greater increase in plasma PYY than an isocaloric high fat and high carbohydrate meal in both normal and obese subjects (Batterham et al., 2006). Additionally, mice lacking PYY are resistant to the satiating effects of a high protein diet (Batterham et al., 2006). Carbohydrate ingestion reduces circulating ghrelin more rapidly than protein or fat (Koliaki et al., 2010, Monteleone et al., 2003). However, a protein rich meal has been reported to result in a more sustained decrease in circulating ghrelin (Blom et al., 2006, Foster-Schubert et al., 2008, Tannous dit El Khoury et al., 2006). Acute studies comparing a high protein meal to a normal protein meal have demonstrated that the higher carbohydrate content of the normal protein meal makes it a more potent stimulus of GLP-1 secretion (Smeets et al., 2008). Conversely, in a chronic

high protein diet study, GLP-1 concentrations were higher in response to a high protein diet than to a normal protein diet (Lejeune et al., 2006). These studies suggest protein is the most satiating macronutrient, inducing the greatest increase in plasma PYY, a more sustained suppression of plasma ghrelin, and variable effects on GLP-1 release dependent on the length of exposure to a high protein diet. Additionally, different protein sources differentially affect these satiety hormones (Veldhorst et al., 2007, Diepvens et al., 2008, Abou-Samra et al., 2011), likely due to the digestion kinetics of each protein, and therefore it may be appearance of specific amino acids in the gut or in the circulation that modulate gut hormone release and satiety (Anderson et al., 2004).

Indeed, high protein diets do not only affect gut hormone release but also have actions within the CNS. High protein diets increase the activation of noradrenergic neurons in the NTS and increase the activation of POMC neurons in the ARC compared to a normal protein diets (Faipoux et al., 2008). Within the hypothalamus, high protein diets also decrease activation of orexin neurons in the lateral hypothalamus compared to a normal protein diet (Journel et al., 2012) and, out with the hypothalamus, they are reported to inhibit the activation of opioid and GABAergic neurons in the NAcc and thus to reduce the hedonic response to food (Fromentin et al., 2012).

1.5 Protein digestion

Protein digestion can be categorized into three phases: the gastric phase, the pancreatic phase and the intestinal phase.

1.5.1 Gastric Phase

Protein digestion begins in the stomach, where the protease, pepsin, released from chief cells as the zymogen precursor pepsinogen, cleaves peptide bonds between aromatic and hydrophobic amino acids. This phase of protein digestion generates polypeptides, oligopeptides and a small number of free amino acids (Freeman and Kim, 1978).

1.5.2 Pancreatic Phase

Protein digestion products generated during the gastric phase are secretagogues for the hormones CCK and secretin, which stimulate the release of hydrolytic enzyme precursors

from pancreatic acinar cells (Meyer and Kelly, 1976). Concomitantly, these hormones stimulate the release of the brush-border glycoprotein enzymes such as enteropeptidase from mucosal cells (Freeman and Kim, 1978). Enteropeptidase cleaves the pancreatic zymogen trypsinogen to generate trypsin. Enteropeptidase and trypsin then activate a number of other pancreatic zymogens. In the duodenum, endopeptidases including trypsin (which cleaves peptides at the carboxyl side of arginine or lysine), chymotrypsin (which cleaves peptides at the carboxyl side of large hydrophobic amino acids) and elastase (which cleaves peptides at the carboxyl side of small hydrophobic amino acids) further digest the poly- and oligo-peptides generated during the gastric phase. This phase generates small oligopeptides and free amino acids (Freeman and Kim, 1978).

1.5.3 Intestinal Phase

The final stages of protein digestion occur at the brush border and the cytoplasmic membranes of intestinal mucosal cells where amino- and carboxy- peptidases cleave the amide- and carboxyl terminal amino acids from the majority of oligopeptides remaining. The jejunum also has a small degree of amino peptidase activity and is the major site of amino acid and peptide absorption. Amino acids are the predominant products generated during protein digestion. However, a small number of oligopeptides do escape complete digestion and can be absorbed into the portal circulation (Freeman and Kim, 1978).

1.6 Amino Acids

Following intestinal absorption, amino acids enter the portal circulation and are delivered to all tissues, where they can serve as building blocks for protein synthesis, precursors for a wide variety of bioactive molecules or as substrates for energy yielding processes.

There are hundreds of amino acids found in our diet. However, only 20 contribute towards protein synthesis and are thus classified as proteinogenic amino acids. Proteinogenic amino acids can be classified as essential or non-essential (Table 1.1). Essential amino acids are classified as those that cannot be synthesised within the human body, whereas non-essential amino acids can be synthesised. However, a subset of these non-essential amino acids are classified as conditionally-essential, as at certain stages of life *de novo* synthesis

does not meet the body's requirements. These conditionally-essential amino acids are L-arginine, L-cysteine and L-glutamine (Wu, 2009).

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

Table 1.1 Essential and Non-essential amino acids
Essential and non-essential amino acids with their three letter and one letter codes. *Conditionally-essential amino acids.

Amino acids can also be classified in a number of ways according to the properties of their side chains (Table 1.2).

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

Table 1.2 Classification of amino acid side chains
Amino acids can be classified into groups according to the properties of their side chains.

1.6.1 Amino acids and appetite

1.6.1.1 Amino acids and satiety

As early as 1956, it was suggested that the rise in serum amino acid concentration following a protein load or amino acid infusion correlated with satiety (Mellinkoff et al., 1956). Further, a higher level of circulating plasma amino acids following a whey preload has been implicated in the associated increased satiety with this protein compared to casein (Hall et al., 2003). In addition, a series of studies by Velhorst and colleagues comparing the effect of low and high (i) soy, (ii) whey and (iii) casein on satiety suggested that the increase in satiety following the high protein loads correlated with the appearance of specific amino acids in the plasma. Increased satiety following a high soy load correlated with the apperance of taurine (Veldhorst et al., 2009e), high whey with serine, threonine, alanine and isoleucine (Veldhorst et al., 2009a), and high casein with branched chain amino acids (Veldhorst et al., 2009c).

1.6.1.2 Amino acids and central mechanisms of appetite regulation

Certain amino acids acts act as, or are precursors for, neurotransmitters. Glutamate and aspartate are excitatory amino acids which activate the N-methyl-D-aspartate receptor, and glycine is a co-activator of this receptor. Tryptophan is a precursor for serotonin, and tyrosine is a precursor for dopamine; these two monoamine products are involved in a number of regulatory processes, including energy homeostasis (Volkow et al., 2011, Garattini et al., 1988). In addition, histidine is a precursor for histamine which has also been implicated in appetite regulation (Goto et al., 2007, Gotoh et al., 2007, Bassil et al., 2007) and leucine, a branched chain amino acid, is known suppress appetite through central mechanisms which are discussed in section 2.1.4.3.1.(Cota et al., 2006, Blouet et al., 2009).

1.6.1.3 Amino acids and gut hormone release

Chapter 1 introduced the hypothesis that the sensing of individual amino acids generated during protein digestion, and the subsequent effect on gut hormone release, may be involved in protein induced satiety. Reimer *et al* demonstrated that a mixture of essential amino acids was better at stimulating GLP-1 release from the human enteroendocrine cell line, NCI-H716, than a mixture of non-essential amino acids (Reimer, 2006). The number of

studies looking at the effect of individual amino acids on gut hormone release is limited. However, it has been demonstrated that the branched chain amino acids, L-leucine and L-isoleucine, stimulate GLP-1 release in a dose responsive manner from NCI-H716 cells (Chen and Reimer, 2009*)*. The amino acids L-asparagine, L-methionine, L-leucine, L-alanine, L-serine, L-glutamine and L-glutamate have also been shown to stimulate GLP-1 release from the murine GLP-1 secreting GLUTag cell line (Reimann et al., 2004). More recently, L-glutamine, L-phenylalanine and L-asparagine have also been shown to stimulate GLP-1 release from murine primary colonic cultures (Tolhurst et al., 2011), and the aromatic amino acids L-phenylalanine and L-tryptophan to stimulate the release of CCK from isolated CCK-cells (Wang et al., 2011).

The possible mechanisms by which protein and amino acids modulate appetite are summarised in Fig. 1.4.

1.7 Preliminary data

Initial work from our group examined the effect of peripheral administration of a range of L-amino acids on food intake in rats. Following both oral and intraperitoneal administration, L-cysteine was found to decrease food intake to the greatest extent (Fig. 1.5 A and B respectively) (unpublished).

L-cysteine is a conditionally essential amino acid with many biological functions. L-cysteine is a precursor to a wide variety of bioactive molecules including glutathione, an antioxidant, and hydrogen sulphide, a gasotransmitter and vasodilator.

In addition, L-arginine and L-lysine were found to have significant anorectic effects. However, there was concern that these effects might reflect the high pH of the amino acid solution administered rather than the specific biological properties of the amino acids themselves.

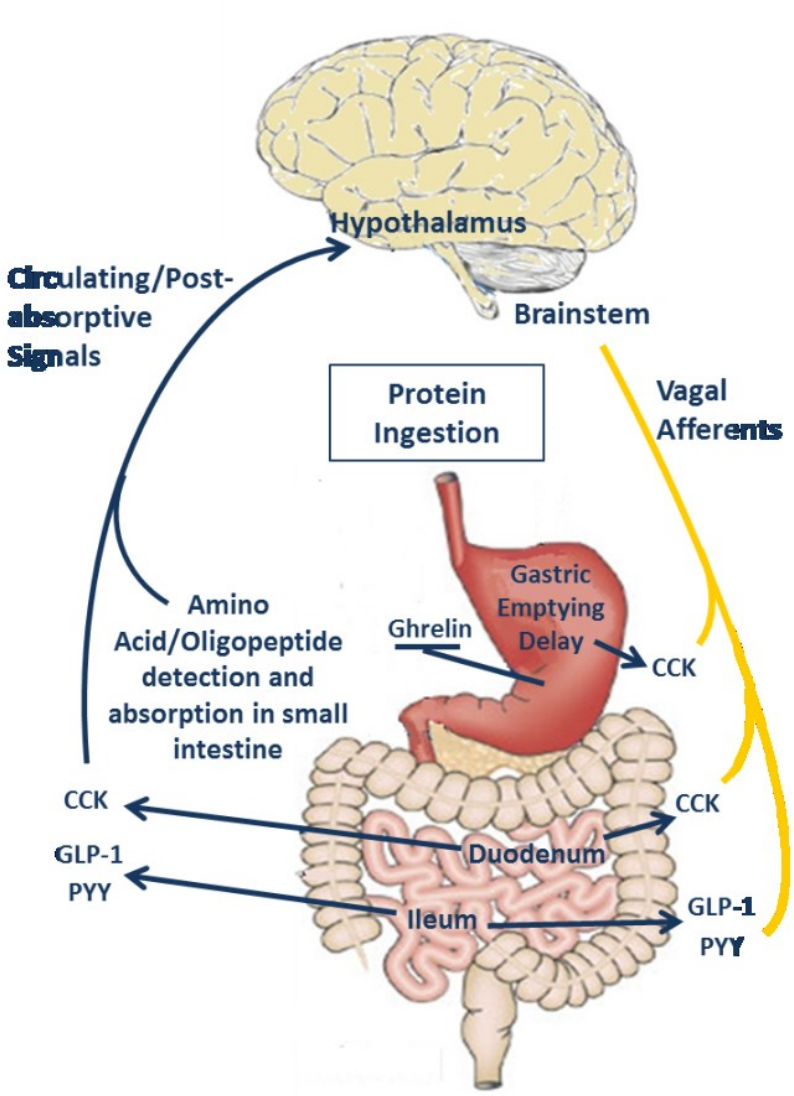
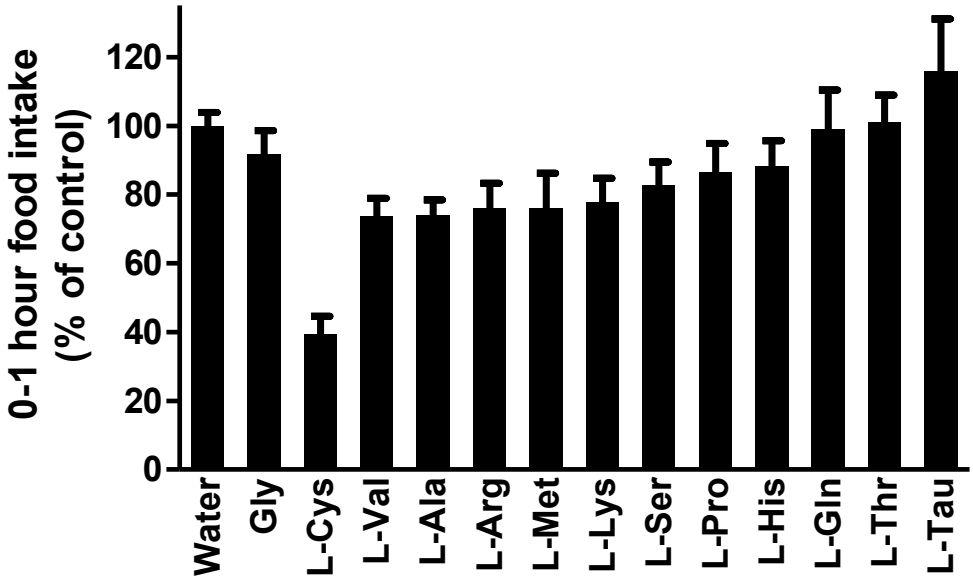


Figure 1.4 Schematic illustrating possible pathways by which amino acids modulate appetite
Protein ingestion stimulates the release of gut hormones, possibly due to amino acid sensing in the gut. These hormones can signal via the vagus, brainstem and hypothalamus to modulate food intake. Additionally, amino acids absorbed from the GI tract may generate post-absorptive signals either peripherally or centrally that modulate food intake. CCK: cholecystokinin, GLP-1: glucagon-like peptide-1, PYY – peptide tyrosine tyrosine.

A



B

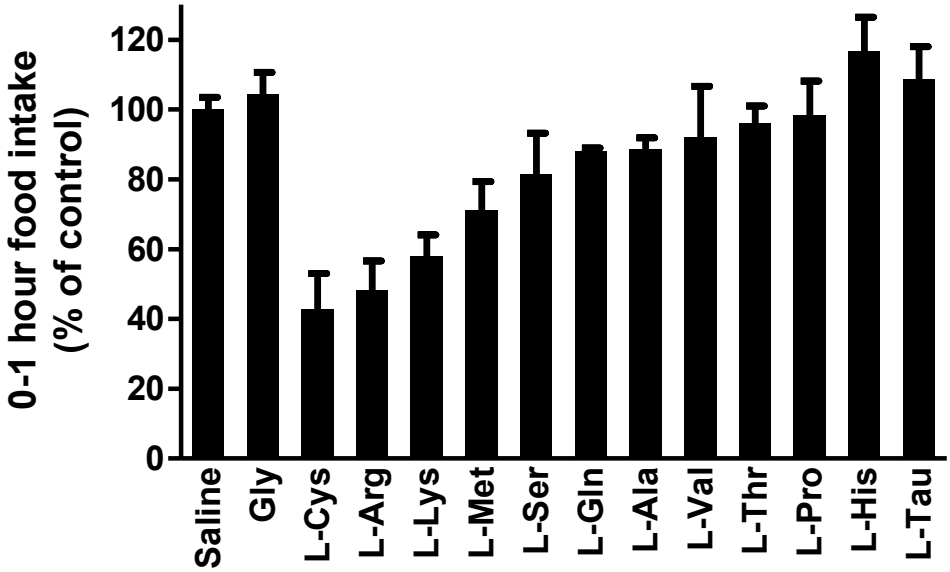


Figure 1.5 The effect of peripheral administration of L-amino acids on food intake in rats
The effect of oral gavage administration of 4mmol/kg L-amino acids on 0-1 hour food intake in the early light phase following an overnight fast (A). The effect of intraperitoneal administration of 2mmol/kg L-Amino acids on 0-1hr food intake in the early light phase following an overnight fast (B). Data expressed as mean ± SEM percentage of vehicle control. L-Glu, and L-Asp were not investigated due to acidity and L-Trp, L-Tyr, L-Phe, L-Iso, L-Leu and L-Asn were not investigated due to poor solubility.

1.8 Hypothesis and General Aims

High protein diets can be an effective weight loss and weight management solution when adhered to, but compliance is an issue. Current understanding of the mechanisms by which high protein diets can facilitate weight loss and promote satiety is limited. Different types of protein have varying satiating effects, and the particular amino acid composition of the protein may be an important contributing factor to the success of these diets. Understanding the mechanisms involved in protein induced satiety and weight loss may provide additional targets for the treatment of obesity. Preliminary data from our lab investigated the effect of a range of amino acids on food intake in rats and identified L-cysteine as the most anorexigenic amino acid.

Hypothesis: L-cysteine reduces food intake by modulating gut hormone release and/or vagal signalling from the gut to the brain, and represents a putative therapy for obesity.

Therefore the general aims of this thesis were to:

1) Characterise the anorectic effects of L-cysteine.
2) Determine the mechanism by which L-cysteine modulates food intake.
3) Investigate the therapeutic potential of L-cysteine as an anti-obesity agent.

CHAPTER 2: THE EFFECT OF L-CYSTEINE ON FOOD INTAKE

2.1 Introduction

2.1.1 Amino Acid Sensing

2.1.1.1 The promiscuous amino acid sensing receptors

Amino acids are sensed by a number of class C G-protein coupled receptors (GPCR). One subgroup of these is the promiscuous amino acid sensing receptors consisting of the G-protein coupled receptor group C member 6A (GPRC6A), the calcium sensing receptor (CaSR) and the heterodimer of taste receptor type 1 member 1 and taste receptor type 1 member 3 (T1R1/T1R3). Class C GPCRs have large extracellular domains consisting of a neurotransmitter or nutrient binding N-terminal Venus Flytrap (VFT) domain and a cysteine rich domain, which couples the VFT domain to a seven transmembrane (7TM) domain. The 7TM domain is coupled to an intracellular C-terminal signaling domain that binds G-proteins. The three promiscuous amino acid sensing receptors are discussed in detail below, and their expression in specific gastrointestinal cell types is detailed in Table 2.1.2.

2.1.1.1.1 GPRC6A

The human G protein coupled receptor family C group 6 member A (hGPRC6A) was first cloned in 2004 (Wellendorph and Brauner-Osborne, 2004) and identified as a receptor for L-amino acids in 2005 (Wellendorph et al., 2005). Three GPRC6A isoforms were identified: isoform 1, the longest and most abundant, is expressed widely, isoform 2 is predominantly expressed in the kidney, and isoform 3 is expressed at much lower levels in the brain, skeletal muscle and kidney (Wellendorph and Brauner-Osborne, 2004). Subsequently, the mouse orthologue was cloned and also characterized as an L-amino acid sensing receptor (Wellendorph et al., 2005) with 80% homology with hGPRC6A. The rat orthologue was cloned and characterized in 2007, however only one rat isoform was identified (Wellendorph et al., 2007). The L-amino acid specificity for each orthologue is summarized in Table 2.1.3. L-ornithine, a non-proteinogenic amino acid, is the most potent amino acid agonist of the mGPRC6A and rGPRC6A, whereas L-arginine is the most potent agonist of hGPRC6A. In addition to amino acids, calcium, other divalent cations, calcimimetics and osteocalcin act as agonists or allosteric modulators of GPRC6A (Pi et al., 2005). It has been

proposed that GPRC6A couples to Gαq and therefore signals through the activation of phospholipase C (Kuang et al., 2005) (Fig 2.1.1).

	hGPRC6A[a]	mGPRC6A[b]	rGPRC6A[c]
	EC_{50} (μM) (pEC_{50} ± SEM)		
L-Orn	112 (3.96 ± 0.05)	**63.6 (4.2 ± 0.001)**	**264 (3.59± 0.05)**
Gly	263 (3.58 ± 0.04)	538 (3.3 ± 0.09)	455 (3.36 ± 0.1)
L-Cit	287 (3.56 ± 0.09)	> 1000	615 (3.21 ± 0.03)
L-Ser	623 (3.21 ± 0.07)	1160 (2.94 ± 0.03)	859 (3.08 ± 0.07)
L-Lys	169 (3.77 ± 0.05)	135 (3.97 ± 0.18)	> 1000
L-Ala	173 (3.76 ± 0.02)	486 (3.41 ± 0.18)	> 1000
L-Arg	**44.1 (4.38 ± 0.11)**	284 (3.58 ± 0.08)	> 1000
L-Met	854 (3.07 ± 0.04)	> 1000	> 1000
L-Cys	> 1000	356 (3.46 ± 0.09)	> 1000
L-Gln	590 (3.23 ± 0.05)	> 1000	> 1000

Table 2.1.1 Ligand specificity for human, mouse and rat GPRC6A orthologues
Data were derived from expression in tsA cells. a. EC_{50} values for human GPRC6A were determined through transfection of hGPRC6A/5.24 chimera and measurement of intracellular calcium levels in response to all 20 amino acids and ornithine (Wellendorph and Brauner-Osborne, 2004). b. EC_{50} values for mouse GPRC6A were determined through transfection of mGPRC6A coexpressed with $G\alpha_q$(G66D) and measurement of agonist induced inositol phosphate accumulation. (Christiansen et al., 2007) c. EC_{50} values for rat GPRC6A were determined through transfection of rGPRC6A coexpressed with $G\alpha_q$(G66D) and measurement of agonist induced inositol phosphate accumulation (Christiansen et al., 2007). All proteinogenic amino acids not shown had EC_{50} values >1000 μM. Bold values represent most potent amino acid agonist of each receptor orthologue.

GPRC6A is expressed in numerous tissues, including taste tissue, pancreatic islets, liver, skeletal muscle, and regions of the GI tract and the brain, suggesting it may have diverse roles. The physiological roles of this receptor are subject to debate. Two knockout models have been generated by two different groups. $GPRC6A^{(-/-)}$ mice generated by Pi and colleagues (Pi et al., 2008) had a selective deletion of Exon 2, the coding region of the venus fly trap domain. This knock out model was similar in appearance, body weight and body length to wildtype littermates, but exhibited a number of abnormalities, including feminization of the males, abnormal renal handling of calcium and phosphorus, defective osteoblast-mediated bone mineralization, adiposity and glucose intolerance. The bone and glucose handling phenotype of this knockout was further characterized and the group also identified an association between hGPRC6A polymorphisms and bone mineral density (Pi et

al., 2010) and a role for this receptor in L-arginine mediated insulin secretion from primary β-cells (Pi et al., 2012). The second knockout model generated by Wellendorph and colleagues (Wellendorph et al., 2009b) had a selective deletion of exon 6, the coding region of the entire 7 transmembrane (7TM) and c-terminal domains. In contrast to the model generated by Pi et al, this model exhibited normal bone mineralization and normal L-arginine stimulated insulin secretion (Smajilovic et al., 2013). The absence of a phenotype in this second model suggests that the requirement of the 7TM domain and C-terminal domain is not essential. It also supports the theory that GPRC6A may function as a heterodimer, which would explain difficulties in surface expression of this receptor in cultured cells (Wellendorph et al., 2005). Collectively, these knockout models suggest that the extracellular domain of GPRC6A has a prominent role in metabolism, whereas the role of the intracellular signalling domain is partially redundant and thus the receptor may mediate its effects through the intracellular signalling domain of an as yet unidentified subunit. However, more recent studies in this second model have observed a mild metabolic phenotype when metabolically challenged. GPRC6A$^{exon6(-/-)}$ are more susceptible to diet induced obesity and show increased fat mass, decreased lean mass and decreased insulin sensitivity when exposed to a high fat diet (Clemmensen et al., 2013), a metabolic phenotype similar to that of GPRC6A$^{exon2(-/-)}$. Whether GPRC6A is involved in amino acid/ protein induced satiety remains to be determined. One study has suggested a role for this receptor in L-ornithine induced GLP-1 secretion from GLUTag cells, an immortalized murine cell line that expresses the proglucagon gene and secretes glucagon-like peptides (Oya et al., 2013). However, this effect could not be replicated in primary murine small intestinal cultures (Oya et al., 2013) , calling into question its physiological relevance.

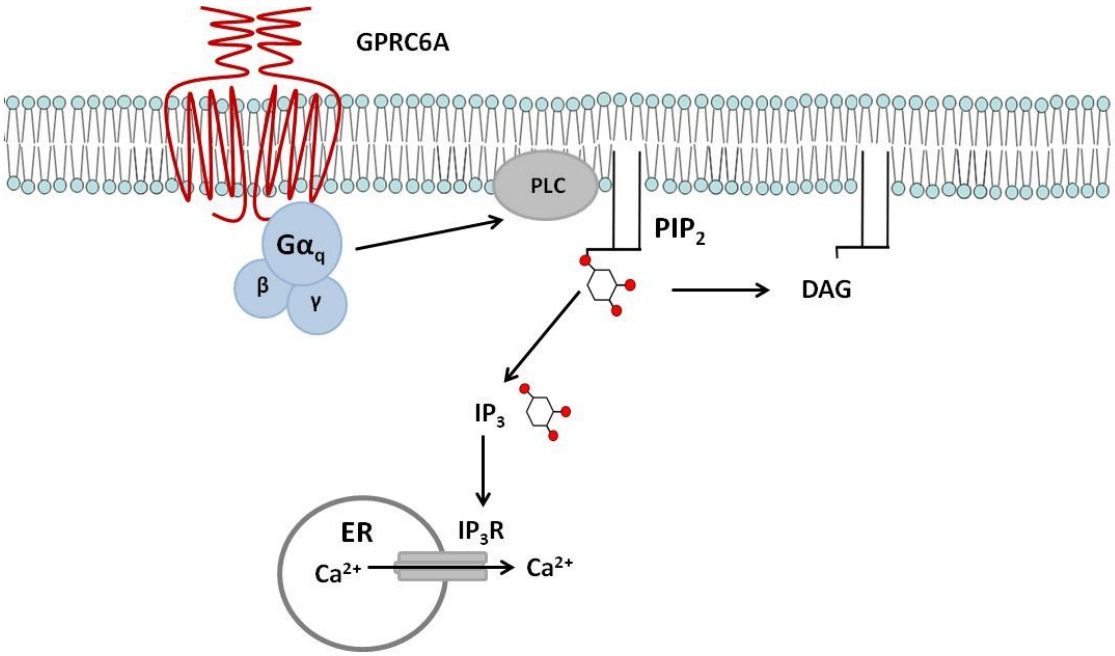


Figure 2.1.1 GPRC6A signalling pathway

GPRC6A signals through Gαq which activates phospholipase C β2 (PLCβ2), leading to the hydrolysis of phosphatidylinositiol -4,5 bisphosphate (PIP_2) to Inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptor (IP_3R) channels on the endoplasmic reticulum (ER), stimulating the release of Ca^{2+}.

2.1.1.1.2 CaSR

The CaSR is expressed in a number of tissues and is involved in calcium homeostasis and the regulation of a variety of cellular processes including proliferation, differentiation, gene expression, ion channel function and hormone secretion. At physiological levels of Ca^{2+}, the receptor is responsive to pH, other divalent cations, L-amino acids (aromatic>aliphatic> polar, see Fig. 2.1.5) and di- and tri-peptides, in an allosteric manner (Brown and MacLeod, 2001, Conigrave et al., 2002, Conigrave et al., 2000). The receptor is normally expressed at the cell surface as a homodimer (Bai et al., 1998) and has been proposed to signal predominantly through both $G\alpha_q$ (Handlogten et al., 2001) but also through $G\alpha_{i/o}$ or $G\alpha_s$ in a cell-specific manner (Chen et al., 1989, Chang et al., 1998, Handlogten et al., 2001) (Fig 2.1.2).

The predominant role of the CaSR is in systemic calcium homeostasis. However, with regards to energy homeostasis, the CaSR has been implicated in hormone release from the gastrointestinal tract and pancreas. Expressed in pancreatic acinar and ductal cells and islets of Langerhans (predominantly α- and β-cells), the CaSR has been shown to regulate the secretion of digestive enzymes, insulin and glucagon (Bruce et al., 1999, Rasschaert and

Malaisse, 1999, Squires et al., 2000). The activity of L-amino acids on the pancreatic CaSR has not been widely investigated. However, one study has suggested L-histidine attenuates glucose stimulated insulin release through the CaSR. (Parkash and Asotra, 2011). In the gastrointestinal tract, CaSR is expressed in G-cells (gastrin producing) (Ray et al., 1997), I-cells (CCK-producing) (Liou et al., 2011, Wang et al., 2011, Haid et al., 2012) and D-cells (somatostatin producing) (Haid et al., 2012). The aromatic amino acids L-phenylalanine and L-tryptophan stimulate the release of CCK from isolated CCK-cells (Wang et al., 2011). A number of amino acids: L-phenylalanine, L-tryptophan, L-asparagine, L-arginine and L-glutamine also stimulate GIP, GLP-1 and PYY secretion from isolated rat small intestine through a mechanism that involves the CaSR (Mace et al., 2012), and L-phenylalanine, L-tryptophan and L-leucine also stimulate secretion of gastric acid from parietal cells in *ex vivo* stomach preparations through allosteric modulation of the CaSR (Busque et al., 2005). Collectively these studies identify the CaSR as a possible mechanism for amino acid sensing in the gastrointestinal tract that may be linked to appetite regulation. However, *in vivo* studies are still required to assess the physiological impact of CaSR-mediated gut hormone release.

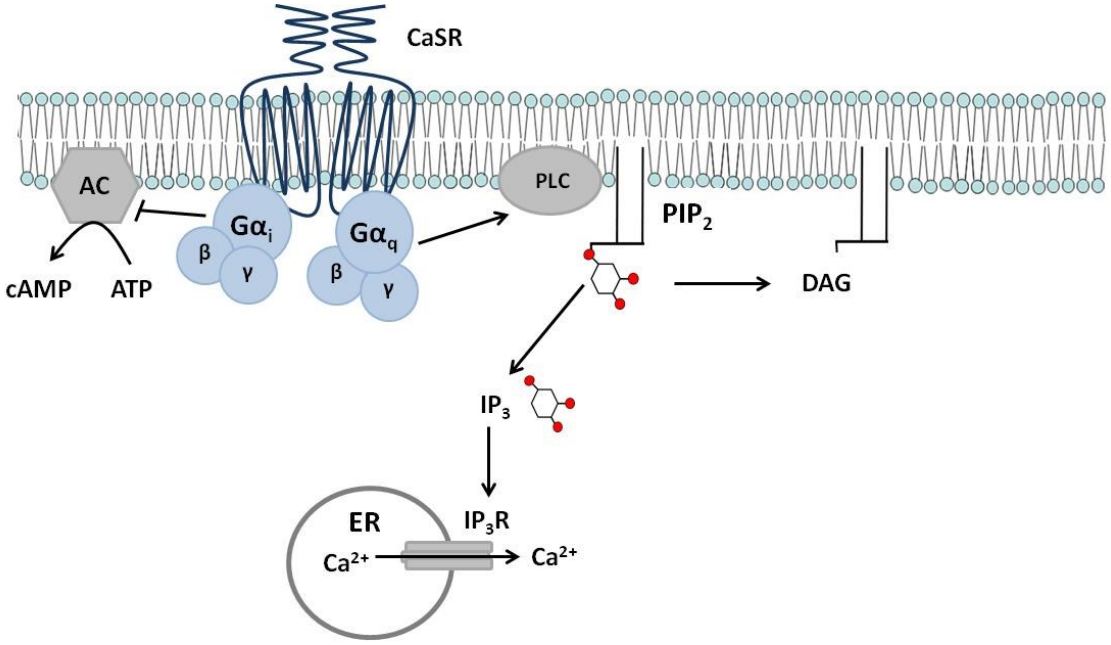


Figure 2.1.2 CaSR signalling pathway
CaSR is hypothesised to signal through 2 G-proteins: Gαi which inhibits adenylate cyclase (AC) thereby reducing cyclic adenosine monophosphate (cAMP), and Gαq which activates phospholipase C β2 (PLCβ2), leading to the hydrolysis phosphatidylinositol- 4,5 bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 binds to IP_3 receptor (IP_3R) channels on the endoplasmic reticulum (ER) stimulating the release of Ca^{2+}.

2.1.1.1.3 T1R1/T1R3

Three T1R taste receptors, T1R1, T1R2 and T1R3, have been identified and characterized. The T1R receptors function as heterodimers, with T1R2 and T1R3 forming a sweet taste receptor that is activated by a broad range of sweet compounds, whereas T1R1 and T1R3 form the umami taste receptor that is activated by mono-sodium glutamate (the umami taste) and a range of L-amino acids. Additionally, T1R3 has been implicated in calcium sensing and calcium taste (Tordoff et al., 2008, Tordoff et al., 2012). However, there is only approximately 70% homology between rodent and human T1Rs, which results in differences in agonist sensitivity: L-glutamate elicits the greatest response at the hT1R1/T1R3 (Li et al., 2002), whereas L-cysteine elicits the greatest response at the mT1R1/T1R3 (Nelson et al., 2002). Additionally, the mouse T1R1/T1R3 receptor appears more promiscuous, responding to a larger number of L-amino acids (Fig. 2.1.4) than the human orthologue (Nelson et al., 2002, Li et al., 2002).

T1R1/T1R3 signalling was first characterized in the taste buds of the tongue, where the receptor mediates its effects in a transient receptor potential cation channel subfamily M member 5 (TRPM5) dependent mechanism through the heterotrimer G-protein gustducin (Fig 2.1.3) (Zhang et al., 2003b, Ruiz et al., 2003). The receptors have since been localized to a number of non-gustatory tissues including the GI tract, pancreas, brain and skeletal muscle (Wauson et al., 2012), but their role in these tissues remains to be fully elucidated. One study has linked the T1R1/T1R3 receptor to CCK secretion, demonstrating that knockdown of T1R1 in STC-1 cells, an immortalized intestinal enteroendocrine cell line, attenuated the stimulatory effect of L-phenylalanine, L-leucine and L-glutamate on CCK release. Furthermore, positive and negative allosteric modulators (IMP and gumarin, respectively) of the receptor also modulated CCK secretion in response to these amino acids from mouse small intestine tissue explants (Daly et al., 2013). A recent study also reported that L-glutamate and L-arginine stimulate T1R1/T1R3 to promote insulin secretion from MIN-6 cells, an immortalized mouse pancreatic β-cell line (Oya et al., 2011). Additionally, isolated islets from $T1R3^{(-/-)}$ mice exhibit delayed insulin secretion kinetics (Geraedts et al., 2012). However, Wauson and colleagues suggest that rather than having a role in insulin secretion, T1R1/T1R3 is involved in regulating insulin content of pancreatic β-cells (Wauson et al., 2012). They propose a mechanism by which amino acids signal via T1R1/T1R3 to

ERK1/2 and mTORC1 and thus control mRNA translation initiation, such that increased extracellular amino acid availability activates mTORC1, which subsequently initiates mRNA translation of gene transcripts and promotes cell growth. In contrast, they propose a decrease in extracellular amino acid availability decreases mTORC1 activity, decreases translation and promotes autophagy.

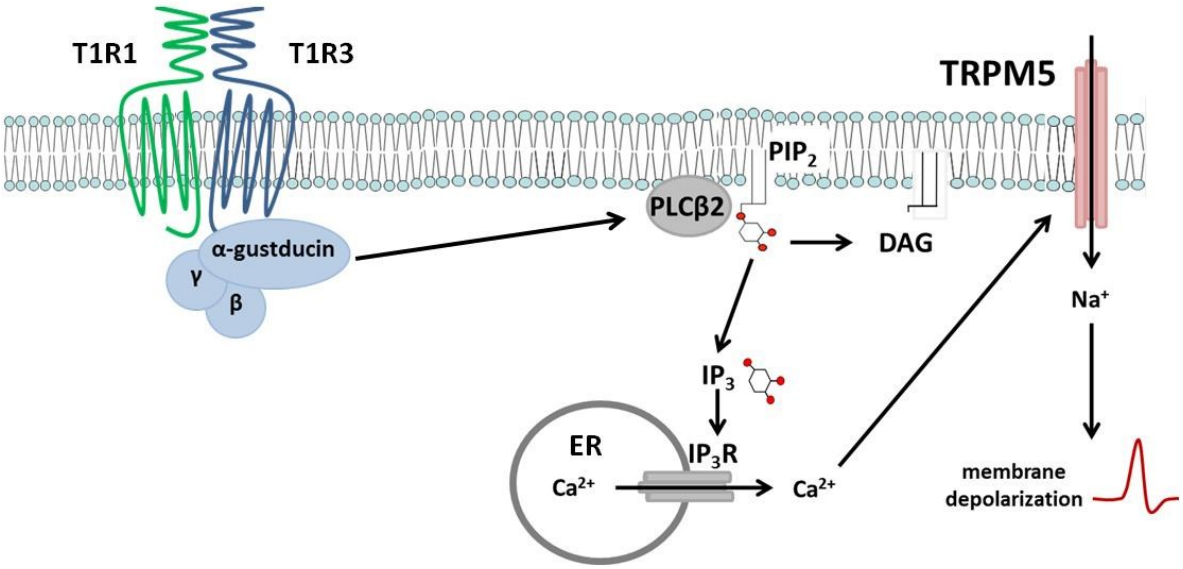


Figure 2.1.3 T1R1/T1R3 signalling pathway in gustatory tissue
Binding of an agonist to the T1R1 Venus Fly Trap (VFT) domain, triggers a conformational change within the receptor activating the G-protein gustducin. This permits the dissociation of the α- and βγ-subunits. The βγ-subunit activates phospholipase C β2 (PLCβ2) which hydrolyses phosphatidylinositol-4,5 bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacyleglycerol (DAG). IP_3 binds to IP_3 receptor (IP_3R) channels on the endoplasmic reticulum (ER) stimulating the release of Ca^{2+}. This increase in intracellular Ca^{2+}opens Ca^{2+} sensitive TRPM5 channel leading to an influx of Na^{+} and membrane depolarization.

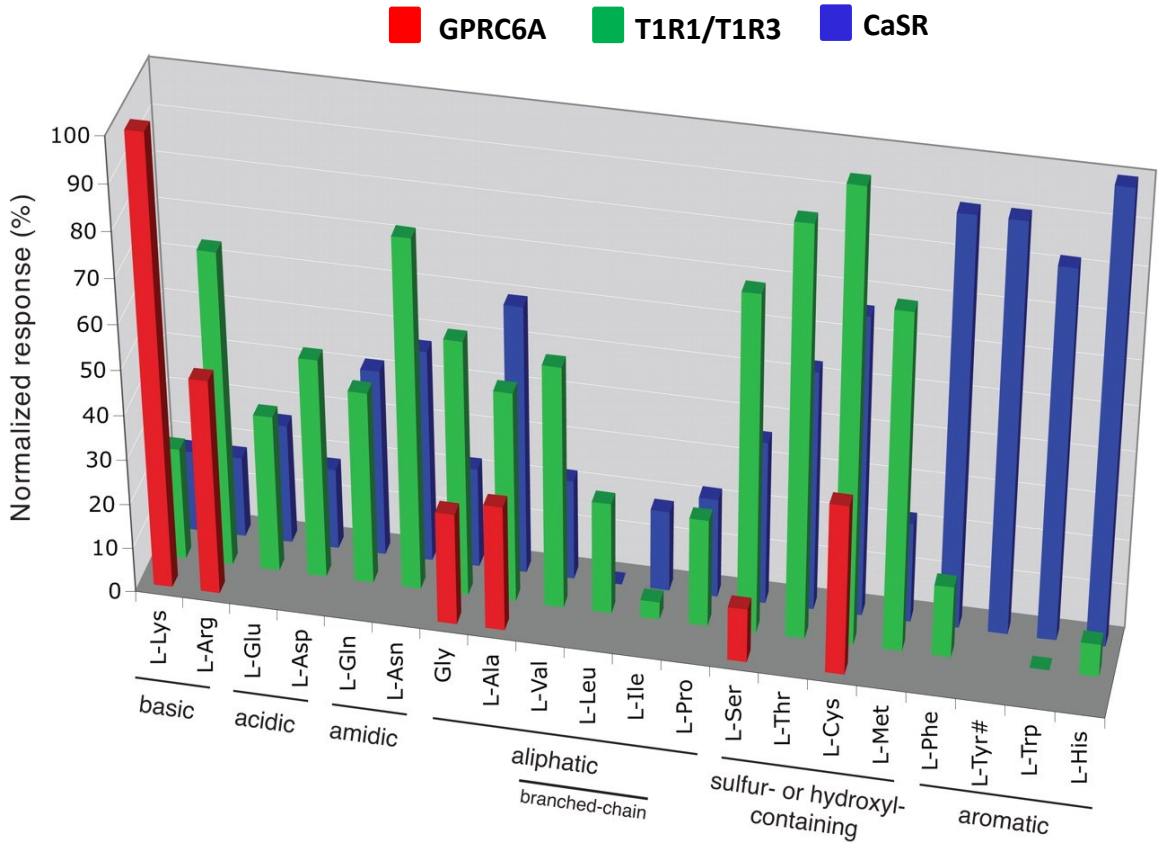


Figure 2.1.4 L-amino acid selectivity profile for mouse CaSR, T1R1/T1R3 and GPRC6A
Effect of the 20 amino acids at 10mM on the Ca^{2+} response at the mCaSR expressed as percentage response relative to L-histidine and on the mT1R1/T1R3 response in the presence of 2.5mM IMP expressed as a percentage normalized to L-cysteine response (Tyrosine was not tested at this receptor). mGPRC6A values are based on EC_{50} values of the 20 amino in the presence of 1mM Ca^{2+} and 1mM Mg^{2+} relative to L-lysine. Reviewed by (Wellendorph et al., 2009a)

Cell Type	Chemosensory Signalling Element	Species	Localization	References
Brush Cells	α-gustducin	Rat, mouse	S, SI	1-4, 6, 7
	α-gustducin	Mouse	C	2
	No α-gustducin	Mouse	O	4
	TRPM5	Mouse	S,D, C	2, 4
	T1R3	Mouse	S, D	2, 5
	No T1R3	Mouse	C	2
	No T1R3	Rat	J	7
	T1R1	Rat, Mouse	D	2, 7
	PLCβ2	Rat	J	7
	No PLCβ2	Mouse	S,D,C	2, 4
Ghrelin (P, X/A cells)	α-gustducin	Mouse	S	6
	T1R3	Mouse	S,D	5
	No PLCβ2	Human	S	12
	No TRPM5	Human	S	12
	PLCβ2	Mouse	S	4
Gastrin (G cells)	No PLCβ2	Human, Mouse	S	16
	PLC β3	Human, Mouse	S	16
	No T1R3	Mouse	S	15
	CaSR	Human, Swine, Mouse	S	13-16
	GPRC6A	Human, Swine, Mouse	S	15-16
	GPR92	Swine, Mouse	S	16
CCK (I cells)	CaSR	Mouse	D	17-18
Somatostatin (D cells)	PLCβ2	Human, Swine, Mouse	S	16
	CaSR	Human, Swine, Mouse	S	16
	GPRC6A	Human, Swine, Mouse	S	15-16
	GPR92	Swine, Mouse	S	16
GLP-1 (L-cells)	α-gustducin	Human	D, C	8-10
	α-gustducin	Mouse	J	3
	PLCβ2	Human	D	10
	TRPM5	Human	D	10
	T1R3	Human	D, C	9, 10
PYY (L-cells)	α-gustducin	Human	C	8
GIP (K cells)	α-gustducin	Human, Rat	D	10, 11
5-HT (EC cells)	No α-gustducin	Human	C	8
	α-gustducin	Mouse	J	3
	No α-gustducin	Mouse	S	4
	No α-gustducin	Rat	D	1

Table 2.1.2 Expression of chemosensory signalling elements in gastrointestinal cells
Abbreviations: C- colon, D- duodenum, J- jejunum, S- Stomach, SM- small intestine, O – oesophagus
References: **1**-(Hofer et al., 1996), **2**-(Bezencon et al., 2007), **3**-(Sutherland et al., 2007), **4**-(Hass et al., 2007), **5**-(Hass et al., 2010), **6**-(Janssen et al., 2011), **7**-(Mace et al., 2007), **8**-(Rozengurt et al., 2006), **9**-(Steinert et al., 2011), **10**-(Jang et al., 2007), **11**-(Fujita et al., 2009), **12**-(Widmayer et al., 2012), **13** - (Ray et al., 1997), **14**-(Feng et al., 2010), **15**-(Haid et al., 2011), **16**-(Haid et al., 2012), **17**-(Wang et al., 2011), **18**-(Liou et al., 2011)

2.1.1.2 Amino acid transporters/transceptors

The absorption of amino acids from the gut lumen and the supply of amino acids to all tissues are dependent on amino acid transporters. Amino acid transporters are organized into families that form part of the solute-linked carrier (SLC) group of membrane transport proteins. These amino acid transporters, much like the receptors previously discussed, are promiscuous, with each system transporting a range of amino acids and a degree of overlap existing between the systems. Despite this overlap, defects in some of these transporter systems are associated with multifactorial diseases, illustrating their importance. The transporter systems and their substrates, as well as the disorders associated with defects in these systems are summarized in Table 2.1.3.

Furthermore, in recent years evidence has emerged to suggest that in addition to their transport role, amino acid transporters may also have receptor like functions, sensing changes in extracellular amino acid availability and regulating nutrient-induced signals. As a relevant example, SNAT2 has been implicated as an amino acid transceptor. SNAT2 functions as a secondary active transporter, coupling the transfer of amino acids against their concentration gradient with the inward movement of Na^+ down its electrochemical gradient in a rheogenic manner. This transport mechanism has been implicated in the stimulation of gut hormone release through consequential increases in intracellular Ca^{2+} (Young et al., 2010), and specifically in glutamine stimulated GLP-1 release from intestinal L-cells (Tolhurst et al., 2011).

Family	System	Transporter	Substrates	Mechanism	Ion Dependence	Defects
SLC3/7 - Glycoprotein-associated AA transporters (gpaAT)	L	4F2hc/LAT1	H, M, L, I, V, F, Y, W	Antiport		
		4F2hc/LAT2	AA^0 except P	Antiport		
	y+L	4F2hc/ y+LAT1	K, R, Q, H, M, L	Antiport	Na^+ (Symport-AA^0)	Lysinuric protein intolerance
		4F2hc/ y+LAT2	K, R, Q, H, M, L, A, C	Antiport	Na^+ (Symport-AA^0)	
	x^-_c	4F2hc/xCT	E, cystine	Antiport		
	asc	4F2hc/asc1	G, A, S, C, T	Antiport		
	$b^{0,+}$	rBAT/ $b^{0,+}$AT	R, K, O, cysteine	Antiport		Cystinuria
SLC38 -Small neutral AA transporters	N	SNAT3	Q, N, H	Symport	Na^+ (Symport), H^+ (Antiport)	
		SNAT5	Q, N, H, S, G	Symport	Na^+ (Symport), H^+ (Antiport)	
	A	SNAT2	G, P, A, S, C, Q, N, H,M	Symport	Na^+	
		SNAT4	G, A, S, C, Q, N, M, AA^+	Symport	Na^+	
SLC7 - Cationic AA transporter	y+	CAT1-3	R, K, O, H	Uniport		
SLC1 - Excitatory AA transporter	X^-_{AG}	EAAT1-5	E, D	Symport	Na^+, H^+ (Symport), K^+ (Antiport)	
	ASC	ASCT1	A, S, C	Antiport	Na^+	
		ASCT2	A, S, C, T, Q	Antiport	Na^+	
SLC16 - Monocarboxylate transporters	T	TAT1	F, Y, W	Uniport		Blue Diaper syndrome
SLC6 - Neurotransmitter transporters	Gly	GLYT1,2	G	Symport	Na^+, Cl^-	
	$B^{0,+}$	$ATB^{0,+}$	AA^0, AA^+	Symport	Na^+, Cl^-	
	B^0	B^0AT1	AA^0	Symport	Na^+	Hartnup Disorder
		B^0AT2	P, L, V, I, M	Symport	Na^+	

Table 2.1.3 Amino acid transporters and their substrates.
Summary of 6 amino acid transporter families, their substrates (denoted by their one letter code), their mechanism of transport and ion dependence, and the names of disorders associated with defects in these systems. (Broer, 2008)

2.1.2.3 Cellular amino acid sensing

2.1.2.3.1 mTORC1

Mammalian target of rapamycin complex 1 (mTORC1, complex of mTOR, Raptor and mLST8) is a serine/threonine protein kinase that functions as a master regulator of metabolism and growth. It acts as a convergence point for a vast signalling network that reflects an organism's physiological state, including its nutritional status. Amino acids have proven to be essential for mTORC1 activation but the mechanism by which amino acids activate mTORC1 remains to be fully elucidated. However, it is clear that the GTPase Rheb, when it is in its GTP bound state, is a direct activator of mTORC1 (Stocker et al., 2003) and that Rheb is essential for mTORC1 activation. The activity of Rheb is regulated by the tuberous sclerosis (TSC) complex , which contains the GTPase activating protein TSC2 (Inoki et al., 2003, Zhang et al., 2003a). The TSC complex retains Rheb in its GDP bound state preventing the activation of mTORC1. Growth factors inhibit the TSC complex and promote the accumulation of $Rheb^{GTP}$ (Long et al., 2005). Additionally, activation of mTORC1 is dependent on Rag GTPases (Sancak et al., 2008). Amino acid availability promotes the activity of a guanine nucletide exchange factor (GEF) termed Ragulator. The Ragulator complex associates with Rags leading to the accumulation of $RagA/B^{GTP}$-$RagC/D^{GDP}$ (Bar-Peled et al., 2012). The lysosomal membrane protein v-ATPase, a proton pump, associates with the Ragulator-Rag complex and together this complex ultimately governs the spatial regulation of mTORC1. In the presence of amino acids the v-ATPase-Ragulator-Rag complex recruits mTORC1 to the lysosome where it can be activated by Rheb. Many mechanisms by which amino acids regulate mTORC1 activity have been proposed. These include an intralysosomal signal (Zoncu et al., 2011), Leucyl-tRNA synthetase GAP activity (Han et al., 2012), mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) (Findlay et al., 2007), T1R1/T1R3 (Wauson et al., 2012), and inositol polyphosphate multikinase (IMPK) (Kim et al., 2011). It is possible that there are a number of mechanisms through which amino acids regulate basal mTORC1 activity, but what is clear is that amino acids are essential signals in mTORC1 activation by growth factors and are therefore themselves 'master regulators of metabolism and growth'.

Administration of L-leucine into the mediobasal hypothalamus (MBH) activates mTORC1 in arcuate POMC neurons, suppressing food intake and meal size through an MBH-PVN-NTS circuit (Blouet et al., 2009). Additionally, administration of L-leucine into the caudomedial NTS activates mTORC1 in noradrenergic, dopaminergic and POMC neurons in the NTS and suppresses food intake meal size (Blouet and Schwartz, 2012).

2.1.2.3.2 GCN2

The biological requirement for amino acids is exemplified by animal behaviour in response to diets deficient in essential or indispensable amino acids. Animals can detect and reject a diet lacking specific indispensible amino acids (IAA) within 20 minutes and preferentially choose diets containing the limiting IAA (Koehnle et al., 2003) (Koehnle et al., 2004). This adaptive eating behaviour is governed by the anterior piriform cortex (APC) (Leung and Rogers, 1971). Following ingestion of a diet deficient in a specific amino acid, the concentration of that amino acid is reduced in the APC (Koehnle et al., 2004). The reduction in amino acid availability leads to an accumulation of uncharged tRNAs, which subsequently bind to and activate general control nonrepressed 2 (GCN2) which can then phosphorylate eukaryotic initiation factor 2α (eIF2α) leading to a global down-regulation of protein synthesis. The exception to this is the up-regulation of ATF4 and ATF4 responsive genes. ATF4 is a transcriptional activator of amino acid biosynthetic enzymes and transporters including SNAT2 (Hao et al., 2005, Blais et al., 2003, Gietzen et al., 2004) and therefore acts to increase amino acid availability. The sensing of IAA deficiency is believed to be conveyed to feeding centres including the hypothalamus, particularly the LHA, and amygdala through a number of candidate mechanisms including: the rheogenic activity of SNAT1, ERK1/2 signalling (Franchi-Gazzola et al., 1999), increased glutamatergic activity and AMPA receptor activation (Sharp et al., 2004), and loss of GABAergic inhibitory neurotransmission (Truong et al., 2002).

2.1.2.3.3 FTO

The fat mass and obesity associated protein (FTO) is a nucleic acid demethylase (Gerken et al., 2007). Single-nucleotide polymorphisms (SNP's) in this gene are strongly associated with BMI and obesity (Frayling et al., 2007), as a result of increased energy intake (Cecil et al.,

2008, Speakman et al., 2008, Haupt et al., 2009). Within the arcuate nucleus, Fto expression is bi-directionally regulated according to energy availability, such that fasting decreases expression and prolonged high fat feeding increases expression (Tung et al., 2010). Moreover, this regulation appears to be under the influence of amino acid availability, with essential amino acid deprivation leading to a downregulation of Fto mRNA and protein levels (Cheung et al., 2013). Additionally, mouse embryonic fibroblasts (MEFs) derived from $Fto^{(-/-)}$ mice exhibit slower rates of growth and have reduced mRNA translation (Gulati et al., 2013). As previously discussed, amino acids are capable of regulating mTORC1 activity, and mTORC1 is a major regulator of translation initiation and cell growth. Indeed similar to cells lacking the amino acid sensing receptor T1R1/T1R3, cells lacking FTO demonstrate decreased mTORC1 activity, decreased rates of mRNA translation and increased autophagy (Gulati et al., 2013). FTO may function as an amino acid sensor upstream of mTORC1.

2.1.6 Hypothesis and Aims

It is evident that there are many mechanisms by which mammals sense amino acids. However, the role of amino acids in appetite regulation is still unclear.

Hypotheses: The anorectic effect of intraperitoneal administration of L-arginine and L-lysine observed in preliminary studies is not present when they are administered as neutral salts.

L-cysteine decreases food intake by modulating gut hormone release and activating central appetite regulating pathways.

Aims: To investigate

1. The anorectic effect of L-arginine and L-lysine salts.
2. The dose response effect of L-cysteine on food intake in rats and mice.
3. The location at which L-cysteine mediates its anorectic effects.

2.2 Methods

2.2.1 Animals

Male Wistar rats (Charles River, Margate, Kent, UK) weighing between 200-220g were maintained in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to food (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless otherwise stated. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/7062).

Male C57BL/6 mice (Harlan, Bicester, Oxon, UK) weighing between 18-20g were maintained in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to food (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless otherwise stated. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/7062).

Prior to any experimental procedure, animals were acclimatised to the handling and administration procedure. For all studies, animals were randomised by body weight.

2.2.2 The effect of L-arginine.HCl and L-lysine.HCl on food intake

Preliminary data suggested intraperitoneal administration of 2mmol/kg L-arginine and L-lysine significantly reduced food intake during the 0-1 hour period following administration. L-arginine and L-lysine have side chains with basic properties and thus when dissolved form alkaline solutions. To investigate whether the alkalinity of these amino acid solutions was a factor in the reduction in food intake, the effect of the hydrochloride salts of L-arginine and L-lysine on food intake was investigated following intraperitoneal and oral gavage administration in rats and mice.

Rats were fasted overnight and then received an intraperitoneal administration saline, 2mmol/kg L-arginine.HCl or L-lysine.HCl during the early light phase. Subsequently, rats were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

In a second study rats received an oral gavage of water, 4mmol/kg L-arginine.HCl, L-lysine.HCl or L-cysteine, and food intake was measured as before. These two studies were also repeated in mice with an intraperitoneal dose of 4mmol/kg and oral gavage dose of 8mmol/kg.

2.2.3 The effect of OG administration of L- and D-cysteine on food intake in rats during the early light phase

Rats were fasted overnight and then received an oral gavage of water, 1, 2 or 4mmol/kg L-cysteine (Sigma-Aldrich) or 4mmol/kg D-cysteine (Sigma-Aldrich) (n=7-8) during the early light phase. Subsequently, rats were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

2.2.3.1 The effect of OG administration of L-cysteine on behaviour in rats during the early light phase

Following oral gavage administration of water or 4mmol/kg L-cysteine, and upon return to their home cages, animals were observed by an investigator blinded to the experimental treatment. Each animal was observed once every 5 seconds for a period of 15 seconds every 5 minutes up to one hour post administration. Behaviour during each observation was classified into one of twelve categories: feeding, drinking, rearing, locomotion, grooming, pica, bed-making, head-down/hunched, sleeping, tremors, climbing and stationary. These were sub-categorised into six behaviours: Feeding (feeding or drinking), Locomotion (rearing, locomotion, bed-making or climbing), Grooming, Head-down (head-down, hunched or tremors), Pica and Resting (stationary or sleeping) (Ghourab et al., 2011).

2.2.4 The effect of IP administration of L-cysteine on food intake in rats during the early light phase

Rats were fasted overnight and then received an intraperitoneal (IP) administration of saline, 0.5, 1 or 2mmol/kg L-cysteine (n=8) during the early light phase. Subsequently, rats were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

2.2.4.1 The effect of IP administration of L-cysteine on behaviour in rats during the early light phase

Behaviour was monitored following IP administration of saline or 2mmol/kg L-cysteine described in section 2.2.3.1.

2.2.5 The effect of IP administration of L- and D-cysteine on food intake in rats during the early light phase

Rats were fasted overnight and then received an intraperitoneal (IP) administration of saline, 2mmol/kg L-cysteine or 2mmol/kg D-cysteine (n=8) during the early light phase. Subsequently, rats were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

2.2.6 The effect of OG administration of L- and D-cysteine on food intake in mice during the early light phase

Mice were fasted overnight and then received an oral gavage of water, 4 or 8mmol/kg L-cysteine or 8mmol/kg D-cysteine (n=10) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

2.2.7 The effect of IP administration of L- and D-cysteine on food intake in mice during the early light phase

Mice were fasted overnight and then received an intraperitoneal injection of saline, 1, 2 or 4mmol/kg L-cysteine or 4mmol/kg D-cysteine (n=9-12) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

2.2.7.1 The effect of IP administration of L-cysteine on behaviour in mice during the early light phase

Behaviour was monitored following IP administration of saline or 2mmol/kg L-cysteine described in section 2.2.3.1.

2.2.8 The effect of OG administration of L-cysteine on c-Fos-like immuno-reactivity in rats

Rats were fasted overnight and then received an oral gavage of water, 4mmol/kg L-cysteine or 4mmol/kg glycine (n=4-6) during the early light phase. Glycine was used as a negative control, as it had previously been found to have no effect on food intake (Fig 2.1.6 A and B). Two animals were IP injected with hypertonic saline and used as positive controls for the staining procedure.

2.2.8.1 Tissue collection

Ninety minutes post gavage, rats were deeply anaesthetised with an IP injection of 2.5ml pentobarbital (Euthatal, Merial Animal Health Ltd. Harlow, UK). Once anaesthetised, rats were transcardially perfused with 0.01M PBS (appendix 1) to flush the vasculature of blood and subsequently perfused with 4% formaldehyde to fix the neural tissue. Once fixed, the brains were removed and stored in 4% formaldehyde solution overnight at 4°C. Brains were then stored in 40% (w/v) sucrose solution (appendix 1) for 5-7days to dehydrate them for cryopreservation. They were then frozen in isopentane chilled to -80°C and stored at -80°C until sectioning.

Forty micrometre coronal sections of the whole hypothalamus and brainstem were cut using a freezing sled microtome (Shandon Southern Products, Ltd., Runcorn, Cheshire, U.K.). Free-floating sections were stored in antifreeze (appendix 1) at -20°C until immunohistochemistry (IHC) was carried out.

2.2.8.2 c-Fos immunohistochemistry

Free-floating sections were used for c-Fos IHC. Endogenous peroxidase activity was blocked by incubation in 0.6% H_2O_2 for 15 minutes. Sections were then incubated for two hours in blocking solution. Sections were incubated overnight in 1:20,000 dilution of anti-rabbit c-Fos (Merck Chemicals Ltd. Nottingham, UK). Sections were then incubated in a 1:400 dilution of secondary antibody (biotinylated anti-rabbit ImmunoglobinG; Vector Laboratories, Peterborough, UK) for two hours followed by one hour incubation in a 1:200 dilution of avidin-biotin peroxidase/horse radish peroxidase solution. The sections were then immersed in a 1% diaminobenzedine tetrahydrochloride solution for staining. SON/PVN

sections from the positive control animals were included in the staining protocol. Between each step sections were washed in 0.01M PBS. Sections were mounted onto poly-L-lysine-coated slides then dehydrated in ethanol, delipidated in xylene and cover-slipped using DPX mounting medium. All solutions are outlined in appendix 1.

Cell bodies positive for c-Fos-like immunoreactivity, were counted bilaterally from matched sections by an observer blinded to the treatment. The hypothalamic nuclei examined were the arcuate nucleus (ARC), paraventricular nucleus (PVN), anterior hypothalamic area (AHA), ventromedial nucleus (VMN), dorsal medial nucleus (DMN) and lateral hypothalamic area (LHA), and the nucleus tractus solitarius (NTS) and area postrema (AP) were examined in the brainstem. Nuclei were defined in relation to anatomical landmarks according to the rat brain atlas of Paxinos and Watson (Paxinos, 2007).

2.2.9 The effect of OG administration of L-cysteine on conditioned taste aversion

Rodents lack the required anatomy for emesis, it is therefore difficult to determine whether the administration of specific substances induce feelings of nausea. To investigate the possibility that the administration of L-cysteine and the subsequent reduction in food intake were associated with feelings of visceral illness, a conditioned taste aversion protocol was used.

The experimental protocol was adapted from an established method for analysis of conditioned taste aversion using a single-bottle method (Lachey et al., 2005). Adult male Wistar rats underwent a training week followed by test week. Animals were trained daily for one week to consume their daily fluid intake within a one hour period; at all other times water access was restricted. Any animal failing to consume at least 40ml/kg/24hr on two consecutive days within the training period or showing clinical signs of dehydration was removed from the study.

The treatments tested were water, 1, 2 or 4mmol/kg L-Cysteine (n=8-9) and 127mg/kg Lithium Chloride (n=5) was used as a positive control. On Day 1 and 3 of the test week animals were introduced to a novel flavour; Grape Kool-Aid (Northfield, IL, USA) diluted according to manufacturer's instructions, during the one hour fluid access period in place of

water. Introduction to the flavour was immediately followed by an oral gavage of their allocated treatment. On day 2 and 4 all animals received water during the fluid access period. On the final day, day 5, all animals received Kool-Aid, without any subsequent gavage. Fluid intake was compared between groups.

2.2.10 The effect of central administration of L-cysteine on food intake

2.2.10.1 Stereotactic Surgery

Animals were anaesthetised by inhalation anaesthesia (2L/min Oxygen and 4% Isoflurane). Prophylactic antibiotics, flucloxacillin (37.5mg/kg) and amoxicillin (37.5mg/kg) were administered prior to surgery. A stainless steel 22-gauge cannula (Plastics One, Inc., Roanoke, VA) was inserted stereotactically into the lateral ventricle using a Kopf stereotactic frame (Harvard Apparatus); co-ordinates used for the lateral ventricle were 0.5mm posterior from bregma, +1.5mm lateral and 3.5mm ventral. Co-ordinates were taken from the Paxinos and Watson atlas. The cannula was secured using three stainless steel screws that were inserted into the cranium and dental cement. Animals were given buprenorphine (45µg/kg) for analgesia postoperatively and allowed one week to recover. Correct cannula placement was verified with a positive dipsogenic response to Angiotensin II (150ng).

In all ICV feeding studies, test substances were administered in a volume of 5µl at a rate of 120µl/hour to conscious, free moving animals via a 26-gauge stainless steel injector projecting 1mm beyond the tip of the cannula. Following injection, animals were returned to their individual cage with a pre-weighed amount of food and free access to water.

2.2.10.2 The effect of intracerebroventricular administration of L- cysteine on food intake in rats

Overnight fasted rats received a single ICV injection of 0.9% Saline (control), L-Cysteine (1 or 2µmol) or 3nmol NDP-MSH (positive control) during the early light phase between 09.00 and 10.00hr (n=5-8). Food intake was measured 1, 2, 4, 8 and 24 hours after administration.

2.2.10.3 The effect of intracerebroventricular administration of L- and D-cysteine on food intake in rats

Overnight fasted rats received a single ICV injection of 0.9% Saline (control), L-Cysteine (1.5 or 2µmol) or 2µmol D-cysteine during the early light phase between 09.00 and 10.00hr (n=5-8). Food intake was measured 1, 2, 4 and 24 hours after administration.

2.2.11 The effect of L-cysteine on gut hormone release

The effect of oral and IP administration of L-cysteine on plasma levels of GLP-1, PYY and acyl-ghrelin was investigated.

2.2.11.1 The effect of OG administration of L-cysteine on gut hormone release

Male Wistar rats were fasted overnight and then received an oral gavage of water or 4mmol/kg L-cysteine (n=8). Animals were returned to their home cages then thirty minutes post-administration, killed by decapitation and trunk blood collected in lithium heparin tubes containing 0.6mg aprotinin. Plasma was separated by centrifugation and then frozen and stored at -20°C for analysis. After centrifugation an aliquot of plasma was acidified with HCl to a concentration of 1N before freezing.

2.2.11.1.1 GLP-1 RIA

Plasma GLP-1-like immunoreactivity was measured using an established, specific and sensitive radioimmunoassay (Kreymann et al., 1987). The antibody was produced in rabbits against GLP-1 coupled to bovine serum albumin (BSA) and cross-reacts fully with all amidated forms of GLP-1 but does not cross react with glycine extended forms (GLP-1_{1-37} and GLP-1_{7-37}) or any other gastrointestinal peptides. ^{125}I-GLP-1 was prepared by Prof. Mohammad Ghatei using the iodogen method (Wood et al., 1981) and purified by high pressure liquid chromatography (HPLC). The assay was performed in a total volume of 700µl of Veronal buffer (appendix 1) (pH 8) containing 0.3% BSA. The standard curve was constructed by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of GLP-1 at a concentration of 0.5pmol/ml. A sample of 100µl plasma was used and all samples were assayed in duplicate. The assay was incubated over three nights at 4^{o}C before separation of the free from antibody-bound labelled peptide by charcoal adsorption (appendix 1). Free and bound

radioactivity was measured using γ scintillation counters (model NE1600, Thermo Electron Corporation, Ohio, USA).

2.2.11.1.2. PYY RIA

PYY-like immunoreactivity was measured using a specific and sensitive radioimmunoassay (Adrian et al., 1985). The antibody was produced in rabbits against synthetic porcine PYY coupled to BSA by glutaraldehyde and cross-reacts fully with all biologically active forms of PYY (full length PYY_{1-36} and the truncated fragment PYY_{3-36}) but does not cross-react with other gastrointestinal peptides. ^{125}I-PYY was prepared by Professor M. Ghatei using the iodogen method (Wood et al., 1981) and purified by HPLC. The assay was performed in a total volume of 700µl of 0.06M phosphate buffer (appendix 1) (pH 7.3) containing 0.3% BSA. The standard curve was constructed by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of synthetic PYY at a concentration of 0.5pmol/ml. A sample of 100µl plasma was used and all samples were assayed in duplicate. The assay was incubated over three nights at 4^{o}C before separation of the free from antibody-bound labelled peptide by immunoprecipitation using sheep anti-rabbit antibody. Free and bound radioactivity was measured using γ scintillation counters.

2.2.11.1.3 Acylated Ghrelin EIA

Acidified rat plasma samples were used for this assay (1N HCl). A rat/mouse acylated ghrelin enzyme immunoassay kit was used and performed according to manufacturer's instructions (SPI bio bertin, Bertin Pharma, Montigny le Bretonneux, France). The kit had 100% specificity to rat acylated ghrelin.

2.2.11.2 The effect of IP administration of L-cysteine on gut hormone release

Male Wistar rats were fasted overnight and then received an IP injection of saline or 2mmol/kg L-cysteine (n=8). Animals were culled thirty minutes post administration and plasma collected and analyzed as 2.2.9.1.

2.2.12 Statistical analysis

All food intake data is expressed as mean ± SEM and analysed using one-way ANOVA with post hoc Tukey's test where appropriate. Behavioural data is expressed as median and interquartile range and analysed using Mann-Whitney test. C-Fos data was analysed using Kruskall-Wallis one-way ANOVA with Dunn's multiple comparison where appropriate. Gut hormone data is expressed as mean ± SEM and was analysed using an unpaired t-test. Statistical significance was accepted at $p<0.05$. All analysis was carried out using Graph Pad Prism Software, version 5.0.

2.3 Results

2.3.1 The effect of L-arginine.HCl and L-lysine.HCl on food intake

Intraperitoneal administration of 2mmol/kg L-arginine.HCl or L-lysine.HCl did not significantly reduce food intake in the 0-1 hour period following administration rats. Oral gavage administration of 4mmol/kg L-arginine.HCl or L-lysine.HCl did not significantly reduce food intake in the 0-1hour period following administration in rats.

Intraperitoneal administration of 4mmol/kg L-arginine.HCl or L-lysine.HCl did not significantly reduce food intake in the 0-1 hour period following administration mice. Oral gavage administration of 8mmol/kg L-arginine.HCl or L-lysine.HCl did not significantly reduce food intake in the 0-1hour period following administration in mice.

These data suggest that much of the anorectic effect of L-arginine and L-lysine were secondary to the alkalinity of the amino acids in solution, rather than to their specific biological properties.

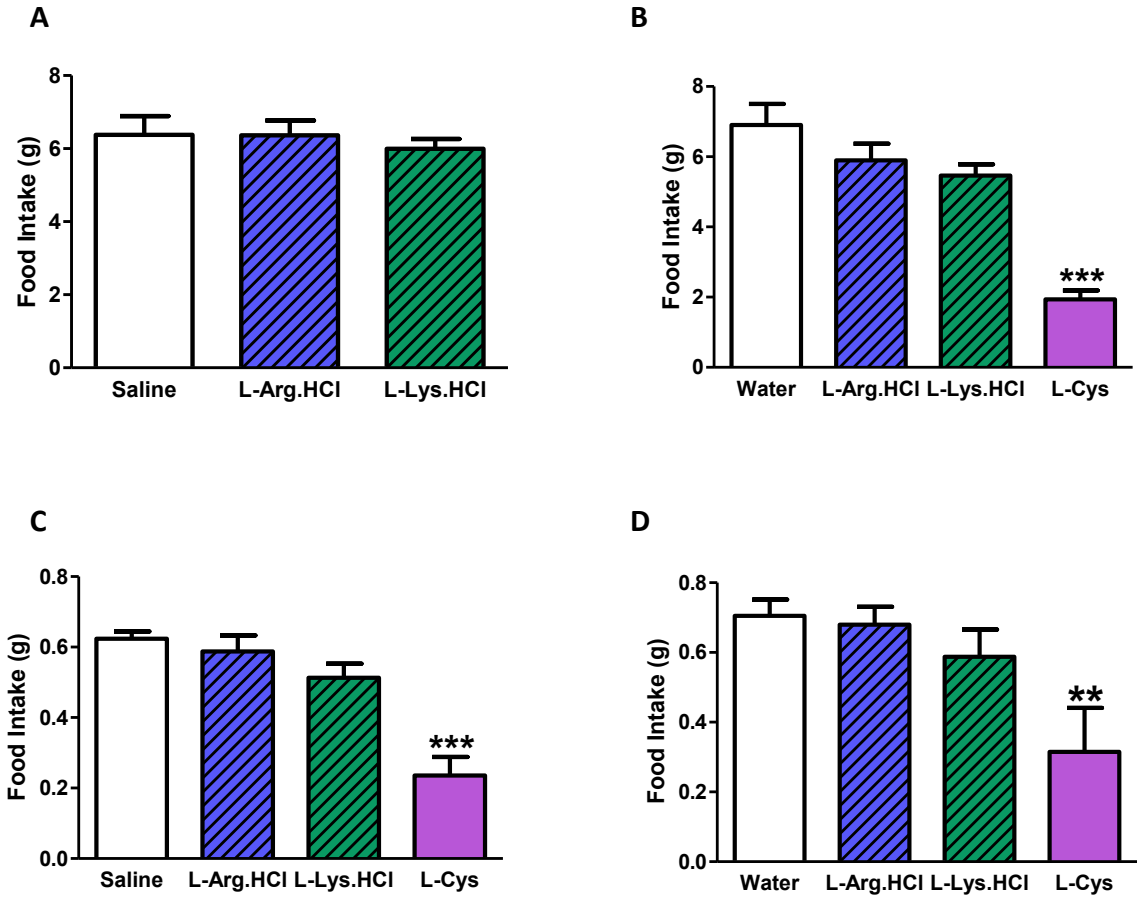


Figure 2.3.1 The effect of L-arginine.HCl and L-Lysine.HCl on food intake

The effect of intraperitoneal administration of saline, 2mmol/kg L-argininie.HCl or L-lysine.HCl (n=7-10) (A), the effect of oral gavage administration of water, 4mmol/kg L-arginine.HCl, L-lysine.HCl or L-cysteine in rats (n=6-12) (B), the effect of intraperitoneal administration of saline, 4mmol/kg L-argininie.HCl, L-lysine.HCl or L-cysteine (n=5-15) (C), the effect of oral gavage administration of water, 8mmol/kg L-arginine.HCl, L-lysine.HCl or L-cysteine in mice (n=4-8) (D) on 0-1 hour food intake following an overnight fast. **p<0.01, ***p<0.001

2.3.2 The effect of OG administration of L- and D-cysteine on food intake in rats during the early light phase

Oral gavage administration of 2 and 4mmol/kg L-cysteine significantly reduced food intake compared to water control in the 0-1 hour period following administration (water: 6.8 ±0.6g vs. 2mmol/kg L-cysteine: 4.3 ± 0.6g, $p<0.05$; vs. 4mmol/kg L-cysteine: 2.7 ± 0.3g, $p < 0.001$, n = 7-8) (Fig.2.3.2A). Food intake following 4mmol/kg L-cysteine was significantly reduced compared to food intake following 4mmol/kg D-cysteine in the 0-1hour period following administration (L-cysteine: 2.7 ± 0.3g vs. D-cysteine: 6.0 ± 0.9g., $p<0.01$) (Fig.2.3.2A). Food intake following administration of 4mmol/kg D-cysteine was not significantly different from water control during any time period.

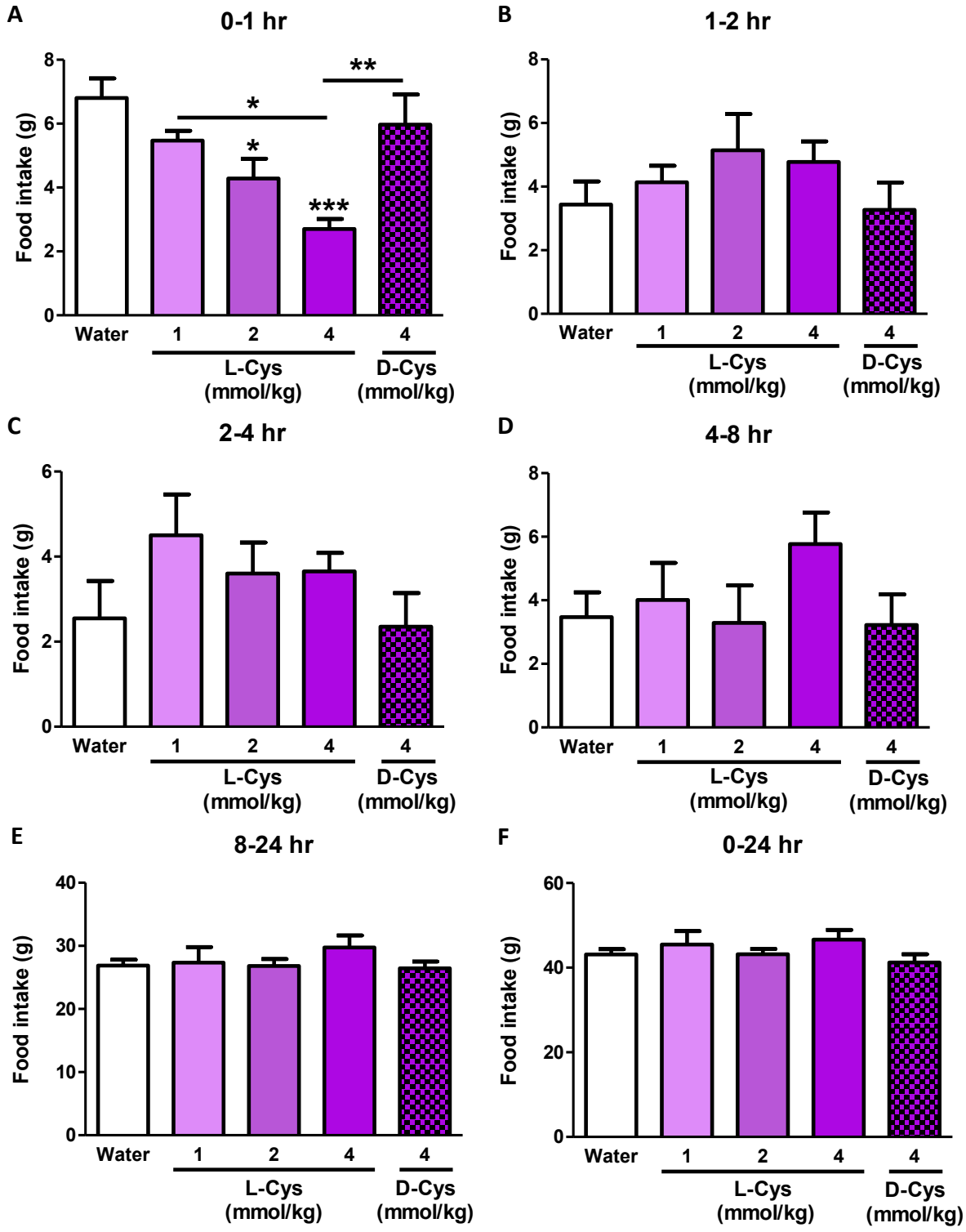


Figure 2.3.2 The effect of OG administration of L- and D-Cysteine on food intake in rats
The effect of an oral gavage of water, 1, 2 or 4mmol/kg L-cysteine, or 4mmol/kg D-Cysteine on food intake following an overnight fast during 0-1hr (A), 1-2hr (B), 2-4hr (C), 4-8 (D), 8-24hr (E), and 0-24hr (F) post administration. Data expressed as mean + SEM. n=7-8. *p<0.05, **p<0.01, ***p<0.001

2.3.2.1 The effect of OG administration of L -Cysteine on behaviour in rats during the early light phase

There was a significant decrease in feeding in the 0-1 hour period following OG administration of 4mmol/kg L-cysteine. There was no significant difference in behaviours indicative of illness or nausea (Table 2.3.1).

	Feeding	**Locomotion**	**Grooming**	**Resting**	**Head Down**	**Pica**
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Water	23.0 (18.75-24.0)	9.0 (6.75-10.0)	2.0 (0.0-3.25)	2.0 (0.0-5.5)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
L-Cys	13.0 (9.75-17.0)*	11.5 (7.0-17.5)	3.0 (0.5-4.75)	2.5 (0.25-12.25)	1.5 (0.0-5.25)	0.0 (0.0-2.25)

Table 2.3.1 Observed behaviour following OG administration of L-cysteine in rats.
Number of observed behaviours expressed as median and interquartile range in rats in the 0-1hr period following oral gavage administration of water or 4mmol/kg L-cysteine. Data expressed as median (interquartile range), n=4-10, *p<0.05

2.3.3 The effect of IP administration of L-cysteine on food intake in rats during the early light phase

Intraperitoneal administration of 2mmol/kg L-cysteine significantly reduced food intake 0-1 hour post-injection compared to saline control (saline: 5.3 ± 0.5g vs. 2mmol/kg L-cysteine: 2.3 ± 0.5g, $p < 0.05$, n=8) (Fig.2.3.3A). Doses of 1mmol/kg and 2mmol/kg L-cysteine significantly increased food intake during the 1-2 hour time period post-injection compared to saline (saline: 3.6 ± 0.5g vs. 1mmol/kg L-cysteine: 5.4 ± 0.6g, $p < 0.05$; 2mmol/kg L-cysteine: 5.7 ± 0.3g, $p < 0.01$) (Fig.2.3.3B).

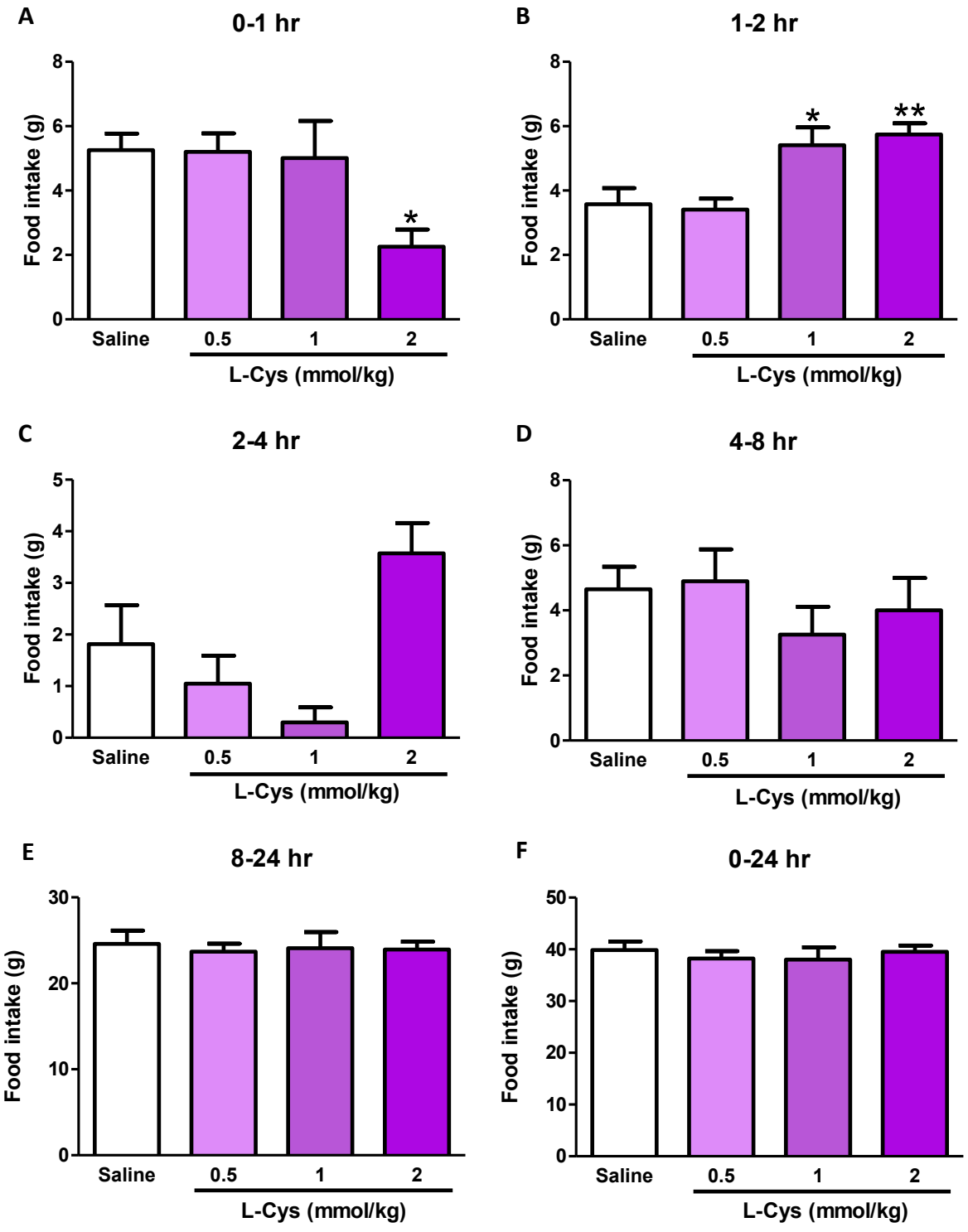


Figure 2.3.3 The effect of IP administration of L-Cysteine on food intake in rats

The effect of IP administration of saline, 0.5, 1 or 2mmol/kg L-cysteine on food intake following an overnight fast during 0-1hr (A), 1-2hr (B), 2-4hr (C), 4-8 (D), 8-24hr (E), and 0-24hr (F) post administration. Data expressed as mean + SEM. n=8. *p<0.05, **p<0.01, ***p<0.001

2.3.3.1 The effect of IP administration of L -Cysteine on behaviour in rats during the early light phase

Intraperitoneal administration of L-cysteine did not cause any significant changes in behaviour in the one hour period following injection (Table 2.3.2).

	Feeding	**Locomotion**	**Grooming**	**Resting**	**Head Down**	**Pica**
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Saline	21.0 (11.5-21.0)	11.0 (5.0-18.5)	0.0 (0-3.25)	3.0 (0.0-6.0)	0.0 (0.0-3.0)	0.0 (0.0-0.0)
L-Cys	17.0 (9.5-22.0)	10.0 (7.0-13.5)	0.0 (0.0-2.0)	3.0 (1.5-7.0)	3.0 (0.0-4.5)	0.0 (0.0-3.0)

Table 2.3.2 Observed behaviour following IP administration of L-cysteine in rats.
Number of observed behaviours expressed as median and interquartile range in mice in the 0-1 hr period following intraperitoneal administration of saline or 2mmol/kg L-cysteine. Data expressed as median (interquartile range), n=9-10

2.3.4 The effect of IP administration of L and D-cysteine on food intake in rats during the early light phase

Intraperitoneal administration of 2mmol/kg L-cysteine significantly reduced food intake compared to saline control and 2mmol/kg D-cysteine in the 0-1 hour period following administration (L-cysteine: 2.42 $\pm$ 0.4g vs. Saline: 5.56 $\pm$ 0.66g, $p<0.01$, vs. D-cysteine: 4.96 $\pm$ 0.45g, $p<0.01$) (Fig. 2.3.4A). L-cysteine significantly increased food intake in the 1-2 hour period following administration compared to saline and D-cysteine (L-cysteine: 5.02 + 0.76g vs. Saline: 1.52 + 0.88g, $p<0.01$, vs. D-cysteine: 2.30 + 0.48g, $p<0.01$) (Fig. 2.3.4B).

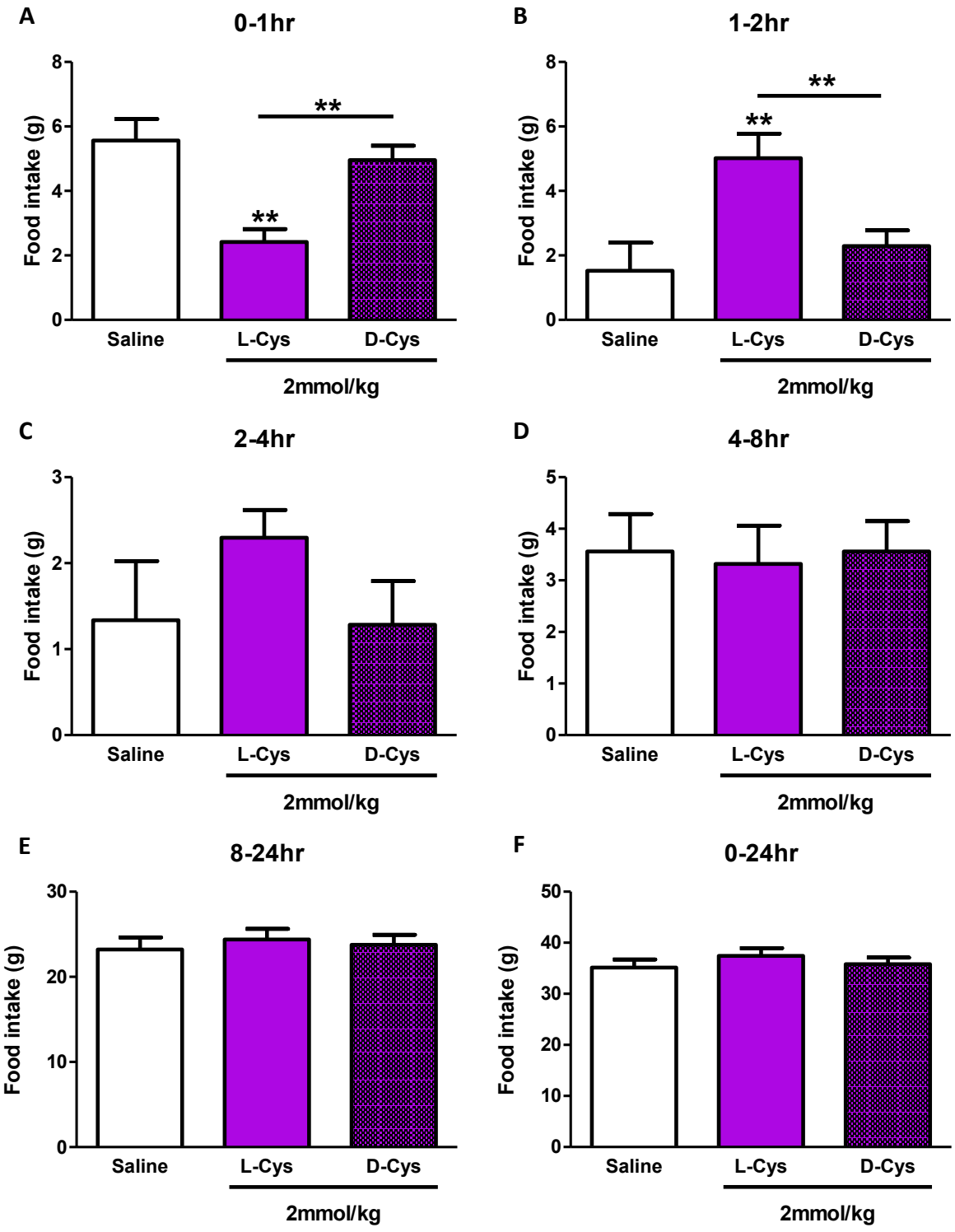


Figure 2.3.4 The effect of IP administration of L- and D-Cysteine on food intake in rats

The effect of IP administration of saline, 2mmol/kg L-cysteine or D-cysteine on food intake following an overnight fast during 0-1hr (A), 1-2hr (B), 2-4hr (C), 4-8 (D), 8-24hr (E), and 0-24hr (F) post administration. Data expressed as mean + SEM. n=8. **p<0.01

2.3.5 The effect of OG administration of L- and D-cysteine on food intake in mice during the early light phase

Oral gavage administration of 4 and 8mmol/kg L-cysteine and 8mmol/kg D-Cysteine significantly reduced food intake compared to water control in the 0-1 hour period following administration (water: 0.65 ± 0.04g vs. 4mmol/kg L-cysteine: : 0.28 ± 0.08g, $p < 0.001$; vs. 8mmol/kg L-cysteine: 0.18 ± 0.04g, $p < 0.001$; vs. 8mmol/kg D-cysteine: 0.09 ± 0.03g, $p<0.001$. n = 7-10) (Fig.2.3.5A). Food intake following 8mmol/kg L-cysteine was not significantly different from food intake following 8mmol/kg D-cysteine in the 0-1hour period following administration (L-cysteine: 0.18 ± 0.04g vs. D-cysteine: 0.09 ± 0.03g) (Fig.2.3.5A). However, during the 2-4 hour period following administration food intake was significantly decreased by 8mmol/kg D-cysteine compared to 8mmol/kg L-cysteine. (L-cysteine: 0.47 ± 0.09g vs. D-cysteine: 0.19 ± 0.08g, $p<0.05$)(Fig.2.3.5C). Cumulative food intake 24 hours after administration of 8mmol/kg L-cysteine and 8mmol/kg D-cysteine was significantly reduced compared to water control (Water: 5.14 ± 0.09g vs. 8mmol/kg L-cysteine: 4.70± 0.06g, $p<0.05$; vs. 8mmol/kg D-cysteine: 3.99 ± 0.13 g, $p<0.001$). Food intake following 8mmol/kg D-cysteine was also significantly reduced compared to 8mmol/kg L-cysteine during this time period (L-cysteine: 4.70 ± 0.06g vs. D-cysteine: 3.99 ± 0.13g, $p<0.001$) (Fig.2.3.5F)

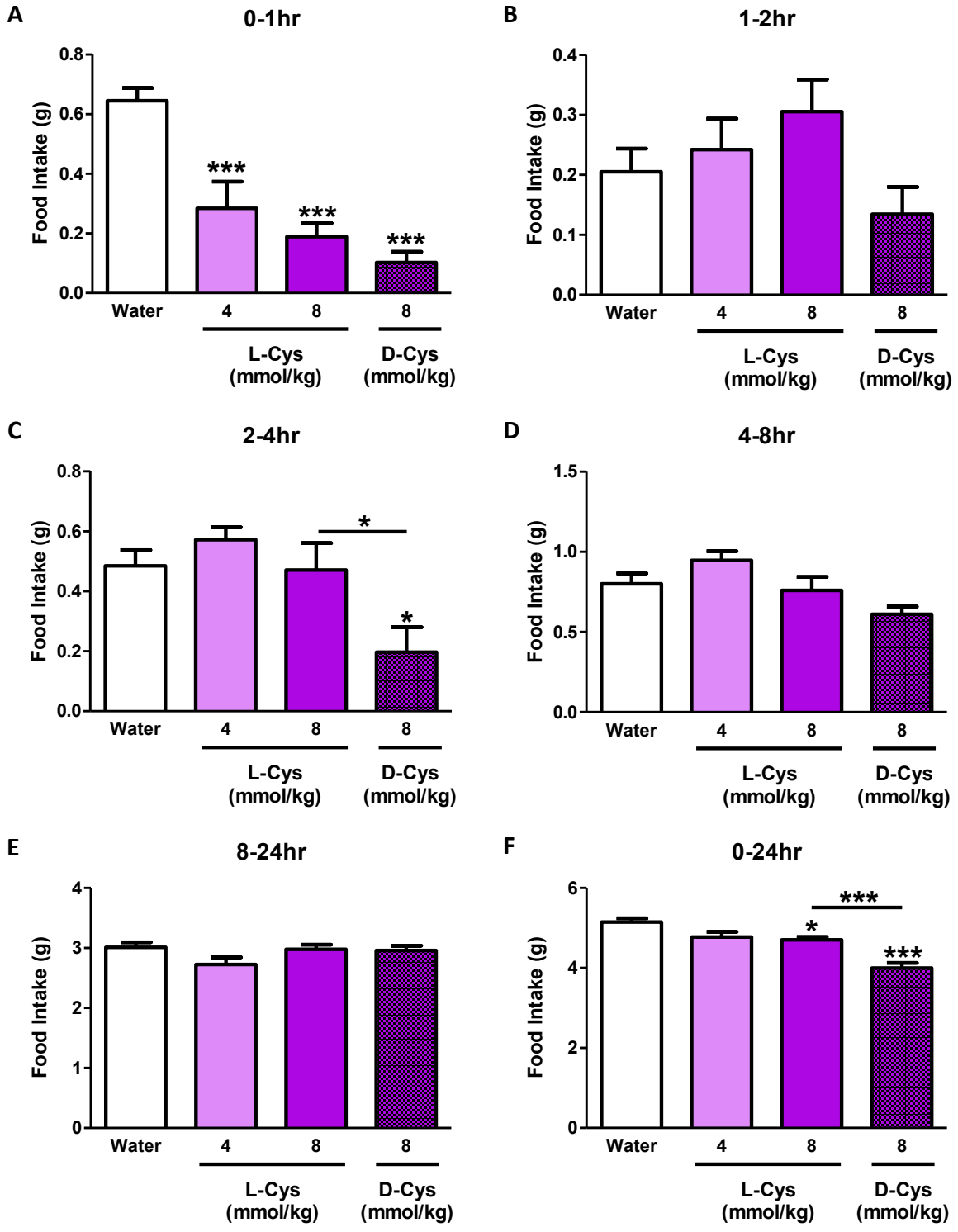


Figure 2.3.5 The effect of OG administration of L- and D-cysteine on food intake in overnight fasted mice

The effect of oral gavage of water, 4, 8mmol/kg L-cysteine or 8mmol/kg D-cysteine on food intake 0-1hr (A), 1-2hr (B), 2-4hr (C), 4-8 (D), 8-24hr (E), and 0-24hr (F) after administration. Data expressed as mean + SEM. n=9-12. *p<0.05, ***p<0.001

2.3.6 The effect of IP administration of L- and D-Cysteine on food intake in mice during the early light phase

Intraperitoneal administration of 2 and 4mmol/kg L-cysteine and 4mmol/kg D-Cysteine significantly reduced food intake compared to saline control in the 0-1 hour period following administration (saline: 0.66 ± 0.04g vs. 2mmol/kg L-cysteine: 0.28 ± 0.07g, $p < 0.001$; vs. 4mmol/kg L-cysteine: 0.16 ± 0.04g, $p < 0.001$; vs. 4mmol/kg D-Cysteine: 0.10 ± 0.01g, $p<0.001$. n = 7-10) (Fig.2.3.6A). Food intake following 4mmol/kg L-cysteine was not significantly different from food intake following 4mmol/kg D-cysteine in the 0-1 hour period following administration (L-cysteine: 0.16 ± 0.04g vs. D-cysteine: 0.10 ± 0.01g) (Fig.2.3.6A). During the 1-2 hour period food intake in the 2mmol/kg L-cysteine group was significantly increased compared to saline control (saline: 0.14 ± 0.02g vs. 2mmol/kg L-cysteine: 0.36 ± 0.06g, $p<0.05$) and food intake in the 4mmol/kg D-cysteine group was significantly decreased compared to 4mmol/kg L-cysteine (4mmol/kg L-cysteine: 0.31 $\pm$ 0.02g vs. 4mmol/kg D-cysteine: 0.09 $\pm$ 0.06g, $p<0.05$) (Fig 2.3.6B). During the 4-8 hour period food intake in the 4mmol/kg D-cysteine group was significantly decreased compared to saline control (saline: 0.71 ± 0.07g vs. 4mmol/kg D-cysteine: 0.42 ± 0.05g, $p<0.05$) (Fig.2.3.6D). Food intake 24 hours after administration of 4mmol/kg D-cysteine remained significantly reduced compared to saline control and 4mmol/kg L-cysteine (D-cysteine: 3.53 ± 0.19g vs. saline: 4.6 ±0.13g, vs. 4mmol/kg L-cysteine: 4.71 ± 0.20g, $p<0.001$) (Fig. 2.3.6F).

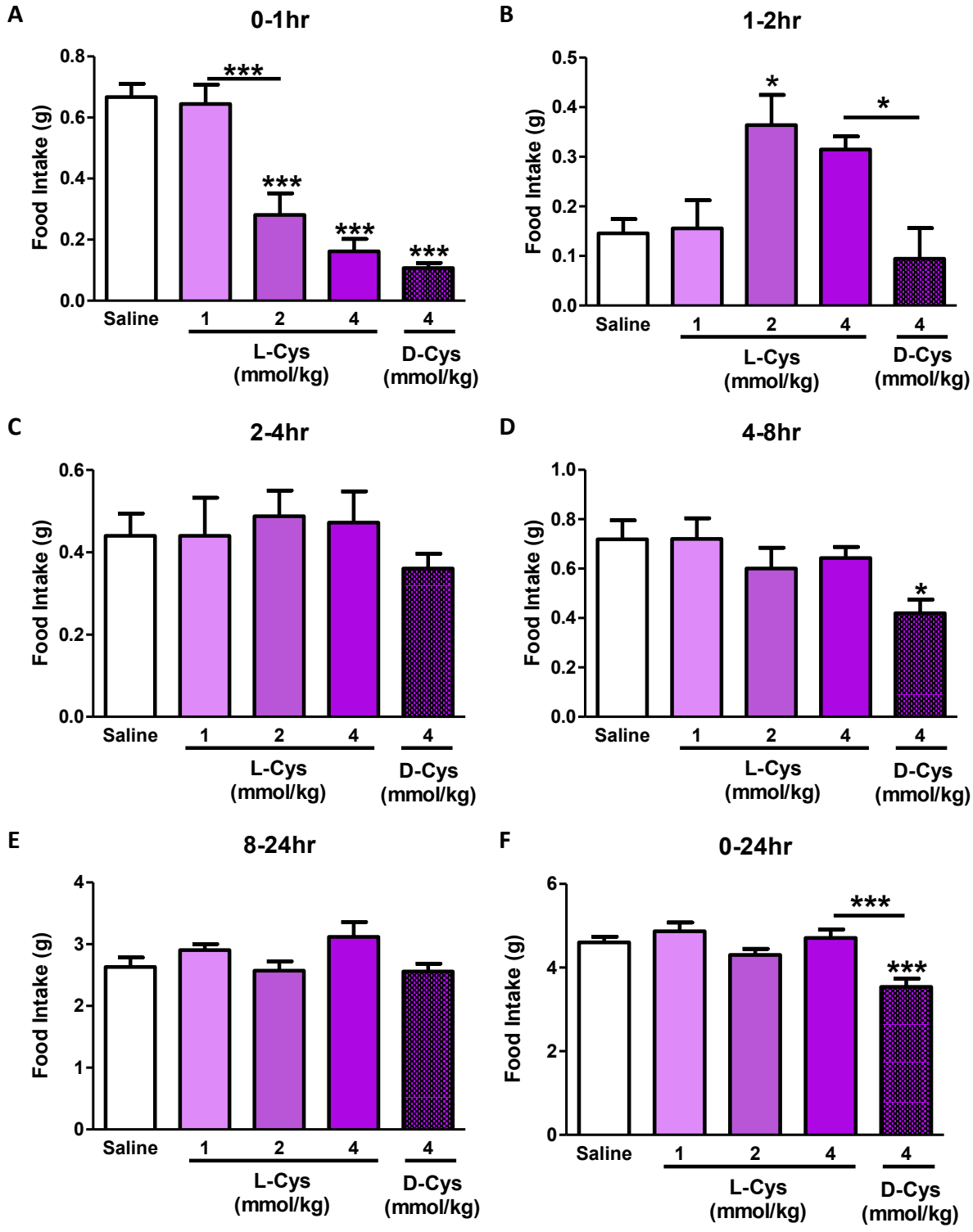


Figure 2.3.6 The effect of IP administration of L- and D-cysteine on food intake in overnight fasted mice

The effect of IP administration of saline, 1, 2 or 4mmol/kg L-cysteine or 8mmol/kg D-cysteine on food intake 0-1hr (A), 1-2hr (B), 2-4hr (C), 4-8 (D), 8-24hr (E), and 0-24hr (F) after administration. Data expressed as mean + SEM. n=7-10. *p<0.05, ***p<0.001

2.3.6.1 The effect of IP administration of L -Cysteine on behaviour in mice during the early light phase

There was a significant decrease in feeding in the 0-1 hour period following IP administration of L-cysteine and a significant increase in resting. There were no behaviours observed that would indicate illness or nausea (Table 2.3.3).

	Feeding	**Locomotion**	**Grooming**	**Resting**	**Head Down**	**Pica**
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Saline	14.0 (11.25-19.25)	11.0 (7.0-13.25)	2.0 (1.75-8.25)	3.0 (2.75-9.25)	0.0 (0.0-0.0)	0.0 (0.0-2.0)
L-Cys	4.0 (1.0-8.0)**	11.0 (5.0-13.5)	4.0 (2.0-8.5)	15.0 (10.5-20.0)**	0.0 (0.0-0.0)	0.0 (0.0-0.0)

Table 2.3.3 Observed behaviour following IP administration of L-cysteine in mice.
Number of observed behaviours expressed as median and interquartile range in mice in the 0-1 hr period following intraperitoneal administration of saline or 2mmol/kg L-cysteine. Data expressed as median (interquartile range), n=7-8, **p<0.01

2.3.7 The effect of OG administration of L-cysteine on c-Fos like immunoreactivity in rats

2.3.7.1. The effect of OG administration of L-cysteine on c-Fos like immunoreactivity in hypothalamic nuclei

Oral administration of 4mmol/kg L-cysteine significantly reduced cFLI in the lateral hypothalamic area (LHA) (Water: 32 (26-60) positive cells vs. L-cysteine: 6.5 (0-24) positive cells, $p<0.05$) (Fig. 2.3.6A). However there was no significant difference in cFLI between L-cysteine and glycine treated animals in the LHA (Fig. 2.3.7A).

2.3.7.2 The effect of OG administration of L-cysteine on c-Fos like immunoreactivity in brainstem nuclei

Oral administration of 4mmol/kg L-cysteine significantly increased cFLI in the area postrema compared to glycine treated controls (L-cysteine: 55 (49-58) positive cells vs. glycine 7 (6-8) positive cells, $p<0.05$) (Fig. 2.3.7A, representative sections 2.3.7B-D). L-cysteine also significantly increased cFLI in the medial NTS (water: 15.5 (14-18.75) positive cells vs. L-cysteine: 31.5 (21-60) positive cells, $p<0.05$) (Fig. 2.3.7A, representative sections 2.3.7E-F).

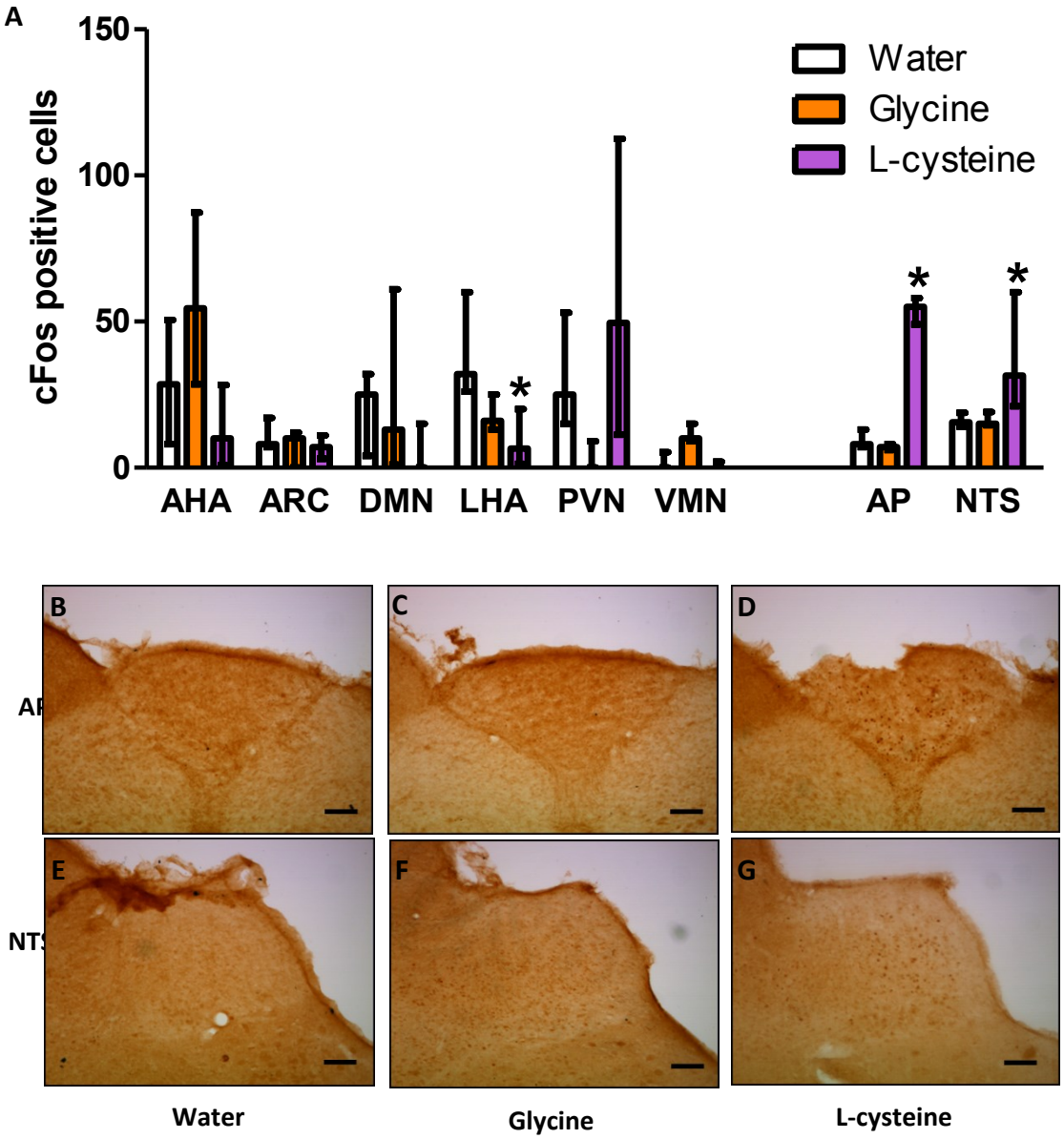


Figure 2.3.7 The effect of OG administration of L-cysteine on c-Fos Like Immunoreactivity in the hypothalamus and brainstem

The effect of oral gavage of water, 4mmol/kg glycine or 4mmol/kg L-cysteine on cFLI in hypothalamic and brainstem nuclei 90 minutes after administration (A) Expressed as median and interquartile range. n=3-5. *p<0.05. Representative sections from the area postrema of water treated (B), glycine treated (C), cysteine treated (D) and from the nucleus tractus solitarius (E-G) respectively. Scale bar = 100µm AHA: anterior hypothalamic area, ARC: arcuate nucleus, DMN: dorsomedial nucleus, LHA: Lateral hypothalamic area, PVN: paraventricular nucleus, VMN: ventromedial nucleus, AP: area postrema, NTS: nucleus tractus solitarius

2.3.8 The effect of OG administration of L-cysteine on conditioned taste aversion

Oral administration of 127mg/kg lithium chloride (positive control) resulted in conditioned aversion to the novel substance, with animals drinking significantly less fluid than water administered controls (water: 14.64 ± 0.74 ml vs. LiCl: 0.22 ± 0.4ml, $p<0.001$). L-cysteine did not result in conditioned taste aversion at any dose tested (1, 2 and 4mmol/kg) compared to water controls (Fig.2.3.8A).

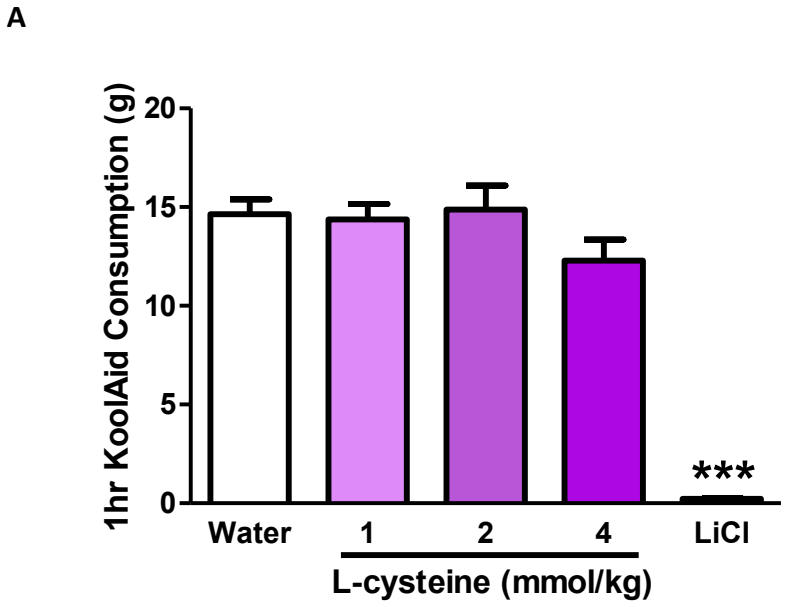


Figure 2.3.8 The effect of OG administration of L-Cysteine on KoolAid consumption as a measure of conditioned taste aversion

The effect of oral administration of water, 1, 2 or 4mmol/kg L-cysteine or 127mg/kg LiCl on Kool Aid consumption in conditioned rats. Data expressed as mean + SEM. n=5-9. ***p<0.001

2.3.9 The effect of intracerebroventricular administration of L-cysteine on food intake

ICV administration of 2µmol L-cysteine significantly decreased food intake compared to saline control during the 0-1hour period post injection (saline: 6.08 ± 0.73g vs. 2µmol L-cysteine: 2.42 ± 0.58g, $p<0.01$) (Fig.2.3.9A). However, from approximately 40 minutes post administration, mild seizure like behaviour was observed in this group. Food intake remained significantly reduced up to 4 hours post administration (0-4hr Saline: 10.75 ± 1.14g vs. 2µmol L-cysteine: 7.61 ± 0.91g, $p<0.05$, data not shown). Administration of 3nmol NDP-MSH (positive control) significantly reduced food intake compared to saline-injected controls during the 0-1hour time period post injection (saline: 6.08 ± 0.73g vs. 3nmol NDP-MSH: 1.45 ± 0.31g, $p<0.001$) (Fig2.3.9A). NDP-MSH significantly reduced food intake up to 8 hours post administration (0-8hrs - Saline: 15.33 ± 1.23g vs. NDP-MSH: 7.98 ± 0.59g, $p<0.001$, data not shown).

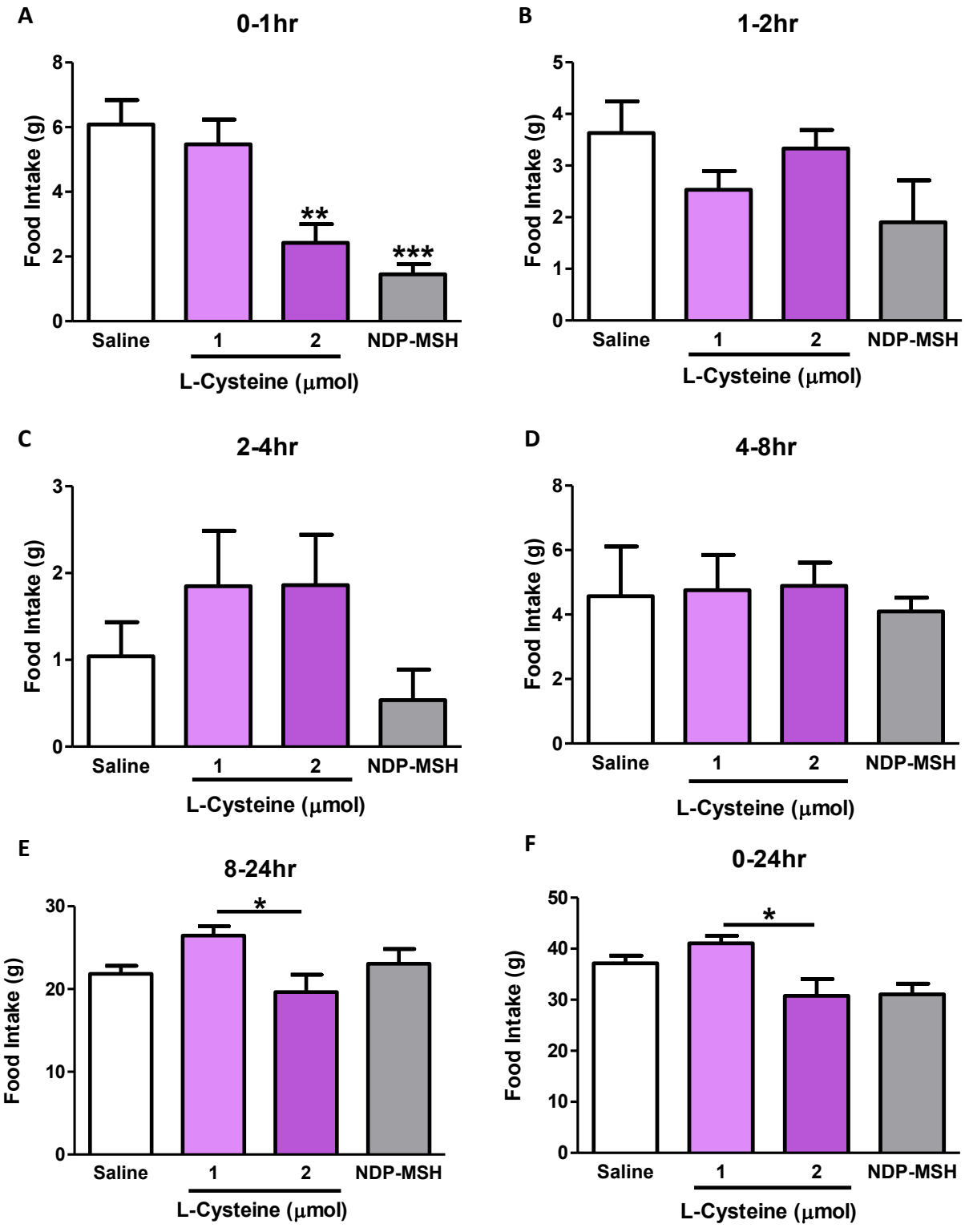


Figure 2.3.9 The effect of intracerebroventricular administration of L-cysteine on food intake in rats

The effect of intracerebroventricular administration of saline, 1 or 2 μmol L-cysteine or 3nmol NDP-αMSH on food intake following an overnight fast in rats 0-1hr (A), 1-2hr (B), 2-4hr (C), 4-8 (D), 8-24hr (E), and 0-24hr (F) after administration. Data expressed as mean + SEM. n=5-8. *p<0.05, **p<0.01, ***p<0.001

2.3.10 The effect of intracerebroventricular administration of L- and D-cysteine on food intake in rats

ICV administration of 2µmol L-cysteine significantly decreased food intake compared to saline control during the 0-1hour period post injection (saline: 6.15 $\pm$ 0.33g vs. 2µmol L-cysteine: 2.14 $\pm$ 0.74g, $p<0.01$) (Fig.2.3.10A). Administration of 2µmol D-cysteine did not significantly affect food intake compared to saline controls. D-cysteine treated animals consumed more food within the 0-1hour period post injection compared to L-cysteine treated animals, but this effect did not reach statistical significance. Cumulative food intake in the 2µmol L-cysteine group remained significantly reduced up to 4 hours post administration (saline: 13.36 $\pm$ 0.52g vs. 2µmol L-cysteine: 7.74 $\pm$ 0.74g, $p<0.05$).

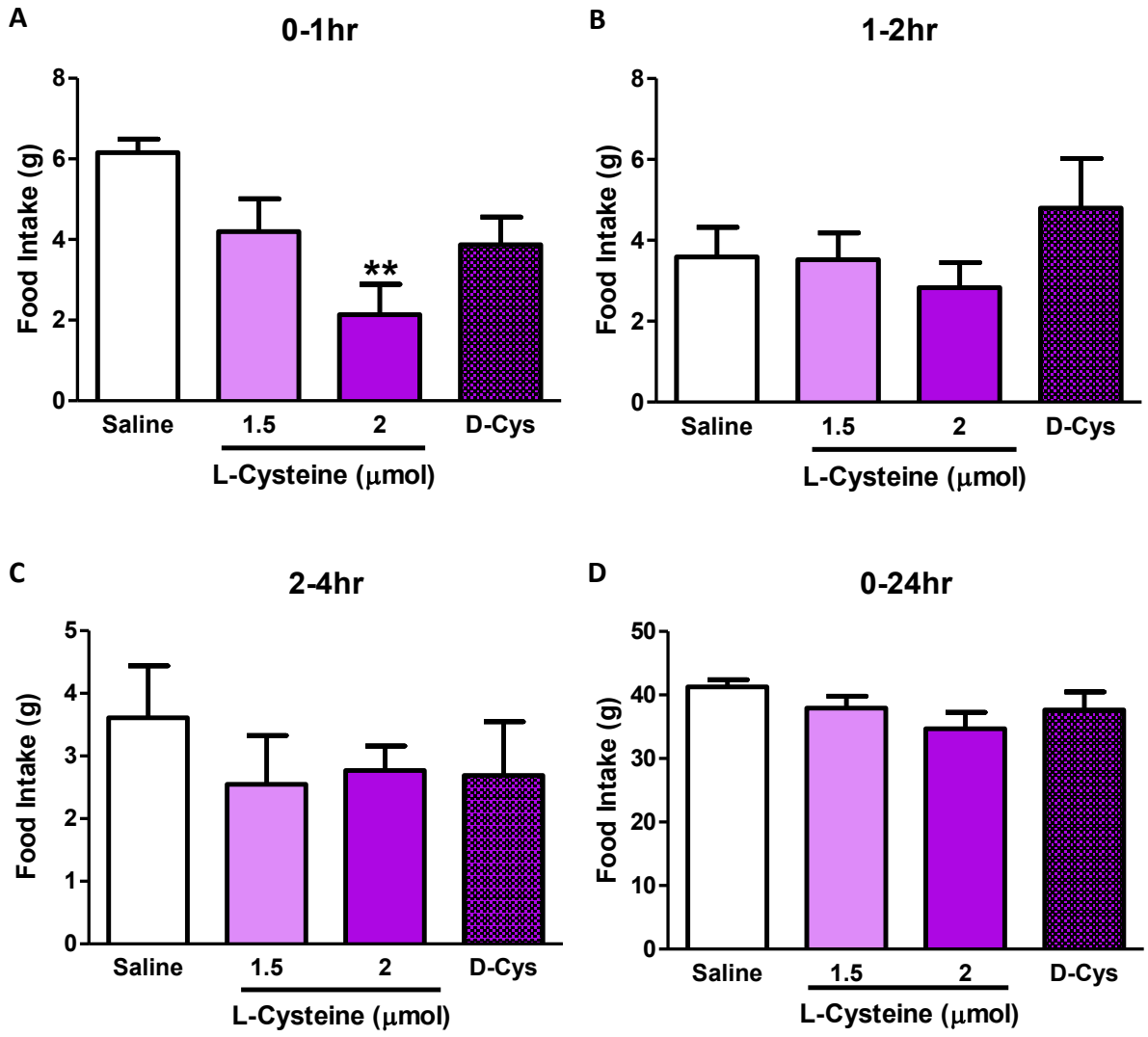


Figure 2.3.10 The effect of intracerebroventricular administration of L-and D-cysteine on food intake in rats

The effect of intracerebroventricular administration of saline, 1.5 or 2μmol L-cysteine or 2μmol D-cysteine on food intake following an overnight fast in rats 0-1hr (A), 1-2hr (B), 2-4hr (C), 0-24hr (D) after administration. Data expressed as mean + SEM. n=6-9. **p<0.01

2.3.11 The effect of L-cysteine on gut hormone release

2.3.11.1 The effect of OG administration of L-cysteine on gut hormone release

Thirty minutes after oral administration of 4mmol/kg L-cysteine, plasma levels of acyl-ghrelin were significantly reduced compared to water-treated animals (water: 8.83 ± 1.63 pmol/l vs. L-cysteine: 3.63 ± 0.91 pmol/l, $p<0.05$) (Fig. 2.3.11A). L-cysteine had no effect on plasma GLP-1 or PYY levels (Fig, 2.3.11C and E, respectively)

2.3.11.2 The effect of IP administration of L-cysteine on gut hormone release

Thirty minutes after intraperitoneal administration of 2mmol/kg L-cysteine, plasma levels of acyl-ghrelin were significantly reduced compared to saline-treated animals (saline: 22.3 ± 1.2pmol/l vs. L-cysteine: 9.2 ± 2.4pmol/l, $p<0.001$) (Fig. 2.3.11B). L-cysteine had no effect on plasma GLP-1 or PYY levels (Fig. 2.3.11 D and F, respectively).

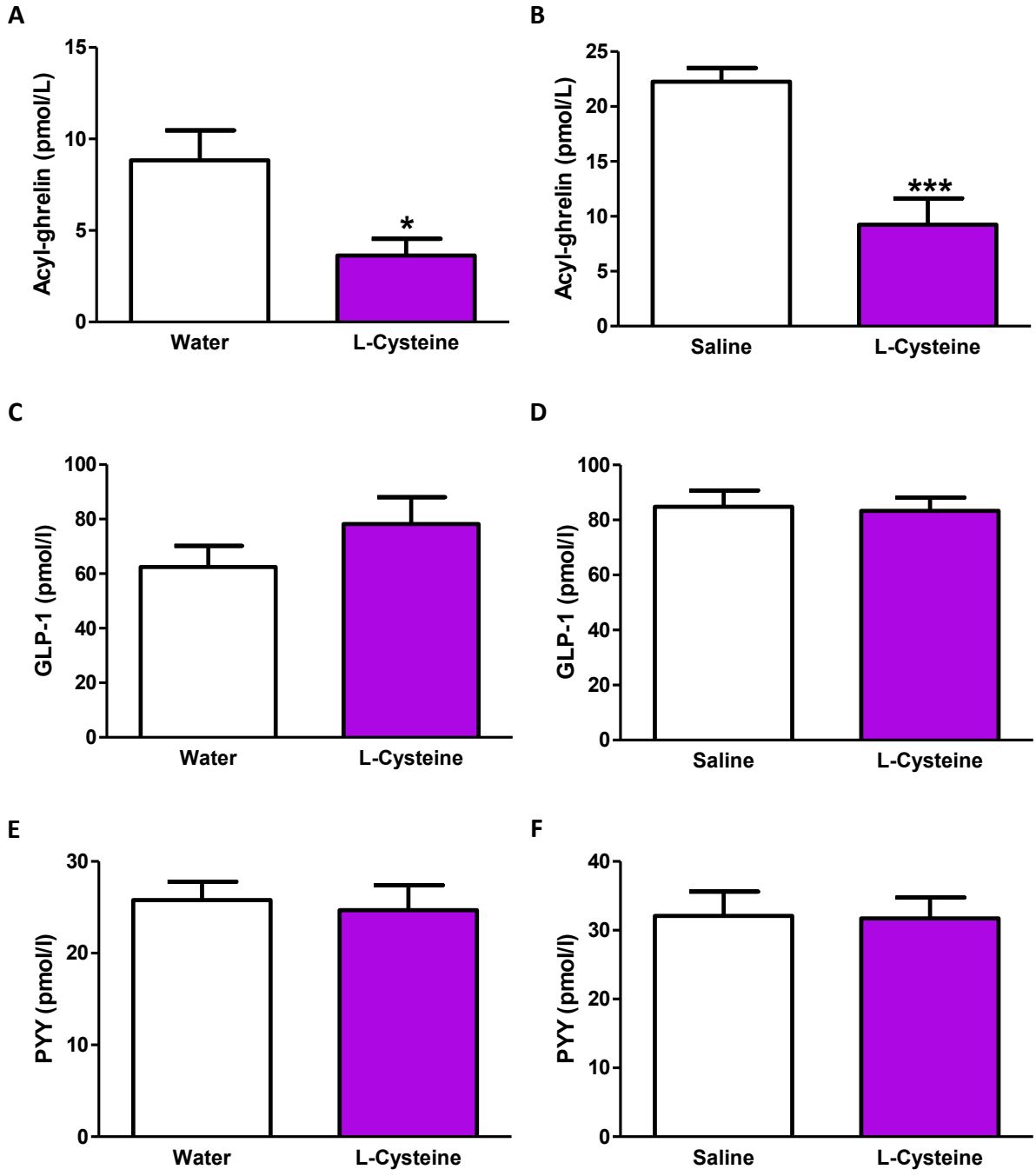


Figure 2.3.11 The effect of L-cysteine on gut hormone release

The effect of oral gavage of water or 4mmol/kg L-cysteine on plasma acyl-ghrelin (A), GLP-1 (C) and PYY (E) (n=8) and intraperitoneal administration of saline or 2mmol/kg L-cysteine on plasma acyl-ghrelin (B), GLP-1 (D) and PYY (F) 30minutes after administration following an overnight fast (n=9-10). Data expressed as mean + SEM. *p<0.05, ***p<0.001

2.4 Discussion

This chapter describes experiments investigating the effect of L-cysteine on food intake in two rodent species and investigates its site of action. Preliminary data had identified L-cysteine as the amino acid with the most potent anorectic effects following oral gavage and intraperitoneal administration in rats. This data also identified L-arginine and L-lysine as amino acids with potent anorectic effects following intraperitoneal administration. However, initial studies in this chapter demonstrate that this effect may have been dependent on the pH of these amino acids, as neutral salts of these amino acids had no significant effect on food intake, and thus this thesis focussed on the effects of L-cysteine on food intake. This chapter presents novel data demonstrating that L-cysteine dose dependently reduces food intake in rats and mice following oral and intraperitoneal administration. This reduction in food intake was not secondary to any adverse effects.

In rats, oral gavage administration of L-cysteine dose-dependently reduced food intake. However, D-cysteine, at a dose equivalent to the highest dose of L-cysteine tested, had no effect on food intake. The promiscuous amino acid receptors, GPRC6A, CaSR and T1R1/T1R3 are stereoselective for L-amino acid enantiomers and both CaSR and T1R1/T1R3 have been localised to the rat GI tract (Cheng et al., 1999, Bezencon et al., 2007, Brown and MacLeod, 2001). L-cysteine activates the mouse orthologue of all three of these receptors and is a weak agonist of the rat GPRC6A receptor (Wellendorph et al., 2007). However, no published studies have investigated the effect of L-cysteine on the rat CaSR or rat T1R1/T1R3. Additionally, IP administration of L-cysteine also dose-dependently reduced food intake, whereas D-cysteine had no effect on food intake. Comparing the anorectic response of L-cysteine at isomolar doses following oral or IP administration, food intake was reduced to a greater extent following IP administration. It is possible the effect of oral administration of L-cysteine on food intake is mediated through a post absorptive mechanism. Our group has shown that plasma cysteine levels in humans begin to increase within 30 minutes following an oral protein load and within 15 minutes following an oral L-cysteine load (unpublished). Importantly, oral or IP administration of L-cysteine did not cause any behaviour indicative of illness or nausea. In addition, oral administration of anorectic doses of L-cysteine did not result in conditioned taste aversion. Collectively these results suggest the anorectic effect of L-cysteine is not secondary to side effects or nausea.

To further assess the anorectic potential of L-cysteine, its effect on food intake in mice was investigated. Similar to rats, oral administration of L-cysteine dose-dependently reduced food intake. However, higher doses were required to achieve a similar anorectic response to that achieved in rats. Intraperitoneal administration of L-cysteine also dose dependently reduced food intake. The anorectic potential of isomolar doses following IP administration between rats and mice was similar. If L-cysteine is mediating its effect via a post absorptive mechanism, the difference in the anorectic response following oral administration in mice may suggest disparities in absorption kinetics or a more efficient first pass metabolism in mice. However, in contrast to rats, D-cysteine also significantly reduced food intake in mice to a similar extent as L-cysteine in the hour after administration following both oral and IP administration. Furthermore, the anorectic effect of D-cysteine was still evident 24 hours after administration. Considering the lack of a stereoselective effect in mice and that this characteristic has been most widely studied in the mouse orthologues, these studies would suggest that it is unlikely the promiscuous amino acid sensing receptors are mediating the effect of L-cysteine on food intake in mice. However, it is still possible that D-cysteine is mediating its effects through a different mechanism from L-cysteine. Oral administration of D-cysteine to rats leads to a far greater increase in serum cystine than oral administration of L-cysteine, whereas L-cysteine leads to a greater increase in serum taurine levels (Krijgsheld et al., 1981). The reason for the differential effect seen between rats and mice following administration of D-cysteine is unclear. However, a study in mice similar to that of Krijgsheld *et al,* may help determine whether there are differences in utilization of D-cysteine between species that may account for the differences seen here.

Hypothesising, that L-cysteine was mediating its effect via a post-absorptive mechanism, the effect of oral administration of L-cysteine on cFos-like immunoreactivity (cFLI) in the hypothalamus and brainstem was examined. L-cysteine significantly decreased cFLI in the lateral hypothalamic area (LHA). The LHA is associated with orexigenic signals. This characteristic was demonstrated by crude lesioning experiments which showed destruction of this area resulted in decreased food consumption and a lean phenotype (Anand and Brobeck, 1951). Additionally, an intragastric load of protein suppresses the activity of orexin neurons in the LHA (Journel et al., 2012). However, oral administration of glycine, which was used as a negative control as it did not have any effect on food intake, also decreased cFLI in

this area. There was no significant difference between cFLI in the LHA between the L-cysteine and glycine treated animals, suggesting this reduction was not specific to the anorectic effect of L-cysteine. Surprisingly, there were no significant changes in any of the other hypothalamic nuclei examined.

To confirm that L-cysteine was not acting directly on hypothalamic appetite centres, I investigated the effect of intracerebroventricular (ICV) administration of L-cysteine on food intake. Central administration of L-cysteine did reduce food intake. However, it is likely this reduction in food intake was secondary to adverse effects, as mild seizure like behaviour was noted following administration of 'anorectic' doses. L-cysteine and some of its metabolites are reported to have NMDA receptor activity (Parsons et al., 1998). This receptor and its role in L-cysteine induced anorexia is discussed further in Chapter 3. These studies suggest that it is unlikely the peripheral effects of L-cysteine on food intake are mediated by a change in central concentrations of L-cysteine. However, L-cysteine significantly increased cFLI in the area postrema and nucleus tractus solitarius. As discussed in chapter 1, the area postrema is sensitive to circulating peripheral factors and the NTS is the terminal for vagal afferents, suggesting L-cysteine may be mediating its effect either directly in the area postrema and/or via the area postrema through a secondary mediator or via vagal afferents. Additionally, the area postrema is a region associated with sensing toxic substances and mediating emetic responses. However, as rodents lack the required anatomy for emesis, it can be difficult to assess this behaviour. As discussed, cysteine did not induce conditioned taste aversion, suggesting it was not causing nausea. It is therefore unlikely, that the increase in cFLI in the area postrema was a signal of nausea.

The brainstem integrates numerous signals originating in the periphery, including gut hormones. I therefore investigated the effect of L-cysteine on gut hormone release. L-cysteine significantly decreased plasma levels of acyl-ghrelin, an orexigenic hormone following both oral and IP administration. Ghrelin is secreted from X/A cells which are predominantly closed-type cells without contact to the lumen. Therefore, the reduction in ghrelin following oral administration is likely to be a systemically mediated effect. This is supported by the similar reduction in ghrelin seen following IP administration. Ghrelin secretion is inhibited by a number of factors including somatostatin, CCK, GLP-1 and insulin (Stengel et al., 2010). L-cysteine did not significantly affect plasma GLP-1 levels, thus it is

unlikely the suppression in ghrelin is secondary to a rise in GLP-1. The sympathetic nervous system and the vagus nerve have also been implicated in the regulation of ghrelin secretion. Norepinephrine, a sympathetic nervous system neurotransmitter stimulates the release of ghrelin from rat primary stomach cultures (Gagnon and Anini, 2012). Vagotomy also increases plasma ghrelin levels (Lee et al., 2002), suggesting the vagus nerve is involved in the regulation of ghrelin release.

The work described in this chapter has demonstrated that L-cysteine reduces food intake in both rats and mice following peripheral administration and that this reduction in food intake is not secondary to adverse behaviour. The mechanism by which L-cysteine reduces food intake requires further investigation. However, I show here that L-cysteine reduces plasma acyl-ghrelin and increases neuronal activation in the brainstem. The mechanisms by which L-cysteine reduce food intake are further explored in the next chapter.

CHAPTER 3: INVESTIGATING THE MECHANISMS MEDIATING THE ANORECTIC EFFECT OF L-CYSTEINE

3.1 Introduction

Chapter 2 described the anorectic effect of L-cysteine in rodents. Peripheral administration of L-cysteine reduced food intake in rodents without any detectable side effects. L-cysteine also decreased circulating levels of the orexigenic hormone acyl-ghrelin and increased neuronal activation in the area postrema and medial nucleus tractus solitarius. Elucidating the mechanism by which L-cysteine reduces food intake may aid our understanding of amino acid sensing and protein induced satiety. Moreover, it may help to identify potential targets for anti-obesity agents.

3.1.1 L-cysteine and its metabolites

L-cysteine is one of only two sulphur containing proteinogenic amino acids. Much of L-cysteine's biological activity is due to its thiol group (carbon bonded sulfhydryl group C-SH). Thiols are potent nucleophiles meaning they are readily oxidized. L-cysteine can be oxidized to the disulphide derivative cystine. L-cysteine is a glucogenic amino acid that is also a major precursor for glutathione, taurine and hydrogen sulphide.

3.1.1.1 Glutathione

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), synthesised from L-glutamine, L-cysteine and glycine in a two-step enzymatic process involving γ-glutamyl cysteine synthetase (γGCS) and glutathione synthetase (Fig. 3.1.1). L-cysteine is the rate-limiting precursor in glutathione synthesis (Lu, 2009).

Glutathione has a number of roles within the body, most importantly providing protection against reactive oxygen and nitrogen species (ROS and RNS respectively). Glutathione interacts with these reactive species generating thiyl radicals, which can react with each other to form glutathione disulphide (GSSG) (Lushchak, 2012).

3.1.1.2 Taurine

Taurine is a conditionally essential non-proteinogenic amino acid. It is synthesised from cysteine through a series of enzymatic steps involving cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (Fig 3.1.1). Cysteine sulfinate decarboxylase is dependent on Pyridoxal 5'-phoshate as a cofactor for enzymatic activity, but cysteine dioxgenase is not.

Taurine has numerous physiological functions, which may be reflected in the fact that it is the most abundant intracellular amino acid (Lourenco and Camilo, 2002). Taurine conjugates with bile acids and this conjugation is essential for bile acid solubility. Conjugation also helps to limit bile-acid hepatotoxicity (Lourenco and Camilo, 2002). Additionally, taurine-conjugated bile acids have a choleretic effect and prevent cholestasis, a condition in which flow of bile from the liver to the duodenum is obstructed (Belli et al., 1991). Taurine has also been reported to have positive cardiovascular effects including antiarrhythmic, chronotropic and inotropic effects (Nittynen et al., 1999, Sole and Jeejeebhoy, 2000). Taurine also appears essential for normal vision (Lima, 1999) and has been implicated in the maintenance of blood glucose levels through enhancing insulin receptor signalling (Hansen, 2001).

3.1.1.3 Hydrogen Sulphide

Hydrogen Sulphide (H_2S) is a gasotransmitter synthesised from cysteine through enzymatic pathways. There are at least four enzymatic pathways which can result in the production of H_2S from L-cysteine, and which involve the enzymes (i) cystathionine β synthetase (CBS), (ii) cystathionine γ lyase (CSE, also known as γ-cystathionase), (iii) cysteine amino transferase (CAT) and 3 mercaptopyruvate sulfurtransferase (3-MST), and (iv) cysteine lyase (CL) (Li et al., 2011), summarized in Figure 3.1.1. These enzymes are all Pyridoxal 5'-phosphate dependent.

H_2S has many roles. It functions as a neuromodulator, facilitating long-term potentiation (LTP) by enhancing the activity of N-methyl-D-aspartate (NMDA) receptors in neurons (Abe and Kimura, 1996, Nagai et al., 2004). It also functions as a glial mediator, for example eliciting direct activation of Ca^{2+} influx in astrocytes (Nagai et al., 2004). Thirdly, H_2S functions as a smooth muscle relaxant, particularly in the thoracic aorta, portal vein and ileum (Hosoki et al., 1997). This H_2S-induced relaxation is suggested to occur as a result of ATP-dependent K^+ channel opening (Zhao et al., 2001). Additionally, H_2S also functions as a neuro- and cardio-protectant by enhancing glutathione production and thus protecting against oxidative stress (Kimura and Kimura, 2004, Geng et al., 2004).

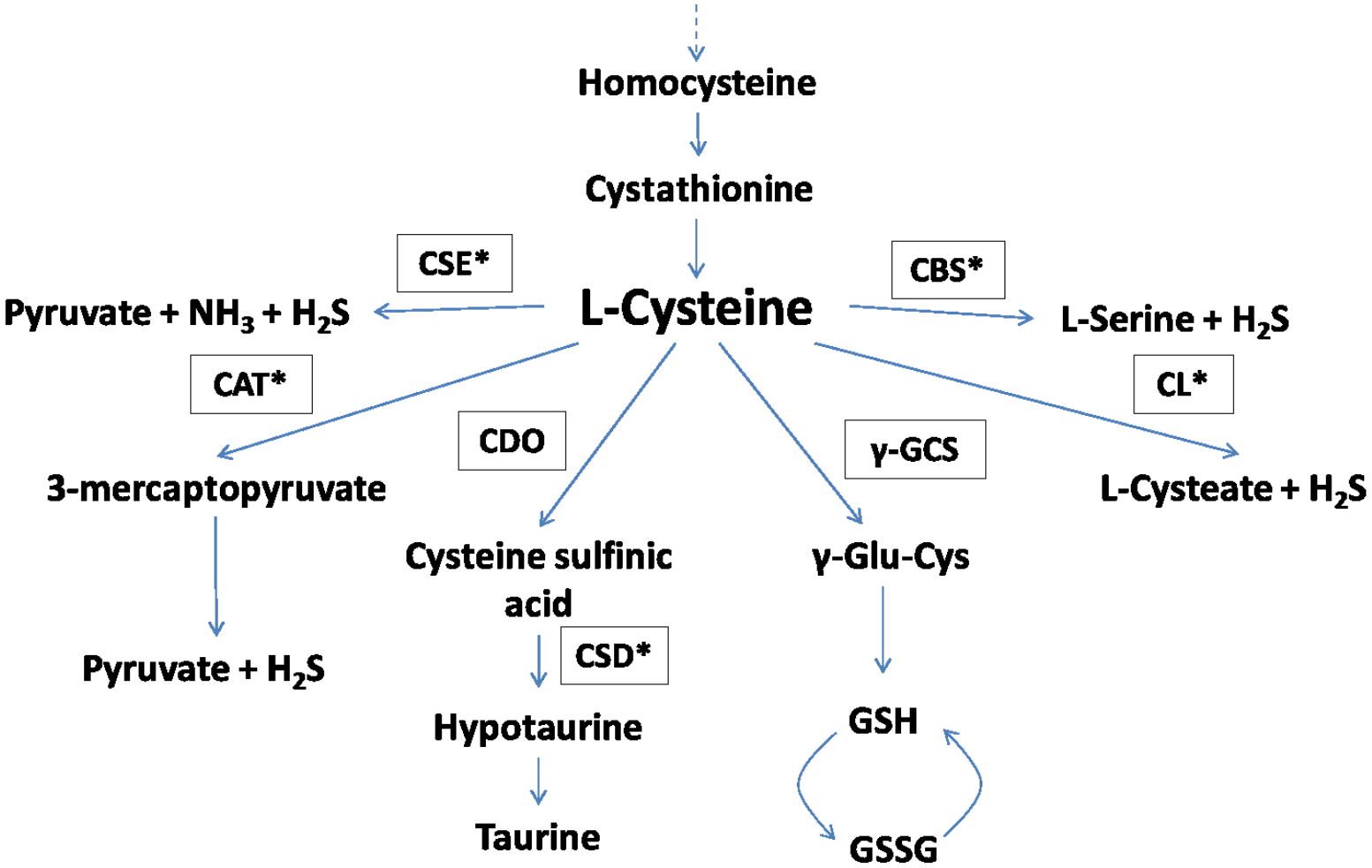


Figure 3.1.1 L-cysteine metabolism

Diagram illustrating the various pathways through which L-cysteine can be metabolised *in vivo*. CSE: γ-Cystathionase, CAT: Cysteine aminotransferase , CDO: Cysteine Dioxygenase, CSD: cysteine sulfinate decarboxylase, γ-GCS: γ-Glutamyl cysteine synthetase , CL: Cysteine Lyase, CBS: Cystathionine-β-synthase . *Pyridoxal 5'-phosphate dependent enzymes. NH_3: ammonia, H_2S: Hydrogen sulphide, GSH: Glutathione, GSSG: oxidized glutathione. (Li et al., 2011)

3.1.3 L-cysteine receptors

3.1.3.1. Promiscuous amino acid sensing receptors

L-cysteine is an agonist/modulator of all three of the promiscuous amino acid sensing receptors: T1R1/T1R3, GPRC6A and CaSR, discussed in chapter 2. Of the 20 proteinogenic amino acids, L-cysteine is reportedly the most potent agonist of mT1R1/T1R3 (Nelson et al., 2002), the 5th most potent agonist of mGPRC6A (Christiansen et al., 2007) and 5th most potent modulator of the mCaSR (Conigrave et al., 2000).

3.1.3.2 N-Methyl D-Aspartate Receptor

The NMDA receptor is an ionotropic glutamate receptor that is widely expressed in the central and peripheral nervous systems. NMDA receptors are tetrameric receptors made up of different subunits from three families: GluN1, GluN2 (of which there are 4 isoforms: NR2A-D) and GluN3 (of which there are 2 isoforms: NR3A-B). The presence of GluN1 is thought to be essential for receptor function, and this subunit forms heterotetrameric complexes with GluN2 and ternary tetrameric complexes with GluN2 and GluN3. Activation of the receptors requires the simultaneous binding of two agonists: glutamate and glycine (or D-serine). NR1 and NR3 subunits bind glycine whereas NR2 subunits bind glutamate. NR2 subunits also have binding sites for allosteric modulators such as Zn^{2+} in their N-terminal domains. Agonist binding leads to channel opening and an influx of Ca^{2+}, leading to membrane depolarization (Paoletti and Neyton, 2007) (Fig. 3.1.2).

L-cysteine and some of its metabolites can act as excitotoxins via NMDA receptor-dependent mechanisms (Parsons et al., 1998, Sakata et al., 1999, Pace et al., 1992). Systemic administration of NMDA receptor blockers or antagonists has previously been shown to increase food intake (Burns and Ritter, 1997, Guard et al., 2009). This increase in food intake is mediated by NMDA receptors in the dorsal hindbrain (Treece et al., 1998), and the increase is dependent upon intact central vagal afferent terminals (Treece et al., 2000). Furthermore, NMDA receptors have been implicated in mediating the anorectic effect of CCK (Guard et al., 2009). CCK is an anorexigenic gut hormone (discussed in section 1.2.3.3.2) that predominantly mediates its effect through vagal afferents.

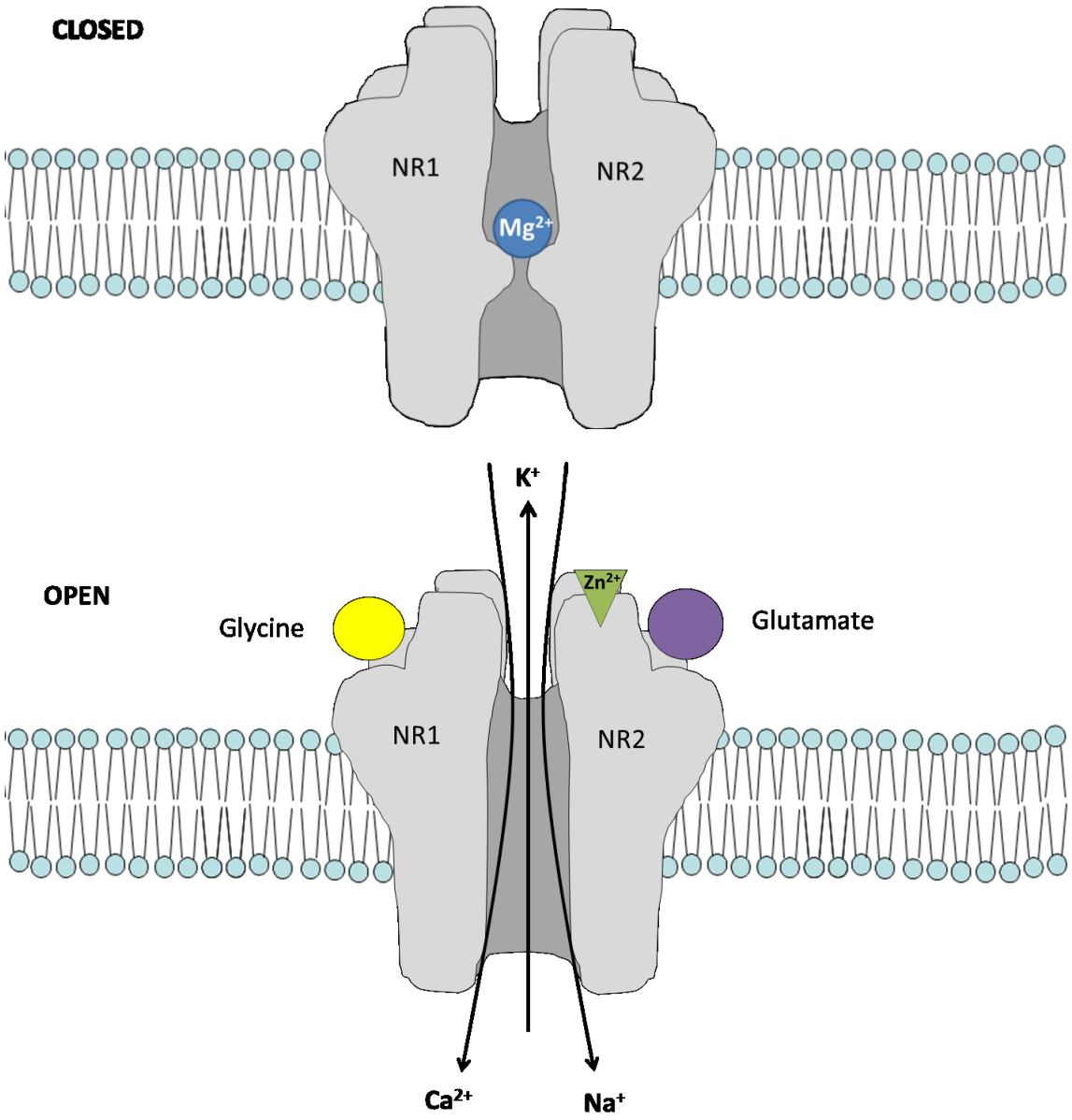


Figure 3.1.2 The NMDA receptor

The NMDA receptor is a tetrameric ionotropic glutamate receptor. Upon agonist and co-agonist binding the receptor undergoes a confirmation change, displacing the Mg^{2+} ion blocking the channel, allowing flow of Ca^{2+} and Na^{+} ions into the neuron and K^{+} out of the neuron.

3.1.4 Protein, amino acids and vagal nerve signalling

The vagus nerve conveys information between the gut and the brainstem and plays a major role in the control of postprandial gastrointestinal function and satiety by macronutrients. In addition to the GI tract, the subdiaphragmatic branch of the vagus also innervates the liver, portal vein, biliary system and pancreas (Berthoud and Neuhuber, 2000). Proteins, peptides and amino acids are believed to elicit a visceral vagus-mediated activation of neurons in the nucleus of the solitary tract (NTS). Acute and chronic high protein feeding increases cFos expression in the caudal NTS (Faipoux et al., 2008). Small intestinal infusion of protein activates vagal afferents in rats, predominantly through a CCK-dependent mechanism (Tome et al., 2009, Eastwood et al., 1998). The proton-coupled oligopeptide transporter PepT1 has also been implicated in this pathway (Darcel et al., 2005). Additionally, intragastric infusion of L-glutamate activates gastric vagal afferents and induces an autonomic reflex with subsequent activation of vagal and splanchnic efferents (Kitamura et al., 2011). Amino acid induced gastric vagal afferent activity seems to be specific to L-glutamate (Uneyama et al., 2006), but hepatic vagal afferents are sensitive to a wider range of amino acids, with certain amino acids increasing and others suppressing hepatic vagal afferent discharge following intraportal administration (Niijima and Meguid, 1995), as summarized in Table 3.1.2. This may represent a post-absorptive amino acid sensing mechanism. Notably, the responses did not seem to be specific to particular amino acids or classes or characteristics of amino acids. Of note, DL-cysteine had an overall inhibitory effect on vagal afferent discharge; the effects of L-cysteine alone were not reported.

Hepatic vagal afferent discharge	
Excitatory AAs	**Inhibitory AAs**
L-Alanine	DL-Cysteine
L-Arginine	Glycine
L-Histidine	L-Isoleucine
L-Leucine	L-Methionine
L-Lysine	L-Phenylalanine
L-Serine	L-Proline
L-Tryptophan	L-Threonine
L-Valine	

Table 3.1.1 The effect of 15 amino acids on hepatic vagal afferent activity
The effect of intraportal administration of 10mM amino acid in a volume of 0.1ml of vagal afferent discharge in rats. Data from (Niijima and Meguid, 1995)

L-cysteine is known to induce NMDA-receptor mediated responses and as described in chapter 2 L-cysteine increases neuronal activation in the caudal NTS. Protein stimulates CCK release, vagal afferents and neuronal activation in the caudal NTS. Additionally, the NMDA receptor has been implicated in CCK-vagal afferent mediated anorectic signals.

3.1.5 Hypothesis and Aims

Hypotheses:

- The L-cysteine molecule reduces food intake through a CCK/NMDA receptor-vagal afferent mediated pathway.
- Repeated administration of L-cysteine reduces chronic food intake.

Aims:

To investigate:

1. The role of metabolites of L-cysteine in mediating its effect on food intake.
2. The role of the NMDAR, CCK and vagal afferents in mediating the effect of L-cysteine on food intake.
3. The effect of repeated administration of L-cysteine on food intake and body weight.

3.2 Methods

3.2.1 Animals

Male C57BL/6 mice (Harlan, Bicester, Oxon, UK) weighing between 18-20g were maintained in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to food (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless otherwise stated. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/7062).

Male Wistar rats (Charles River, Margate, Kent, UK) weighing between 200-220g were maintained in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to food (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless otherwise stated. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/7062).

Prior to any experimental procedure, animals were acclimatised to the handling and administration procedure. For all studies animals were randomised according to body weight.

3.2.2 The effect of inhibiting L-Cysteine metabolism on food intake in mice

3.2.2.1 The effect of buthionine sulfoximine (BSO) on L-cysteine induced anorexia

Mice were fasted overnight and then received an intraperitoneal injection of saline or 1mmol/kg BSO (Sigma Aldrich), a γ-glutamyl cysteine synthetase competitive inhibitor that doubles hepatic cysteine levels within 20 minutes of adminstration (Standeven and Wetterhahn, 1991). In the first study this was followed 20 minutes later by an intraperitoneal injection of saline or 2mmol/kg L-cysteine and in a second study followed immediately by an intraperitoneal injection of saline or 2mmol/kg L-cysteine (n=10) during the early light phase. Subsequently, mice were returned to their home cage with a pre-

weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.2.2.2 The effect of glutathione on food intake

Mice were fasted overnight and then received an intraperitoneal injection of saline, 1, 2, 4mmol/kg Glutathione or 4mmol/kg L-cysteine (n=5-11) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.2.2.3 The effect of propargylglycine (PPG) on L-cysteine induced anorexia

Mice were fasted overnight and then received an intraperitoneal injection of saline or 200 µmol/kg PPG in study one and 125µmol/kg PPG in study two (Sigma Aldrich), a cystathionine-γ-lyase non-competitive inhibitor, that results in almost complete inhibition of enzyme activity within 4 hours (Uren et al., 1978, Kim and Kim, 2001). An intraperitoneal injection of saline or 2mmol/kg L-cysteine (n=10) was thus given 4 hours later during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.2.2.4 The effect of O-carboxymethyl hydroxylamine hemihydrochloride (CHH) on L-cysteine induced anorexia

Mice were fasted overnight and then received an intraperitoneal injection of saline or 20mg/kg CHH (Wallace et al., 2009), a PLP-dependent enzyme inhibitor (Geng et al., 1995), Sigma Aldrich) 4 hours prior to an intraperitoneal injection of saline or 2mmol/kg L-cysteine (n=10) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.2.3 The role of the NMDA receptor in mediating the effect of peripheral administration of L-cysteine on food intake in mice

Mice were fasted overnight and then received an intraperitoneal injection of saline or 10µg/kg MK-801 (Tocris), a non-competitive NMDA receptor channel blocker, followed 30 minutes later (Reddy and Kulkarni, 1998) by an intraperitoneal injection of saline or

2mmol/kg L-cysteine (n=6-8) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration. A dose of 10μg/kg MK-801 was used as it had previously been shown to block the effect of an NMDAR-dependent anorectic agent in the 0-1 hour period following administration (Reddy and Kulkarni, 1998).

3.2.3.1 The effect of L-aspartate and L-glutamate on food intake

L-aspartate and L-glutamate are more potent NMDA receptor agonists than L-cysteine. Therefore their effect on food intake was also investigated.

Mice were fasted overnight and then received an IP injection of saline, 4mmol/kg L-aspartic acid sodium salt monohydrate, L-glutamic acid monosodium salt hydrate or L-cysteine (n=10). Mice were returned to their home cages with a pre-weighed amount of chow and food was reweighed 1 hour post-administration.

3.2.4 The role of the NMDA receptor in mediating the effect of central administration of L-cysteine on food intake in rats

The rats used in 2.2.8.2 were also used for this experiment. Rats were fasted overnight and then received a single ICV injection of saline, 200nmol D-AP5 (Tocris), a competitive NMDA receptor antagonist, 2μmol L-cysteine or 200nmol D-AP5 plus 2μmol L-cysteine (n=5-8). Rats were then returned to their home cages with a pre-weighed amount of chow and food was reweighed at 1, 2, 4, 8 and 24 hours post-administration. D-AP5 was used as it has been widely and reliably used as a central NMDA receptor antagonist in behavioural and feeding studies.

3.2.5 The effect of L-cysteine on gastric emptying in rats

Gastric emptying was determined using the methylcellulose-phenol red method (Wang et al., 2012, Gondim et al., 1999). Rats were fasted overnight then received an intraperitoneal injection of saline, 2mmol/kg L-cysteine, 2mmol/kg glycine (negative control) or 10nmol/kg A71623 (CCK-A receptor agonist) (n=4-7) followed immediately by an oral gavage of 2ml of a 1.5% Methylcellulose(4000cP), 0.05% Phenol red solution. Animals were culled by decapitation 30 minutes later and trunk blood was collected. Stomachs were ligated at the

top and bottom then removed and added to 100ml 0.1M NaOH. Stomachs were cut in to small pieces using scissors until homogenous and the mixture was allowed to settle for 60minutes, then 5ml of the supernatant was added to 0.5ml 20% trichloroacetic acid and centrifuged at 2800rpm for 20minutes. After centrifugation, 3ml of supernatant was added to 4ml 0.5M NaOH to develop the colour and the absorbance read at 540nm.

Four additional rats were given an intraperitoneal injection of saline followed immediately by an oral gavage of 2ml of the methylcellulose/phenol red mix then culled immediately. These animals acted as a reference for zero emptying. Gastric emptying was then calculated as:

%Gastric Emptying = 1-(absorbance of sample/absorbance of reference)*100

3.2.6 The role of the CCK-1 Receptor in mediating the effect of L-cysteine on food intake

The CCK-1 receptor is responsible for mediating the effects of CCK on food intake (Asin et al., 1992, Kopin et al., 1999). As mentioned, protein, protein hydrolysates and certain amino acids stimulate CCK release. CCK is also a potent inhibitor of gastric emptying (Yamagishi and Debas, 1978) and antagonists of the CCK-1 receptor accelerate gastric emptying (Scarpignato et al., 1993). To establish the role of CCK and this receptor in mediating the effect of L-cysteine on food intake and gastric emptying, pharmacological antagonist of CCK-1R was used.

The CCK-1 receptor antagonist devazepide was used. Its antagonist activity was confirmed by a study investigating its ability to block the anorectic effect of the CCK-1 receptor agonist A71623. Mice were fasted overnight and then given an IP injection of vehicle (saline, 5% DMSO, 5% Tween) or 0.5mg/kg devazepide followed immediately by an IP injection of vehicle (saline, 1% DMSO) or 10nmol/kg A71623. Mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1 hour post administration.

This procedure was repeated with vehicle and 0.5mg/kg Devazepide followed by saline or 4mmol/kg L-cysteine, vehicle and 0.5mg/kg Devazepide followed by saline or 2mmol/kg L-cysteine.

3.2.7 The effect of L-cysteine on food intake in rats that have undergone subdiaphragmatic vagal deafferentation

3.2.7.1 Animals

Male wistar rats weighing 180-200g were maintained in individual cages under controlled temperature (21-23°C) and light (12:12 light-dark cycle, lights on at 0600h) with *ad libitum* access to food (R70, Lactamin, Sweden) and water unless otherwise stated. All procedures were approved by the Gothenburg Animal Review Board (Ethical application number 101505).

3.2.7.2 Subdiaphragmatic vagal deafferentation surgery

This procedure requires the exposure and lesioning of the vagal afferent rootlets on one side, and the sectioning of the subdiaphragmatic vagal trunk that originates from the opposite side. This results in all vagal afferents being cut with half of the vagal efferents remaining intact (Fig. 3.2.1).

Three days prior to surgery animals were adapted to a liquid diet (Nestlé Nutrition, Resource Energy, 1.5kcal/ml, chocolate flavour) which is required for post-operative nutrition, with solid food being removed 1 day prior to surgery. Prophylactic antibiotics (400mg/kg Sulfadiazin + 80mg/kg Trimetoprim) were given on the day prior to surgery, on the day of surgery and one day post-surgery. Liquid diet was removed 4 hours before surgery. Then 15-30minutes prior to anaesthesia 0.05mg/kg Atropin was administered SC. Rats were then anaesthetised using injectable anaesthesia (0.5mg/kg Dormitor + 75mg/kg Ketamine). Perioperative hydration was maintained with an IP injection of Ringers Lactate solution. Anaesthesia was reversed with 1mg/kg antisedan. Post-operative analgesic was administered SC (5mg/kg Carprofen) and was continued as necessary.

For the vagal afferent rootlet surgery, animals were placed in an atraumatic head holder that can be rotated about the animal's long axis. A midline incision was made from the anterior of the mandible caudally towards the manubrium and the skin pulled laterally with retractors. The sternohyoid, omohyoid and digastric muscles were retracted and the left

carotid artery and thyroid artery exposed. Blunt forceps were inserted vertically between the carotid and thyroid arteries and blunt dissection used to expose the occipital bone. Retractors were positioned in a manner in which to maintain this exposure. The section of bone located between the posterior lacerate foramen and the XII cranial nerve was carefully thinned using a dental drill (Buffallo No.220 High speed air turbine) with size 5/0 round bur until it was transparent. The remaining bone was removed with forceps exposing the dura through which the main trunk of the vagus can be visualised. The afferent fibres lie dorsal to fine efferent fibres. These efferent fibres were carefully displaced to expose the afferent rootlets. The afferent rootlets were subsequently avulsed using Dumont forceps. The hole was filled with Gelfoam and the wound closed.

A midline laparotomy was then performed to expose the stomach. The abdominal wall and the xyphoid process were retracted. The liver was displaced using saline soaked gauze and the stomach placed under sufficient traction to expose the oesophagus. The dorsal vagal trunk travelling along the oesophagus was identified and ligated above the first branch using two 3-0 silk sutures and the ligated section was then transected and the wound closed. Sham operations were performed identically except without the lesioning of the rootlets or trunk.

Post-operatively, sham operated animals were given 4-6hours before water and liquid diet were returned. SDA animals were given 6-8 hours before water was returned and liquid diet was retuned the following day. Post-surgery nutrition is outlined in Table 3.2.1.

Post-Surgery Day	Water	Liquid Diet	Wet Mash	Solid Diet
1	X	X		
2	X	X		
3	X	X	X	
4	X	X	X	
5	X	X	X	X
6	X		X	X
7	X			X

Table 3.2.1 Post-surgery diet for subdiaphragmatic vagal deafferentation (SDA) and SDA sham operated rats

Post-surgery rats received combinations of water, liquid diet, wet mash (powdered diet plus water) and solid diet.

Lesion verification was verified at the end of the experimental series microscopically. Any animals in which vagal afferent rootlets were still intact were excluded from all analyses. Ten sham and fourteen SDA operations were performed. Of the ten sham operated, one died during the recovery week. Of the SDA operated, two died during post-operative recovery, one was culled one day post-operatively due to signs of glossopharyngeal nerve damage, and a further animal was culled due to suspected choking and subsequent failure to eat resulting in significant body weight loss. Of the ten SDA operated remaining, three were considered to be incomplete so were excluded from all analyses.

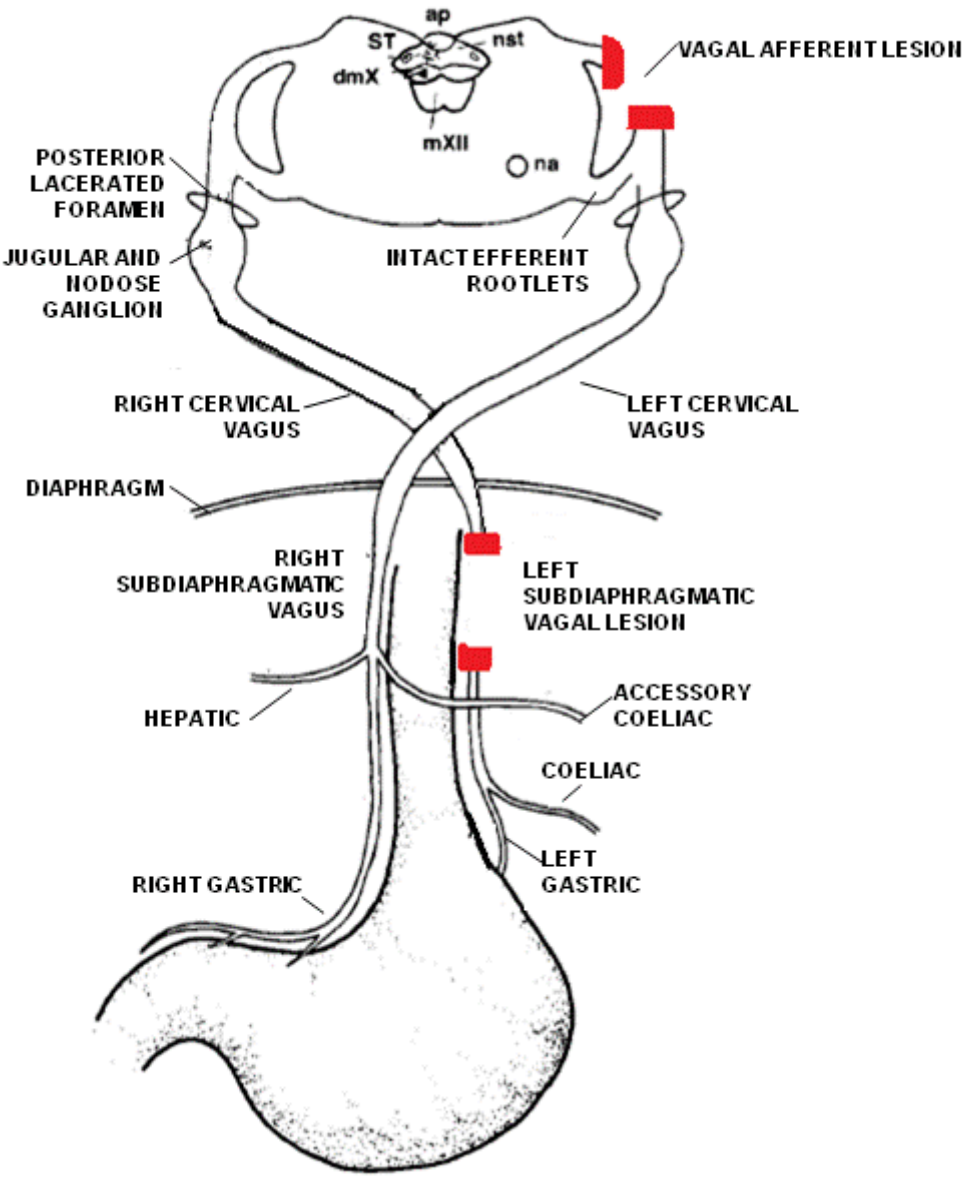


Figure 3.2.1 Surgical preparation for subdiaphragmatic vagal deafferentation
Diagram shows location of vagal lesions required for total vagal deafferentation with 50% vagal efferents remaining intact. Adapted from Smith et al, 1985.

3.2.7.3 The effect of L-cysteine on food intake in rats that have undergone vagal deafferentation

This study was performed as a cross-over study with a 3-day washout period. Rats were fasted overnight then orally gavaged with water or 4mmol/kg L-cysteine during the early light phase. Animals were subsequently returned to their home cages with a pre-weighed amount of chow and food intake was measured 1, 2, 4, 8 and 24 hours post administration. After the washout period the same procedure was repeated with every animal receiving the treatment they did not receive on the first day.

3.2.8 The effect of L-cysteine on food intake in GPRC6A$^{(-/-)}$ mice

3.2.8.1 Animals

GPRC6A$^{(+/-)}$ mice were purchased from the International Knockout Mouse Consortium. Breeding, maintenance and genotyping was performed by Dr James Kinsey-Jones. This knockout consists of a deletion of the whole gene and is thus different from the knockouts discussed previously (Pi et al., 2008, Wellendorph et al., 2009b).

3.2.8.2 The effect of OG administration of L-cysteine on food intake in GPRC6A$^{(-/-)}$ mice

This study was performed as a crossover study. Mice were fasted overnight and then received an oral gavage of water or 8mmol/kg L-cysteine (n=5) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration. This procedure was repeated with every animal receiving the treatment they did not receive on the first day.

3.2.8.3 The effect of IP administration of L-cysteine on food intake in GPRC6A$^{(-/-)}$ mice

This study was performed as a crossover study. Mice were fasted overnight and then received an IP injection of saline or 4mmol/kg L-cysteine (n=6) during the early light phase. Subsequently, mice were returned to their home cage with a pre-weighed amount of chow and food was reweighed 1, 2, 4, 8 and 24 hours post-administration. This procedure was repeated with every animal receiving the treatment they did not receive on the first day.

3.2.9 The effect of repeated OG administration of L-cysteine to rats

Adult male Wistar rats were orally gavaged three times throughout the dark phase, once at onset 19.00, once at 23.00 and once at 03.00, receiving the same treatment for 5 nights. Treatments were water (control), 4mmol/kg L-Cysteine or 4mmol/kg Glycine (negative control) (n=6-9).

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

Body weight and food intake was measured daily at the onset of the dark phase, and food intake was also measured one hour after the first gavage each night.

3.2.9.2 The effect of repeated OG administration of L-cysteine on body composition in rats

At the end of the study rats were culled by CO_2 asphyxiation and the contents of their gastrointestinal tract removed. Animals were then weighed and placed into separate containers. A solution of 3M KOH in 65% ethanol in equal volume to carcass weight was added and the carcass incubated at 70°C for 5 days until dissolved. The resultant liquid was made up to 1L with absolute ethanol and a sample taken for the measurement of protein and fat content.

3.2.9.2.1 Glycerol Assay

A glycerol assay (Assay GY105, Randox Laboratories) was used to determine the fat content of the solutions and thus the percentage fat mass of the animals. The assay is a direct colorimetric procedure for the measurement of glycerol, utilizing a quinoneimine chromogen system in the presence of glycerol kinase, peroxidase and glycerol phosphate oxidase. All reagents were prepared according to manufacturer's guidelines. An 11-point standard curve ranging from 0 – 1M glycerol was created. Samples were diluted 1:100 with dH_2O and 30µl of sample or standard used for the assay. The absorbance of the reactions was read at 520nm using a spectrophotometer. The fat content of each sample was calculated from the standard curve and the carcass fat content was then calculated by assuming that each molecule of triglyceride has a molecular weight of 885g (Bergmeyer, 1974)

3.2.9.2.2 Protein Assay

A modified Lowry Assay (Thermoscientific) was used to determine the protein content of the solutions and thus the percentage protein content. The assay is a colorimetric procedure for the measurement of protein, using a cupric sulphate- tartrate system utilizing Folin-ciocalteu phenol reagent. All reagents were prepared according to manufacturer's guidelines. A 9-point standard curve ranging from 0 - 1500µg/ml bovine serum albumin was created. Samples were diluted 1:100 with dH_2O and 200µl of sample or standard used for the assay. The absorbance of the reactions was read at 750nm using a spectrophotometer. The protein content of each sample was then calculated from the standard curve and carcass protein content calculated accordingly. Lean mass was calculated as total mass- fat mass.

3.2.10 The effect of repeated OG administration of L-Cysteine to diet induced obese mice

Male C57BL/6 mice aged 6 weeks were maintained in group housing with *ad libitum* access to 60% high fat diet (HFD) (Research Diets, New Brunswick, USA) and water for 14 weeks. Animals were then transferred to single housing and allowed 1 week to acclimatise to the new conditions before commencement of experimental procedures.

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

Mice were orally gavaged three times throughout the dark phase, once at onset 19.00, once at 23.00 and once at 03.00 receiving the same treatment for 5 nights. Treatments were water (control), 4mmol/kg L-Cysteine or 4mmol/kg Glycine (negative control) (n=6-9). Food intake and body weight were measured daily at onset of the dark phase.

3.2.11 Statistical Analysis

All data is expressed as mean $\pm$ SEM. Acute food intake and gastric emptying studies were analysed using one-way ANOVA with Tukey's post-hoc test where appropriate. SDA and knockout studies were analysed by two-way repeated measures ANOVA with Bonferroni post hoc test where appropriate. Repeated administration studies were analysed using two-

way repeated measures ANOVA with Bonferroni post hoc test. Statistical significance was accepted at $p<0.05$. All analysis was carried out using Graph Pad Prism Software, version 5.0.

3.3 Results

3.3.1 The effect of inhibiting L-Cysteine metabolism on food intake

Cysteine is metabolised through a number of pathways (Fig. 3.1.1). Inhibiting glutathione production from L-cysteine with the γ-glutamyl cysteine synthetase enzyme inhibitor buthionine sulfoximine (BSO) did not significantly attenuate the effect of intraperitoneal administration of 2mmol/kg L-cysteine on 0-1hr food intake when administered 20 minutes prior (L-cysteine: 0.28 ± 0.05g vs. BSO &L-cysteine: 0.38 ± 0.06g, p=NS) (Fig. 3.3.1 B) or immediately prior (L-cysteine: 0.33 ± 0.07g vs. BSO &L-cysteine: 0.15 ± 0.05g, p=NS) (Fig. 3.3.1C). The effect of glutathione on food intake was also investigated to confirm this was not mediating the effects of L-cysteine on food intake. Intraperitoneal administration of 2 and 4mmol/kg glutathione significantly decreased 0-1hr food intake (Saline: 0.62 $\pm$ 0.04g vs. 2mmol/kg Glutathione: 0.46 $\pm$ 0.03g, $p<0.05$, vs. 4mmol/kg Glutathione: 0.46 $\pm$ 0.02g, $p<0.05$). Intraperitoneal administration of 4mmol/kg L-cysteine was significantly decreased compared to 4mmol/kg glutathione (4mmol/kg Glutathione: 0.46 $\pm$ 0.02g vs. 4mmol/kg L-cysteine: 0.21 $\pm$ 0.08g, $p<0.01$) (Fig. 3.1.1 D)

Inhibiting the enzyme γ-cystathionase (CSE), which catalyzes the production of pyruvate, ammonia and hydrogen sulphide from L-cysteine, with 125 or 200 µmol/kg propargylglycine (PPG) did not attenuate the effect of intraperitoneal administration of 2mmol/kg L-cysteine on 0-1hr food intake. At dose of 200µmol/kg PPG followed by L-cysteine completely inhibited food intake in the first hour (L-cysteine: 0.18 ± 0.07g vs. 200µmol/kg PPG & L-cysteine: 0.00 ± 0.00g) (Fig. 3.3.1 E) and at 125 µmol/kg PPG, the effect of L-cysteine on food intake was also potentiated (L-cysteine: 0.37 ± 0.07g vs. PPG & L-cysteine: 0.19 ± 0.04g, p=NS) (Fig.3.3.1 F).

O-carboxymethyl hydroxylamine hemi-hydrochloride, a general inhibitor of pyridoxal 5' phosphate dependent enzymes, which include cystathionine β-synthetase (CBS), CSE, cysteine amino transferase (CAT), cysteine lyase (CL) and cysteine sulfinate decarboxylase (CSD), potentiated the effect of intraperitoneal administration of 2mmol/kg L-cysteine on 0-1hr food intake (L-cysteine: 0.33 ± 0.05g vs. CHH & L-cysteine: 0.15 ± 0.04g, $p<0.05$). Food intake was significantly reduced following administration of L-cysteine, and PPG with L-

cysteine compared to saline control (saline: 0.75 ± 0.03g vs. L-cysteine: 0.33 ± 0.05g, $p<0.001$; vs. PPG & L-cysteine: 0.15 ± 0.04g, $p<0.001$) (Fig. 3.3.1 G).

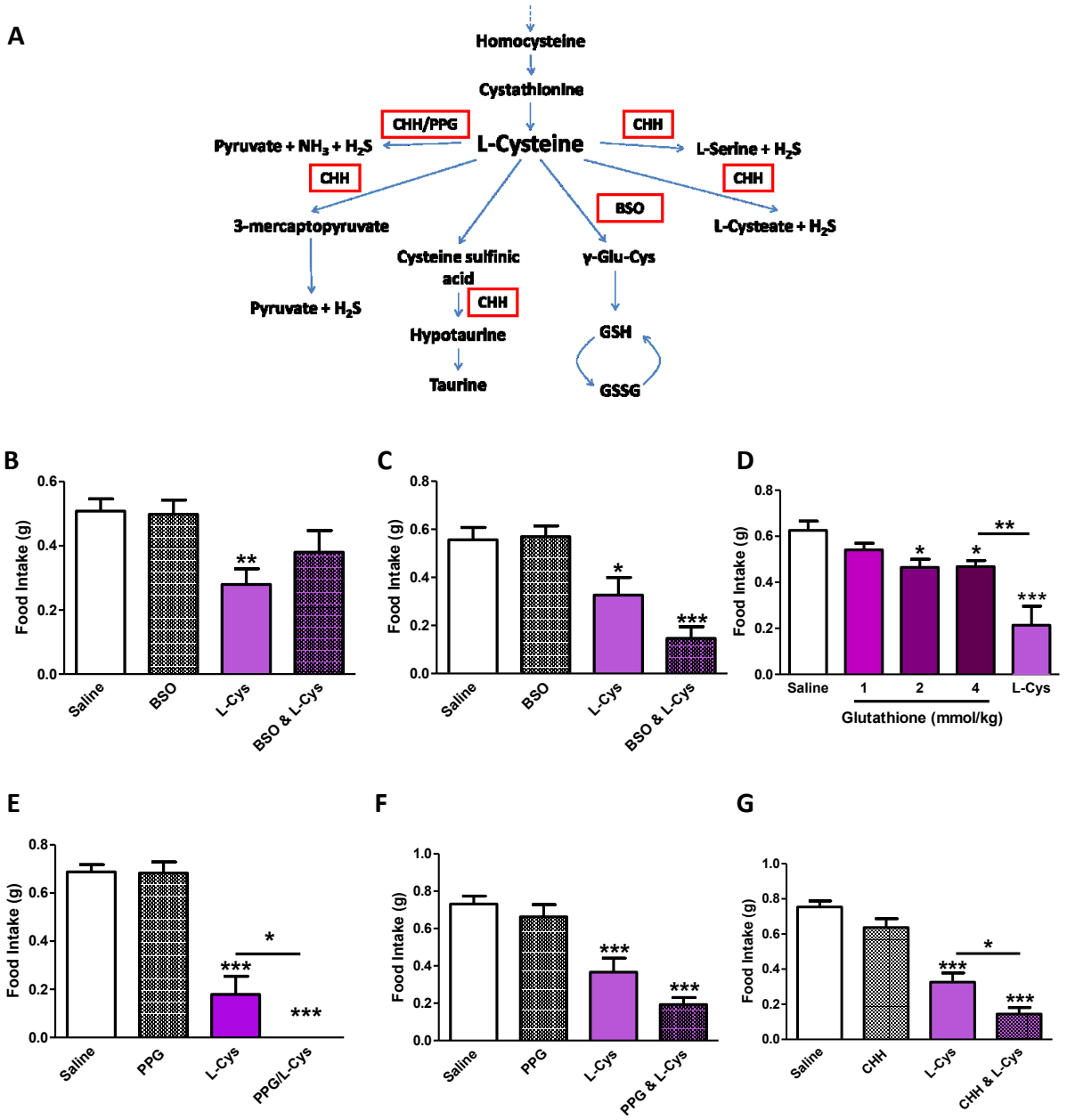


Figure 3.3.1 The effect of inhibiting enzymes involved in the catabolism of L-Cysteine on the anorectic effect of L-cysteine

Cysteine metabolism with red boxes highlighting inhibitors of pathway enzymes (A). The effect of pre-treatment with 1mmol/kg buthionine sulfoximine at t=-20min (BSO; γ-Glutamyl cysteine synthetase inhibitor) (n=9-10) (B), pre-treatment 1mmol/kg buthionine sulfoximine at t=0min (C), saline, 1, 2, 4mmol/kg Glutathione or 4mmol/kg L-cysteine on food intake (n=5-11) (D), pre-treatment with 200µmol/kg propargylglycine (PPG; γ-Cystathionase inhibitor) at t=-4hrs (n=10) (E), pre-treatment with 125µmol/kg propargylglycine (PPG; γ-Cystathionase inhibitor) at t=-4hrs (n=10) (F), pre-treatment with 20mg/kg O-Carboxymethyl-hydroxylamine hemihydrochloride (CHH; PLP-dependent enzyme inhibitor) at t=-4hrs (n=10) (G), on the anorectic effect of 2mmol/kg L-cysteine. Data expressed as mean + SEM. *p<0.05, **p<0.01, ***p<0.001. BSO: buthionine sulfoximine, PPG:proparagylglycine, CHH: O-carboxylmethyl-hydroxylamine hemihydrochloride

3.3.2 The role of the NMDA receptor in mediating the effect of peripheral administration of L-cysteine on food intake in mice

Intraperitoneal administration of 2mmol/kg L-cysteine, and 10µg/kg MK-801 and 2mmol/kg L-cysteine significantly reduced food intake compared to saline control (Saline: 0.72 + 0.06g vs. L-cysteine: 0.3 $\pm$ 0.05g, $p<0.001$, vs. 10µg/kg MK-801 and L-cysteine: 0.26 $\pm$ 0.08g, $p<0.001$). Administration of 10µg/kg MK-801 did not significantly attenuated the effect of L-cysteine on food intake (Fig. 3.3.2 A).

Additionally, L-glutamate and L-aspartate, which also have NMDA receptor activity, did not significantly decrease food intake. Administration of 4mmol/kg L-glutamate actually increased food intake in the first hour post administration (Saline: 0.58 + 0.07g vs. L-glutamate: 0.79 + 0.01g, $p<0.05$) (Fig. 3.3.2B).

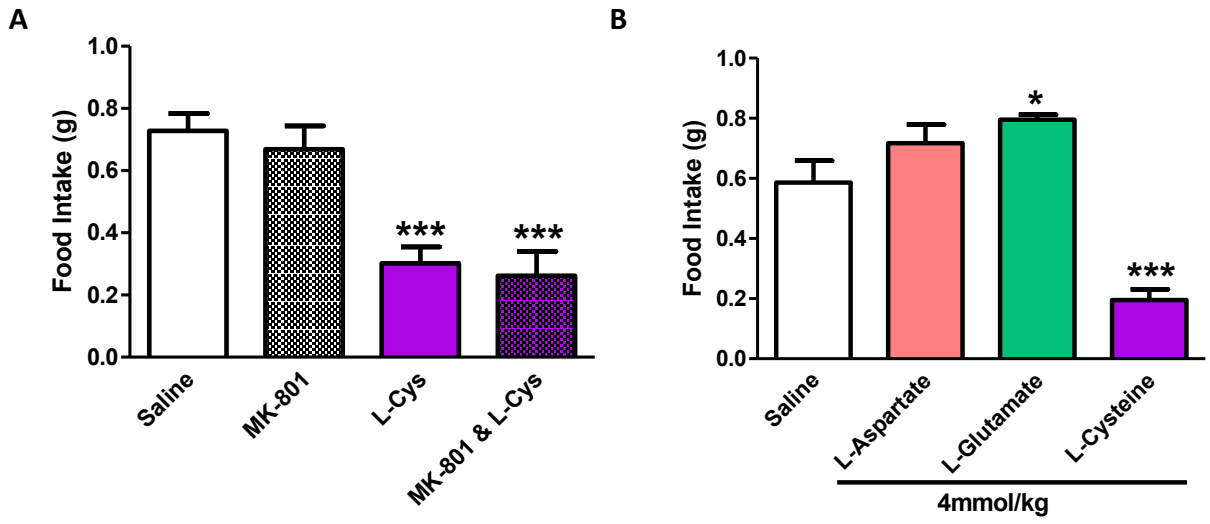


Figure 3.3.2 The role of the NMDA receptor in mediating the effect of peripheral administration of L-cysteine on food intake in mice

The effect of 10µg/kg MK-801 (an NMDA receptor antagonist) on the anorectic effect of IP administration of 2mmol/kg L-cysteine following an overnight fast in mice (n=6-8) (A). The effect intraperitoneal administration of 4mmol/kg L-aspartate, L-glutamate and L-cysteine on 0-1 hour food intake following an overnight fast (n=10) (B). Data expressed as mean + SEM, *p<0.05, **p<0.01, p<0.001.

3.3.3 The role of the NMDA receptor in mediating the effect of central administration of L-cysteine on food intake in rats

Chapter 2 demonstrated that central administration of 2µmol L-cysteine significantly decreased food intake. However, this was associated with behavioural side effects. Here lateral ventricle administration of 2µmol L-cysteine significantly decreased food intake compared to saline control during the 0-1hour period post injection (saline: 6.06 ± 0.33g vs. 2µmol L-cysteine: 2.93 ± 0.94g, $p<0.05$) (Fig.3.3.3A) and similar behavioural side effects were witnessed. Administration of 200nmol D-AP5, an NMDA receptor antagonist, with 2µmol L-cysteine significantly attenuated the effect of L-cysteine on food intake (L-cysteine: 2.93 ± 0.94g vs. L-cysteine + D-AP5: 5.73 + 0.65, $p<0.05$) and behavioural side effects were not observed.

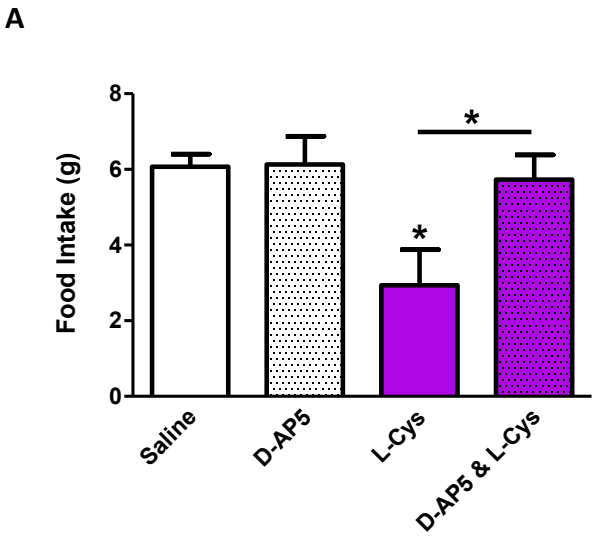


Figure 3.3.3 The effect of NMDA receptor antagonism on L-cysteine induced anorexia following central administration

The effect of intracerebroventricular (lateral ventricle) administration of saline, 200nmol D-AP5, 2µmol L-Cysteine or 200nmol D-AP5 plus 2µmol L-cysteine on 0-1 hour food intake. Data expressed as mean + SEM. n=5-8. *$p<0.05$

3.3.4 The effect of intraperitoneal administration of L-cysteine on gastric emptying in rats

Intraperitoneal administration of 2mmol/kg L-cysteine significantly reduced gastric emptying 30 minutes after administration (Saline: 54.97 ± 11.01% vs. L-cysteine: 2.79 ± 4.91%, $p<0.001$). Intraperitoneal administration of 10nmol/kg A71623, a CCK-A receptor agonist (positive control), also significantly reduced gastric emptying (Saline: 54.97 ± 11.01% vs. A71623: -10.48 ± 4.05%, $p<0.001$). Administration of 2mmol/kg Glycine (negative control) did not affect gastric emptying.

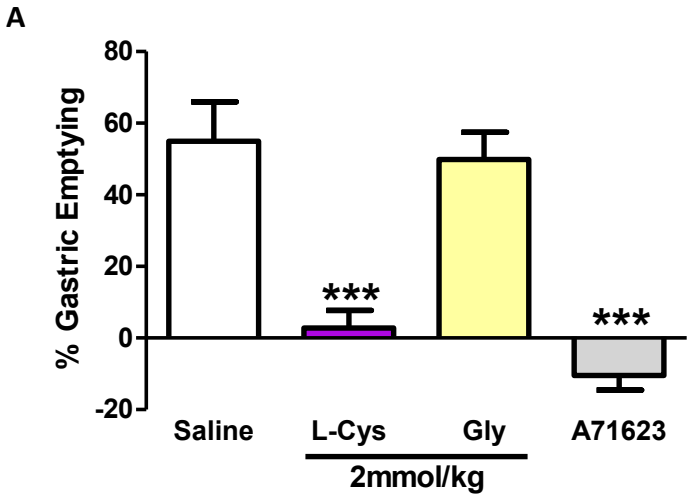


Figure 3.3.4 The effect L-Cysteine on gastric emptying in rats
The effect of IP administration of saline, 2mmol/kg L-cysteine, glycine or 10nmol/kg A71623 on gastric emptying of 2ml of a semi-solid non-nutritive substance given by oral gavage. Data expressed as percentage mean + SEM at 30minutes post injection relative to gastric emptying at time 0. n=4-7, ***p<0.001

3.3.5 The role of CCK-1 receptor in mediating the effect of L-cysteine on food intake in mice

Intraperitoneal administration of 10nmol/kg A71623 significantly reduced food intake (Saline: 0.72 ± 0.02g vs. A71623: 0.33 ± 0.03g. $p<0.001$). A dose of 0.5mg/kg Devazepide significantly attenuated the effect of A71623 on food intake (A71623: 0.33 ± 0.03g vs. Devazepide + A71623: 0.68 + 0.04, $p<0.001$) (Fig. 3.3.5 A).

Intraperitoneal administration of 4mmol/kg L-cysteine significantly reduced food intake (Saline 0.78 ± 0.03g vs. L-cysteine: 0.24 ± 0.02g, $p<0.001$). Intraperitoneal administration of 0.5mg/kg with 2mmol/kg L-cysteine also significantly decreased food intake compared to saline control (Saline 0.78 ± 0.03g vs. Devazepide & L-cysteine: 0.30 ± 0.04g, $p<0.001$). Devazepide did not significantly affect the effect of 4mmol/kg L-cysteine on food intake (Fig. 3.3.5 B).

Intraperitoneal administration of 2mmol/kg L-cysteine significantly reduced food intake (Saline 0.71 ± 0.06g vs. L-cysteine: 0.49 ± 0.03g, $p<0.05$). Intraperitoneal administration of 0.5mg/kg devazepide with 2mmol/kg L-cysteine also significantly decreased food intake compared to saline control (Saline 0.71 ± 0.06g vs. Devazepide & L-cysteine: 0.45 ± 0.07g, $p<0.01$). Devazepide did not significantly affect the effect of 4mmol/kg L-cysteine on food intake (Fig. 3.3.5 C).

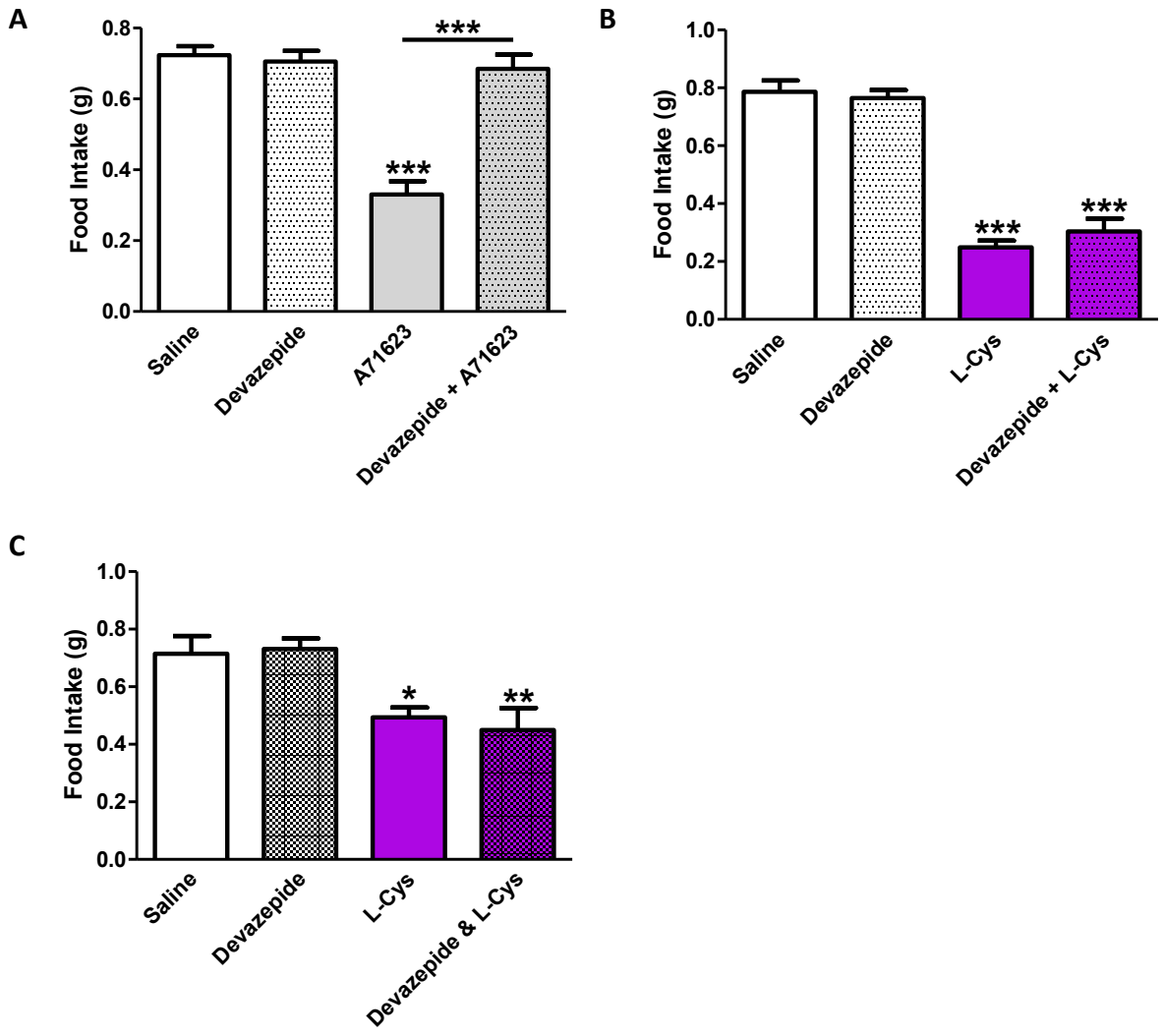


Figure 3.3.5 The role of CCK-1R in mediating the effect of L-cysteine on food intake in mice
The effect of IP administration of 0.5mg/kg devazepide on the anorectic effect of 10nmol/kg A71623 in the 0-1 hour period post administration (n=8-9) (A). The effect of 0.5mg/kg devazepide on the anorectic effect of 4mmol/kg L-cysteine in the 0-1hour period post administration (n=8-9) (B). The effect of 0.5mg/kg devazepide on the anorectic effect of 2mmol/kg L-cysteine in the 0-1hour period post administration (n=8-9) (C). Data expressed as mean + SEM. *p<0.05, **p<0.01, ***p<0.001.

3.3.6 The effect of L-cysteine on food intake in rats that have undergone subdiaphragmatic vagal deafferentation

Oral administration of 4mmol/kg significantly decreased 0-1 hour food intake in Sham operated rats and SDA operated rats compared to water control (Sham: Water: 6.53 + 0.48g vs. L-cysteine: 1.98 + 0.33g, $p<0.001$, SDA: Water: 6.26 + 0.73g vs. L-cysteine: 1.62 + 0.48g, $p<0.001$). There was no significant difference between sham and SDA animals.

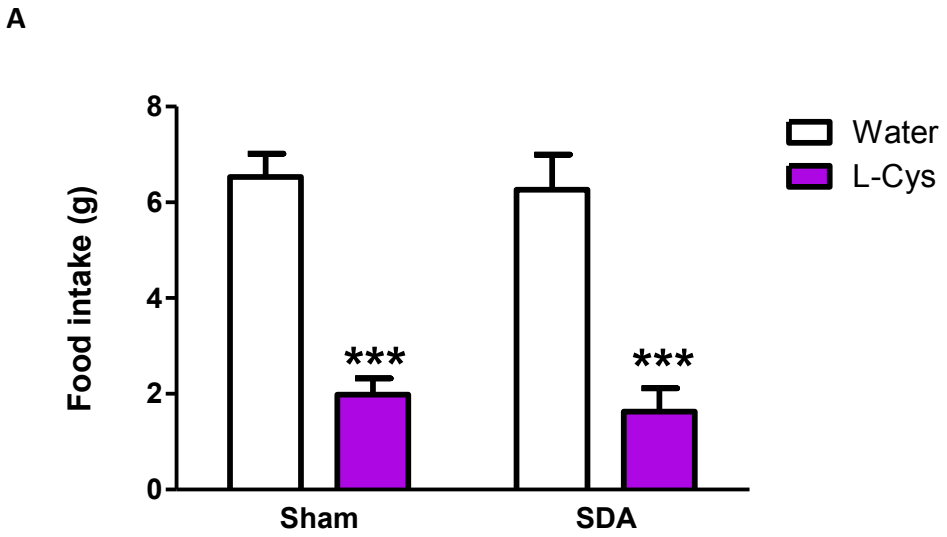


Figure 3.3.6 The effect of oral administration of L-Cysteine in the on food intake in rats that have undergone subdiaphragmatic vagal deafferentation

The effect of oral gavage of water or 4mmol/kg L-cysteine on 0-1 hr food intake in sham and subdiaphragmatic vagal deafferentation (SDA) operated rats following an overnight fast. Data expressed as mean + SEM. n=7-9. ***p<0.001

3.3.7 The effect of L-cysteine on food intake in GPRC6A knockout mice

L-cysteine significantly reduced food intake to a similar extent in both GPRC6a$^{(+/+)}$ and GPRC6a$^{(-/-)}$ mice following oral administration (GPRC6a$^{(+/+)}$ - water: 0.67 ± 0.06g vs. L-cysteine: 0.21 ± 0.09g, p<0.01, GPRC6a$^{(-/-)}$ - water: 0.71 ± 0.02g vs. L-cysteine: 0.36 ± 0.11g, p<0.01, n=5) (Fig 3.3.7 A) and intraperitoneal administration (GPRC6a$^{(+/+)}$ - saline: 0.80 ± 0.03g vs. L-cysteine: 0.25 ± 0.05g, p<0.001, GPRC6a$^{(-/-)}$ - saline: 0.64 ± 0.07g vs. L-cysteine: 0.27 ± 0.05g, p<0.01, n=6) (Fig. 3.3.7 B). It is therefore unlikely that GRPC6a has a role in the effect of L-cysteine on food intake.

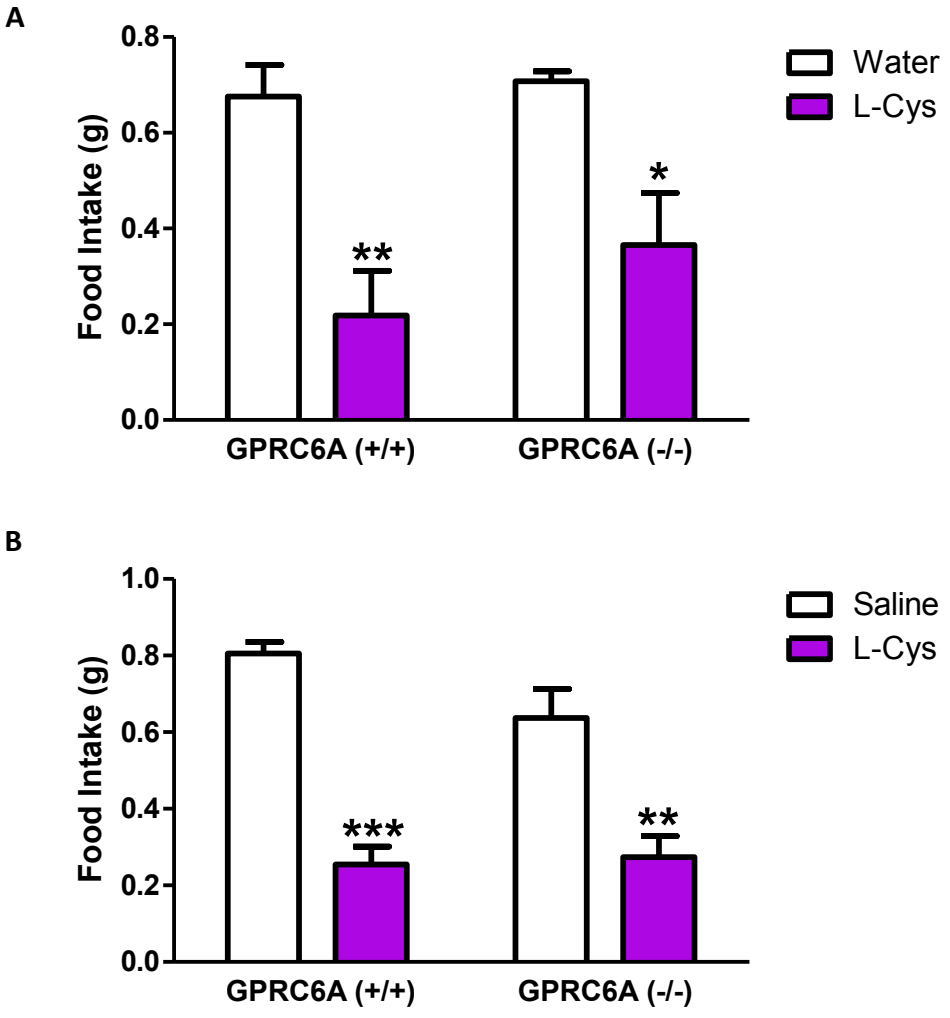


Figure 3.3.7 The effect of L-cysteine on food intake in GPRC6A knockout mice.
The effect of oral gavage of water or 8mmol/kg L-cysteine on 0-1 hour food intake (n=5) (A) and the effect of IP administration of saline or 4mmol/kg L-cysteine on 0-1 hour food intake (n=6) (B) in GRPC6A wildtype and knockout mice. Data expressed and mean + SEM. *p<0.05, **p<0.01, ***p<0.001

3.3.8 The effect of repeated OG administration of L-Cysteine to rats

3.3.8.1 The effect of repeated OG administration of L-cysteine on food intake and body weight in rats

Oral administration of 4mmol/kg L-cysteine at the onset of the dark phase significantly decreased 0-1 hour food intake compared to water control (Water: 2.38 $\pm$ 0.39g vs. L-cysteine: 0.74 $\pm$ 0.17) (Fig. 3.3.8 A). However, after 4 days of repeated administration L-cysteine no longer significantly reduced acute food intake (Fig. 3.3.8 B).

Nonetheless, repeated oral administration of L-cysteine over a period of five nights significantly reduced cumulative food intake compared to water and glycine treated controls. These differences were statistically significant from day 3, and by day 5 there was 14.6g difference in cumulative food intake (Water: 144.7 $\pm$ 3.5g vs. 130.1 $\pm$ 2.5g: L-Cysteine, $p<0.001$) (Fig. 3.3.8 C). There were no significant differences in body weight gain between the groups at any time point observed (Fig. 3.3.8 D).

3.3.8.2 The effect of repeated OG administration of L-cysteine on body composition in rats

Repeated administration of glycine significantly increased percentage fat and significantly decreased percentage lean mass compared to water control (Fat: Water: 14.12 ± 0.76% vs. Glycine: 16.51 ± 0.69% vs, $p<0.05$, Lean: Water: 85.88 ± 0.76% vs. Glycine: 83.49 ± 0.69 %)(Fig. 3.3.8 C). The decrease in lean mass did not appear to be secondary to a decrease in protein mass. Chronic administration of L-cysteine did not result in changes in body composition relative to water treated controls (Fig. 3.3.8 E)

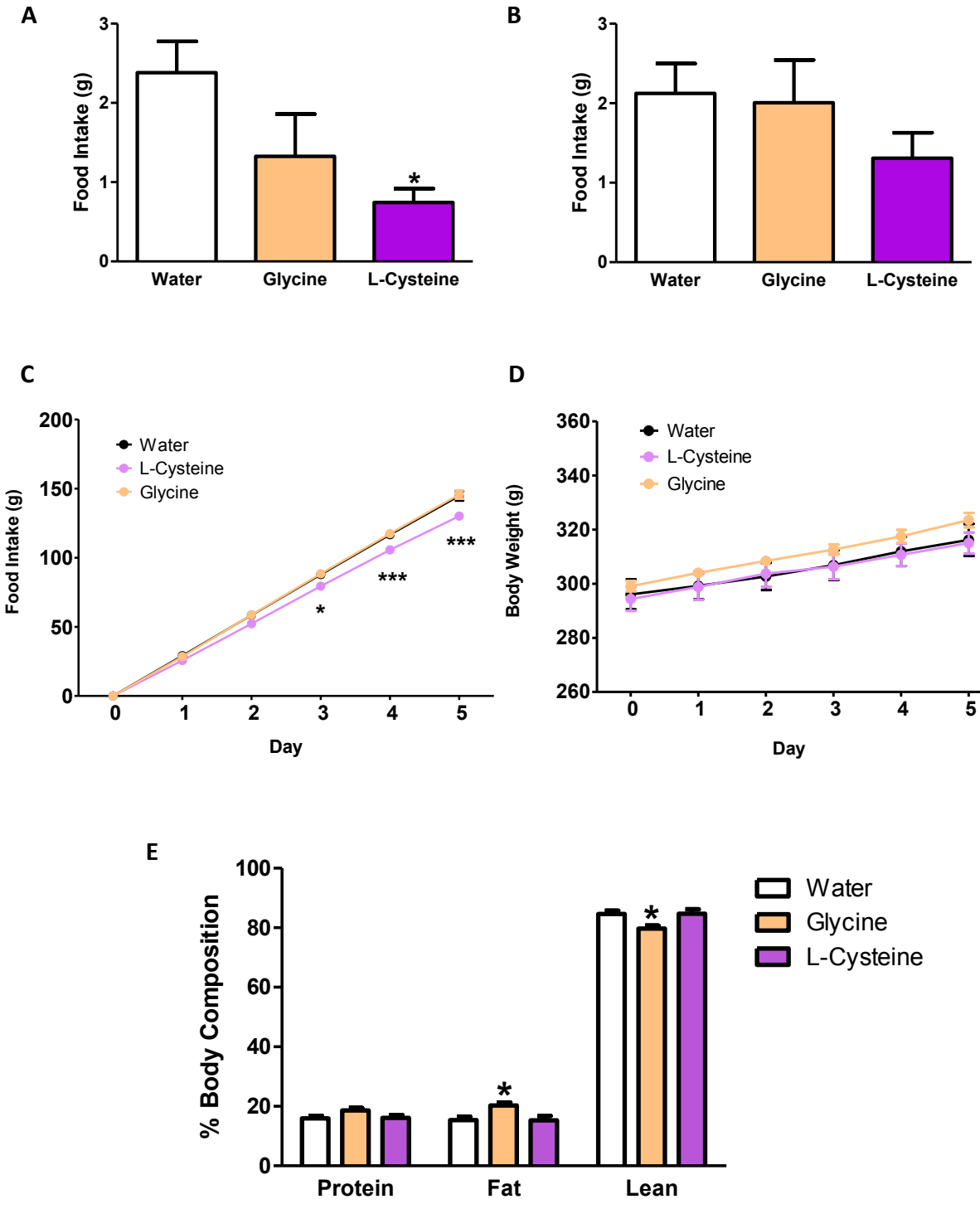


Figure 3.3.8 The effect of repeated OG administration of L-cysteine on food intake, body weight and body composition in rats

The effect of oral administration of water, 4mmol/kg glycine or 4mmol/kg L-cysteine on 0-1 food intake at the onset of the dark phase on day 0 (A) and day 4 of repeated administration (B) and the effect on cumulative food intake (C) body weight (D) and body composition (C). Data expressed as mean ± SEM. n=6-9. *p<0.05, ***p<0.001

3.3.9 The effect of repeated OG administration of L-Cysteine to diet induced obese mice

Oral gavage administration of 4mmol/kg L-cysteine at the onset of the dark phase had no effect on acute food intake in DIO mice (Fig. 3.3.9 A). Surprisingly, on day 4 of repeated administration L-cysteine significantly increased 0-1 hour food intake compared to water control (Water: 0.06 $\pm$ 0.0.01g vs. L-cysteine: 0.18 $\pm$ 0.03g) (Fig. 3.3.9 B). However, acute food intake on day 0 was no different from day 4 in the L-cysteine treated group (Day 0: 0.18 + 0.02g vs. Day 4: 0.18 + 0.03g).

Nonetheless, repeated administration of L-cysteine significantly reduced cumulative food intake between days 2 and 4 compared to water treated controls (Day 2: Water: 4.14 $\pm$ 0.28g vs. L-Cysteine: 2.96 $\pm$ 0.26g, Day 3: Water: 5.82 $\pm$ 0.40g vs. L-Cysteine: 4.47 $\pm$ 0.47g, Day4: Water: 7.64 $\pm$ 0.48g vs. L-Cysteine: 6.46 $\pm$ 0.70g, $p<0.05$) (Fig. 3.3.9 C). There was no significant difference in body weight (Fig 3.3.9 D).

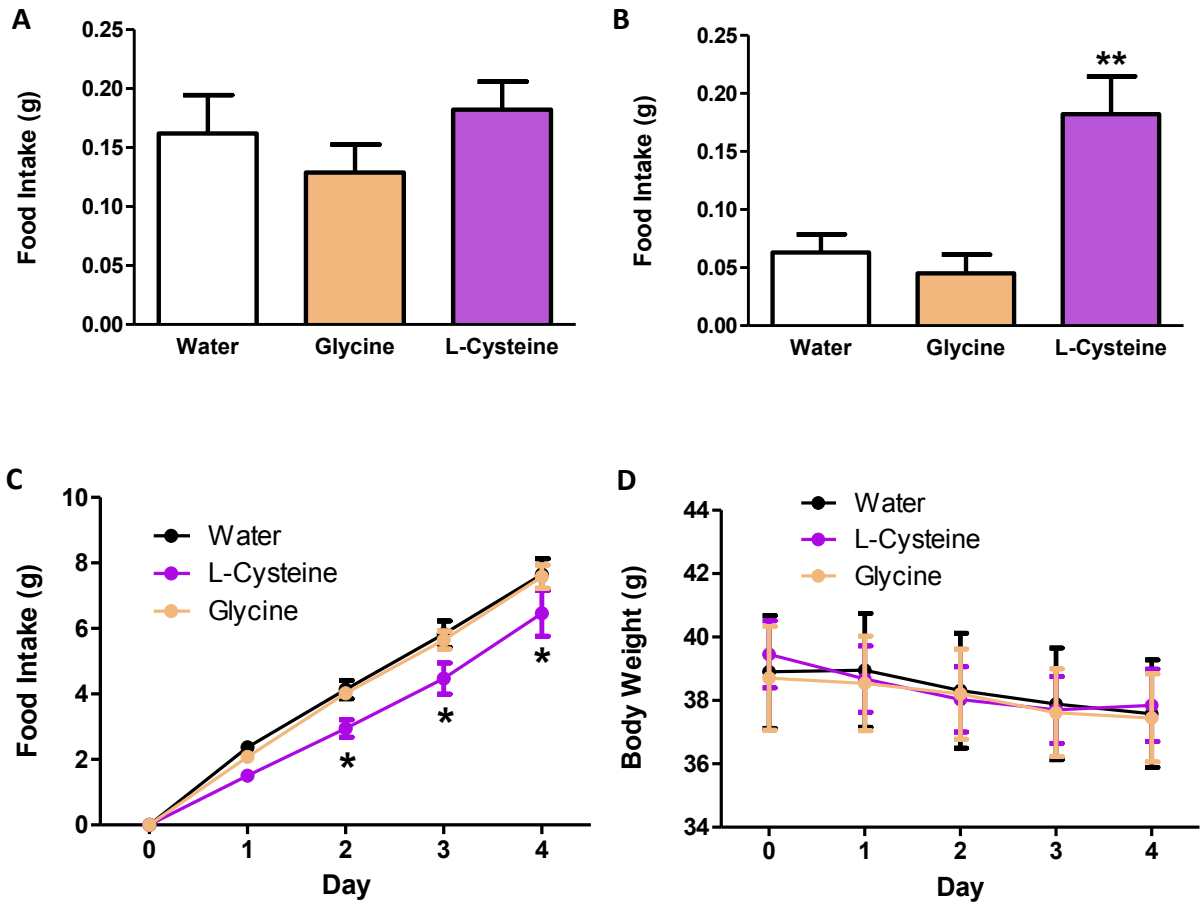


Figure 3.3.9 The effect of repeated OG administration of L-cysteine on food intake and body weight in diet induced obese mice.

The effect of oral administration of water, 4mmol/kg glycine or 4mmol/kg L-cysteine on 0-1 hour food intake at the onset of the dark phase on day 0 (A) and day 4 of repeated administration (B) and the effect on cumulative food intake (C) body weight (D). Data expressed as mean ± SEM. n=8-10. *p<0.05

3.4 Discussion

This chapter describes experiments investigating possible mechanisms through which L-cysteine may mediate its effect on food intake. It presents data suggesting that cysteine, and not one of its metabolites, mediates the effect on food intake when given peripherally. It also shows that the NMDA, GPRC6A and CCK receptors are unlikely to be involved. The effect of repeated administration is also described.

L-cysteine is a major precursor for glutathione, taurine and hydrogen sulphide (H_2S). The enzymes involved in the catabolism of L-cysteine were inhibited to investigate the possibility that a metabolite of L-cysteine was mediating its effect on food intake. Inhibiting the production of glutathione did not attenuate the effect of L-cysteine on food intake, suggesting L-cysteine is not mediating its effect through glutathione. Nonetheless, intraperitoneal administration of glutathione does decrease food intake, albeit not to the same extent as L-cysteine. Glutathione is an allosteric modulator of the CaSR; its potentiating effect is greater than that of the aromatic amino acids (Wang et al., 2006, Broadhead et al., 2011). L-cysteine is also an allosteric modulator of the CaSR. However, it is not as potent as the aromatic amino acids at the CaSR, such that the relative potencies are glutathione>aromatic amino acids>L-cysteine. These experiments suggest that is it unlikely that glutathione or the CaSR are involved in mediating the effect of L-cysteine on food intake. However, the use of an antagonist or negative allosteric modulator of the CaSR may confirm this more conclusively. Additionally, the effect of L-cysteine on food intake was examined in GPRC6A knockout mice. L-cysteine significantly decreased food intake in these knockouts, suggesting this receptor is not necessary to mediate the effect of L-cysteine on food intake. Furthermore, L-cysteine reduced food intake to a greater extent than other, more potent agonists of this receptor, such as arginine and lysine in both rats (Fig. 2.1.6) and mice (data not shown). Despite L-cysteine being considered the most potent amino acid agonist of the T1R1/T1R3 receptor, other potent agonists of this receptor, such as L-threonine, had little or no effect on food intake in both rats (Fig. 2.1.6) and mice (data not shown). Collectively, there is little evidence to suggest these promiscuous amino acid sensing receptors are involved in mediating the effect of L-cysteine on food intake.

CHH is an inhibitor of PLP-dependent enzymes including γ-cystathionase, cysteine aminotransferase, cysteine lyase, cystathionine-β-synthase and cysteine sulfinate decarboxylase, and should thus inhibit the initial step in cysteine catabolism in all other pathways with the exception of the cysteine-taurine pathway. In preliminary studies, peripheral administration of L-taurine had no effect on food intake (Fig. 2.1.6). The synthesis of endogenous taurine from cysteine is controlled by cysteine dioxygenase and cysteine sulfinate decarboxylase. CHH inhibits cysteine sulfinate decarboxylase, the last enzyme in this pathway, and thus endogenous taurine synthesis. However, there are no agents which have been shown to inhibit cysteine dioxygenase *in vivo*. Thus, a role for the intermediate products of the cysteine-taurine pathway in the anorectic action of L-cysteine cannot be ruled out. In addition, the effect of PPG, a specific inhibitor of γ-cystathionase, on the anorectic effect of L-cysteine was also investigated. Inhibiting this pathway did not attenuate the effect of L-cysteine on food intake. Collectively, these studies suggest that L-cysteine itself, rather than downstream products of L-cysteine metabolism, is mediating its effect on food intake although it is still possible that intermediate products in the cysteine-taurine pathway such as cysteine sulfinic acid or hypotaurine may play a role.

L-cysteine and some of its metabolites are excitotoxins and can act through an NMDA receptor- dependent mechanism. Initial studies in this chapter suggested that L-cysteine molecule itself reduces food intake following peripheral administration. L-cysteine is considered a weak activator of NMDA receptor currents (Pace et al., 1992). The exact mechanism remains unclear but L-cysteine's excitatory actions are thought to result from competitive inhibition of tonic reuptake of glutamate and/or heteroexchange with intracellular excitatory amino acids through an EAAT3 transport mechanism (Zerangue and Kavanaugh, 1996), thereby maintaining/increasing extracellular concentrations of the NMDA receptor agonist glutamate. NMDA receptor antagonism did not attenuate the anorectic action of peripherally administered L-cysteine. Furthermore, peripheral administration of the more potent NMDA receptor mediated excitotoxins L-glutamate and L-aspartate did not result in an anorectic effect. NMDA receptor antagonism did, however, attenuate the anorectic action of centrally administered L-cysteine. Collectively, these data suggest the central but not the peripheral effects of L-cysteine on food intake are mediated by the NMDA receptor. In addition, central NMDA antagonism also prevented the

behavioural side effects of centrally administered L-cysteine, suggesting the reduction in food intake may have been a secondary effect. These studies suggest that the NMDA receptor is not involved in the effect of peripherally administered L-cysteine on food intake.

Gastric distension is an important factor in the regulation of satiation during food intake (Wang et al., 2008, Geliebter, 1988). The degree of gastric distension is communicated to the brain predominantly via mechanosensitive vagal afferent fibres. These neural afferent signals can activate neural efferent signals that control gastric emptying. In addition to gastric distension, nutrient detection in the duodenum can also influence gastric emptying through gut hormones and vagal afferent-mediated signals (Moran et al., 1999, Zittel et al., 1994, Raybould and Lloyd, 1994). Two of the major gut hormones involved in delaying gastric emptying are GLP-1 and CCK. The effect of L-cysteine on gastric emptying was investigated to determine whether signals associated with gastric distension might play a role in the associated decrease in food intake. Gastric emptying of a semi-solid non-nutritive bolus was significantly decreased following intraperitoneal administration of L-cysteine. Therefore, changes in gastric distension caused by delayed gastric emptying may contribute towards the acute reduction in food intake observed after administration of L-cysteine. As gastric emptying was investigated in response to intraperitoneal administration, nutrient detection in the duodenum would not be a factor in the delay observed here, though it is possible that oral administration of L-cysteine may modulate gut hormone release through pre- and post-absorptive mechanisms. The effect of oral and intraperitoneal administration of L-cysteine on the release of GLP-1, PYY and ghrelin was investigated in chapter 2, with the results suggesting that L-cysteine does not influence GLP-1 and PYY release, but does suppress ghrelin release. As mentioned earlier, CCK is a potent inhibitor of gastric emptying. Therefore, I investigated the role of CCK in mediating the effect of L-cysteine on food intake. A CCK-1 receptor antagonist, did not attenuate the effect of L-cysteine of food intake, suggesting that either the delay in gastric emptying was not a major factor in the reduction in food intake, or that some other factor was delaying gastric emptying. Furthermore, the anorectic effect of CCK requires NMDA receptor signalling in the hindbrain (Wright et al., 2011) and as previous studies in this chapter suggested, this receptor was not necessary for L-cysteine induced hypophagia, thus a role for CCK seems unlikely. Additionally, it is unlikely vagal afferent fibres play a role in the reduction in food intake as L-cysteine still significantly

reduced food intake in animals that had undergone sudiaphragmatic vagal deafferentation. Collectively, these studies present strong evidence that L-cysteine does not mediate its effect on food intake through a mechanism involving CCK, NMDA receptors or vagal afferents.

Chapter 2 also highlighted an increase in c-Fos in the area postrema. It is plausible that L-cysteine or an unknown mediator is sensed by neurons in the area postrema which can then activate neurons in the NTS and higher brain regions. Amylin, a peptide co-secreted with insulin, is known to delay gastric emptying through an area postrema mediated pathway (Edwards et al., 1998). However, protein and intravenous administration of an amino acid mixture attenuates the action of amylin (Riediger et al., 2009), suggesting it is unlikely that L-cysteine mediates its effect through the action of amylin. Nonetheless, this does highlight the presence of a putative area postrema- gastric emptying/satiety pathway that L-cysteine may activate directly.

L-cysteine reduces food intake following acute administration. A final aim of this thesis was to determine whether this reduction in food intake was preserved following repeated administration. Repeated administration of 4mmol/kg L-cysteine significantly reduced cumulative food intake from day 3 onwards in lean rats. However, there were no observed changes in body weight or body composition compared to water treated controls. To investigate its utility in obesity, a diet induced obese mouse model was used. However, the outcome was similar to that seen in lean rats: repeated administration of 4mmol/kg L-cysteine significantly reduced cumulative food intake from day 2 onwards but had no significant effect on body weight. It should be noted that repeated administration of a higher dose (8mmol/kg) was found to be toxic, and lethal in certain cases when tested (data not shown), and that there was one case of mortality in the 4mmol/kg L-cysteine group which prompted the premature termination of the study at day 4. In contrast, no signs of toxicity were observed in the rat study.

Cysteine is known to be neurotoxic at high doses, particularly in neonates where the blood brain barrier has not sufficiently formed. Our doses ranged from 0.5-4mmol/kg (121-484mg/kg) in acute IP studies, 1-8mmol/kg (242-968mg/kg) in acute oral studies and 3x4mmol/kg (1452mg/kg/day) in the repeated administration studies presented. A

comprehensive dose toxicity study for repeated administration of L-cysteine in adult rats was performed by Sawamoto and colleagues (Sawamoto et al., 2003). They investigated the effect of intravenous 0, 100, 300 and 1000mg/kg/day L-cysteine for four weeks and found that body weight gain was significantly suppressed throughout for the whole course of the study in the 1000mg/kg/day group. In this group there were also observations of tremor and a decrease in spontaneous activity, salivation, stereotypy and ptosis. Histopathology revealed sperm granulomas, necrosis of Purkinje cells and renal tubular basophilia associated with proteinuria. No deaths occurred during their dose toxicity study. The daily dose used here was greater (1452mg/kg vs. 1000mg/kg). However, this was given orally and delivered in three separate boluses. Additionally, this study was of a much shorter duration. Epidemiological studies have identified a positive correlation between plasma total cysteine levels (cysteine and its oxidized derivatives) and BMI (El-Khairy et al., 2003, Elshorbagy et al., 2012). It is possible that the DIO mice may have had a higher level of circulating L-cysteine due to their increased body weight. This putative increase in circulating cysteine may result in desensitization to the anorectic effect of L-cysteine explaining why there was no effect of L-cysteine on acute food intake in DIO mice. Furthermore, repeated administration of L-cysteine, together with the putative increase in circulating cysteine, may make these animals more susceptible to L-cysteine-induced toxicity.

In summary, the work presented in this chapter suggests that L-cysteine itself suppresses food intake. The mechanism or mechanisms mediating this acute anorectic effect are still unclear. However, it is unlikely that the NMDA receptor, CCK-1 receptor, GPRC6A receptor or vagal afferents are involved. Further studies are required to determine the mechanism by which L-cysteine reduces food intake and whether there is potential to exploit this mechanism as a therapeutic approach for weight loss.

CHAPTER 4: GENERAL DISCUSSION

There is currently a lack of safe and effective treatment options for obesity. A high protein diet is an effective weight loss and weight maintenance strategy (Westerterp-Plantenga et al., 2004, Halton and Hu, 2004). However, like many diets, high protein diets can be difficult to adhere to. The mechanisms by which protein exerts its weight-reducing effect remain unclear. Nevertheless, they provide a putative therapeutic target for the treatment of obesity. One hypothesis is that specific amino acids generated during protein digestion contribute towards the satiating effect of high protein diets. Preliminary data from our group investigated the effect of a range of amino acids on food intake in rodents. L-cysteine was identified as the most anorexigenic amino acid. This thesis has investigated the effect of L-cysteine on food intake and explored possible mechanisms by which it mediates its effect.

Oral gavage and intraperitoneal administration of L-cysteine dose dependently suppressed acute food intake in rodents without behavioural side effects. Moreover, anorectic doses did not cause conditioned taste aversion suggesting they are well tolerated and the reduction in food intake is not secondary to feelings of illness. Additionally inhibiting the breakdown of L-cysteine did not attenuate its anorectic effect, suggesting it is L-cysteine and not a derivative that suppresses food intake.

The hypothalamus is an integral site for appetite regulation. However, with the exception of the lateral hypothalamic area, oral administration of L-cysteine did not result in significant changes in c-Fos, a marker of neuronal activation, in the hypothalamic nuclei investigated. L-cysteine significantly decreased neuronal activation in the LHA compared to water treated controls. However, this reduction was not significantly different from the negative control, glycine. Glycine had no effect on food intake, suggesting the reduction in neuronal activation in the LHA was not specific to a process controlling food intake. It is surprising that there were no cysteine specific changes in the hypothalamic nuclei. Peak c-Fos protein levels occur 90 minutes after neuronal activation (Chaudhuri et al., 2000). L-cysteine mediated its effect on food intake within the first hour post-administration. It would be interesting to investigate whether changes in c-Fos could be observed in hypothalamic nuclei between 90 and 150 minutes post-administration to establish whether this 90 minute time point was too premature to pick up any significant differences. For instance if L-cysteine was mediating its effect post-absorptively through direct action on central appetite centres there may be a delay in neuronal activation. However, data from our lab suggest

that in humans, plasma cysteine levels are increased within 15 minutes following an oral cysteine load (unpublished). The effect of L-cysteine on meal size and meal frequency during this first hour post administration may also help to elucidate the mechanisms by which L-cysteine is mediating its effect on food intake. L-leucine, a branched chain amino acid with anorexigenic properties, is sensed in the hypothalamus where it modulates appetite through an mTOR-mediated pathway (Cota et al., 2006, Blouet et al., 2009). Thus to more conclusively determine whether L-cysteine could act on central appetite centres to modulate appetite, its effect on food intake following ICV administration was investigated. L-cysteine did significantly suppress food intake following ICV administration. However, unlike peripheral administration, anorectic ICV doses also induced dramatic side effects, suggesting the reduction in food intake may have been secondary to these side effects. It therefore seems unlikely that the anorectic effect of peripherally administered L-cysteine is mediated through changes in central levels of L-cysteine.

High protein diets are known to increase c-Fos in both the hypothalamus and the hindbrain (Faipoux et al., 2008). Therefore, the effect of L-cysteine on neuronal activation in the brainstem was also examined. Peripheral administration of L-cysteine significantly increased c-Fos in the area postrema and caudal medial nucleus tractus solitarius. The area postrema is an area of the hindbrain devoid of a blood brain barrier and thus able to sense changes in circulating factors such as nutrients, hormones and toxins, whereas the NTS is the terminal for vagal afferent fibres. The location of neuronal activation in the NTS following oral administration of L-cysteine was similar to that observed following high protein feeding (Faipoux et al., 2008). The specific neurons activated in the NTS by the protein feeding include noradrenergic/adrenergic (NA/A) neurons (Faipoux et al., 2008). A subset of these neurons are known to project to the PVN of the hypothalamus (Rinaman, 2003). L-cysteine did increase c-Fos expression in the PVN, but this increase did not achieve statistical significance. It would be interesting to investigate whether the neurons that peripheral L-cysteine activates in the NTS include NA/A neurons. Additionally, these neurons have been strongly implicated in the control of meal size (Rinaman, 2011) and thus, as mentioned, it would also be interesting to investigate the effect of L-cysteine on meal size and meal frequency. There are also GLP-1 and POMC expressing neurons in this region of the NTS that may also be involved (Rinaman, 1999, Seeley et al., 2000, Blouet and Schwartz, 2012).

A number of gut hormones partially mediate their effects through the area postrema and vagal afferent fibres. For example, GLP-1 is known to activate neurons in the area postrema (Zuger et al., 2013) and to mediate some of its effects through vagal afferents (Imeryuz et al., 1997). Additionally, PYY is also thought to mediate some of its anorectic activity through vagal afferents (Koda et al., 2005), and also increases neuronal activation in the caudal medial NTS and area postrema following peripheral administration (Blevins et al., 2008). However, at 30 minutes post administration, neither oral gavage nor intraperitoneal administration of L-cysteine affected plasma levels of GLP-1 or PYY. L-cysteine did suppress plasma levels of the orexigenic hormone acyl-ghrelin. The mechanisms controlling ghrelin release have not been fully elucidated but include neuronal and hormonal signals. Noradrenaline, a sympathetic nervous system neurotransmitter, stimulates ghrelin release suggesting ghrelin release is under some neural control (Gagnon and Anini, 2012). Hormones including somatostatin, CCK, GLP-1 and insulin are known to inhibit its release (Stengel et al., 2010). L-cysteine did not affect GLP-1 release and a CCK-1 receptor antagonist did not attenuate the anorectic effect of L-cysteine, suggesting neither GLP-1 nor CCK is involved. It is, of course, possible that the L-cysteine-induced reduction in ghrelin does not drive the reduction in food intake, and alternative mechanisms are involved. Nevertheless, the mechanism by which L-cysteine suppresses acyl-ghrelin release remains of interest and the fact that both oral and intraperitoneal administration leads to a suppression would suggest it may be a post-absorptive mediated process.

The field of G-protein coupled nutrient sensing receptors has grown over the past 15 years, with the deorphanization of a number of receptors. These receptors hold the potential to act as sensors of food intake and regulators of hormone release and appetite (Wellendorph et al., 2009a). L-cysteine has activity at the mouse orthologue of the three amino acid sensing receptors, GPRC6A, CaSR and T1R1/T1R3, and rat and human orthologues of GPRC6A (Wellendorph et al., 2005, Wellendorph et al., 2007, Conigrave et al., 2000, Nelson et al., 2002). The promiscuous amino acid receptors reportedly respond specifically to L-amino acids and not to the D-enantiomers. L-cysteine but not D-cysteine reduced food intake in rats. However, in mice, both L- and D-cysteine reduced food intake to a similar extent. This highlights species differences, in the response to D-cysteine. The mouse orthologues of these receptors are the best characterized with respect to amino acid

enantiomer specificity, and thus given the lack of stereo-selectivity in mice, it is unlikely that one or more of these receptors are mediating the effect of L-cysteine on food intake. However, it is possible that D-cysteine mediates its anorectic effect through a different mechanism to L-cysteine in mice. Furthermore, L-cysteine reduced food intake in GPRC6A knockout mice to the same extent as in wildtypes, suggesting that this receptor is not necessary for the effect. In addition, other amino acid ligands that are more potent activators of the GPRC6A and CaSR did not significantly affect food intake.

In addition to the promiscuous amino acid sensing G-protein coupled receptors, L-cysteine is also considered an excitatory amino acid that can have biological effects through the NMDA receptor (Pace et al., 1992, Parsons et al., 1998). This receptor is known to be involved in specific appetite pathways (Burns and Ritter, 1997, Guard et al., 2009), and therefore its role in mediating the effect of L-cysteine on food intake was also investigated in this thesis. The effect of central administration of L-cysteine on food intake and behaviour was completely blocked by an NMDA receptor antagonist. However as discussed earlier, it would appear unlikely that cysteine mediates its anorectic effect through changes in central levels as no behavioural side effects are observed following peripheral administration of effective doses, and all centrally administered 'anorectic' doses were associated with side effects. The anorectic effect of peripheral administration of L-cysteine was not blocked by NMDA receptor antagonism, suggesting NMDA receptor signalling is not necessary for the anorectic effect of L-cysteine.

Having established that L-cysteine reduces food intake, increases neuronal activation in the brainstem and suppresses plasma acyl-ghrelin levels, I hypothesised that L-cysteine was having an effect in the stomach that resulted in neural signals to the brainstem to reduce food intake. L-cysteine significantly decreased gastric emptying, and thus may reduce food intake secondary to gastric distension. Furthermore, c-Fos expression in the medial NTS is quantitatively related to the magnitude of gastric distension (Rinaman et al., 1998). Additionally, gastric distension activates catecholaminergic neurons (Rinaman et al., 1998) similarly to high protein feeding (Faipoux et al., 2008) as previously discussed. Gastric distension is predominantly communicated to the brainstem via vagal afferents, thus to investigate whether the gastric distension was inducing the hypophagia following L-cysteine administration, the effect of L-cysteine on food intake was investigated in rats that had

undergone subdiaphragmatic vagal deafferentation. However, L-cysteine reduced food intake in rats that had undergone subdiaphragmatic vagal deafferentation to the same extent as in sham operated rats, suggesting that vagal afferents were not critical to the effect of L-cysteine on food intake, and that the associated gastric distension was unlikely to be an important factor in the hypophagia. Peptone, a protein hydrolysate, is known to delay gastric emptying through mechanisms which do not involve vagal afferents or the CCK-1 receptor (Forster et al., 1990), and it is possible that L-cysteine utilises the same or similar pathways to exert its effect on gastric distension. Additionally, although the vagus nerve has been implicated in protein induced satiety other mechanisms are also involved as evidenced by the protein-induced hypophagia observed in vagotomised rats (L'Heureux-Bouron et al., 2003).

Although the exact mechanism by which L-cysteine modulates food intake is unclear, it is clear that it has a robust effect. To determine whether this reduction could be maintained and provide a potential therapeutic strategy, the effect of repeated administration on food intake and body weight was investigated. In both lean rats and DIO mice, repeated administration of L-cysteine significantly reduced food intake. However, this reduction in food intake did not drive a reduction in body weight. The duration of these studies were relatively short, thus it may be that detectable changes in bodyweight would occur in studies of a longer duration. However, as shown in these studies, cysteine can be toxic at high doses and thus long term repeated administration may not be advisable. However, identifying the mechanism by which L-cysteine reduces food intake may provide a pharmacological target. Additionally, cysteine rich proteins may provide a safer therapeutic strategy (McPherson and Hardy, 2011), and may also avoid the possibility of amino acid imbalance that may occur with the administration of a single amino acid (Harper et al., 1970). Supplementation with the cysteine rich protein α-lactalbumin whey concentrate or the L-cysteine derivative N-acetyl-cysteine alleviates high sucrose diet induced oxidative stress and insulin resistance in rats (Blouet et al., 2007). Additionally, the antioxidant potential of cysteine rich proteins may be beneficial for inflammatory diseases (McPherson and Hardy, 2011) which include obesity. L-cysteine did not significantly affect body weight when given to lean rats or DIO mice. In a similar manner to the high sucrose study, it may be interesting to investigate whether administration of L-cysteine could protect against diet

induced obesity upon exposure to an obesogenic diet such as the 60% high fat diet used in chapter 3. Additionally, one study has investigated the effect of a normal chow diet supplemented with L-cysteine on certain metabolic parameters (Lee et al., 2013). Supplementation with 2% L-cysteine decreased food intake, fat mass, body weight, and serum and liver triglyceride levels. However, in this study supplementation with L-cysteine may have changed the palatability of the diet, which might be responsible for the decreased food intake, with all additional effects being secondary to this reduction (Lee et al., 2013).

Whilst L-cysteine decreased food intake to the greatest extent of all the amino acids tested in our preliminary studies, it must be noted that a recently published study also examined the effect of oral gavage administration of the 20 proteinogenic amino acids on food intake in rats. This study identified L-arginine, L-lysine and L-glutamic acid as the most anorectic amino acids when compared at a dose of 6.7mmol/kg (Jordi et al., 2013). Surprisingly, L-cysteine at a dose of 6.7mmol/kg did not significantly affect food intake in their study. Interestingly, L-arginine, L-lysine and L-glutamic acid did not significantly affect food intake when given at 4mmol/kg in this study, the dose used in our preliminary studies. L-arginine and L-lysine are basic amino acids and L-glutamic acid is acidic. Although not presented in this thesis, additional studies performed by myself during this project suggest L-arginine does not reduce food intake to a significant level at doses equal to or lower than 8mmol/kg when the pH is neutralised (Appendix 2). Thus, with regard to the anorectic effect of L-cysteine and L-arginine, there are discrepancies between results presented in this thesis and those published by Jordi *et al*. It is not unheard of for these kinds of discrepancies to occur (Woods and Langhans, 2012). However, it must be noted that the effect of oral gavage administration of L-cysteine on food intake was consistent in multiple cohorts of rats and in experiments performed at more than one institution.

In summary, this thesis has identified L-cysteine as an amino acid with potent anorectic effects. Although the exact mechanism by which L-cysteine reduces food intake has not been determined, I have demonstrated that L-cysteine supresses plasma acyl-ghrelin levels and delays gastric emptying, both of which may contribute towards L-cysteine induced hypophagia. Additionally, L-cysteine increases c-Fos in the area postrema and nucleus tractus solitarius, suggesting it may be mediating its anorectic effects through the brainstem (Fig. 4.1). Considering that vagal afferents do not appear to be essential for the anorectic

effects of L-cysteine, then L-cysteine may be mediating its effect through the area postrema and signals generated here may activate neurons in the nucleus tractus solitarius and additional higher brain regions. With regard to advancing the work presented in this thesis, future work should focus on identifying the specific neuronal populations activated within the brainstem following L-cysteine administration, and the mechanisms by which L-cysteine suppresses acyl-ghrelin and delays gastric emptying. The role of the aforementioned hormones, insulin and somatostatin, should be investigated as a possible link between L-cysteine and the regulation of ghrelin release. Additionally, *in vitro*/*ex vivo* studies investigating whether L-cysteine can directly modulate acyl ghrelin release would also be of considerable interest.

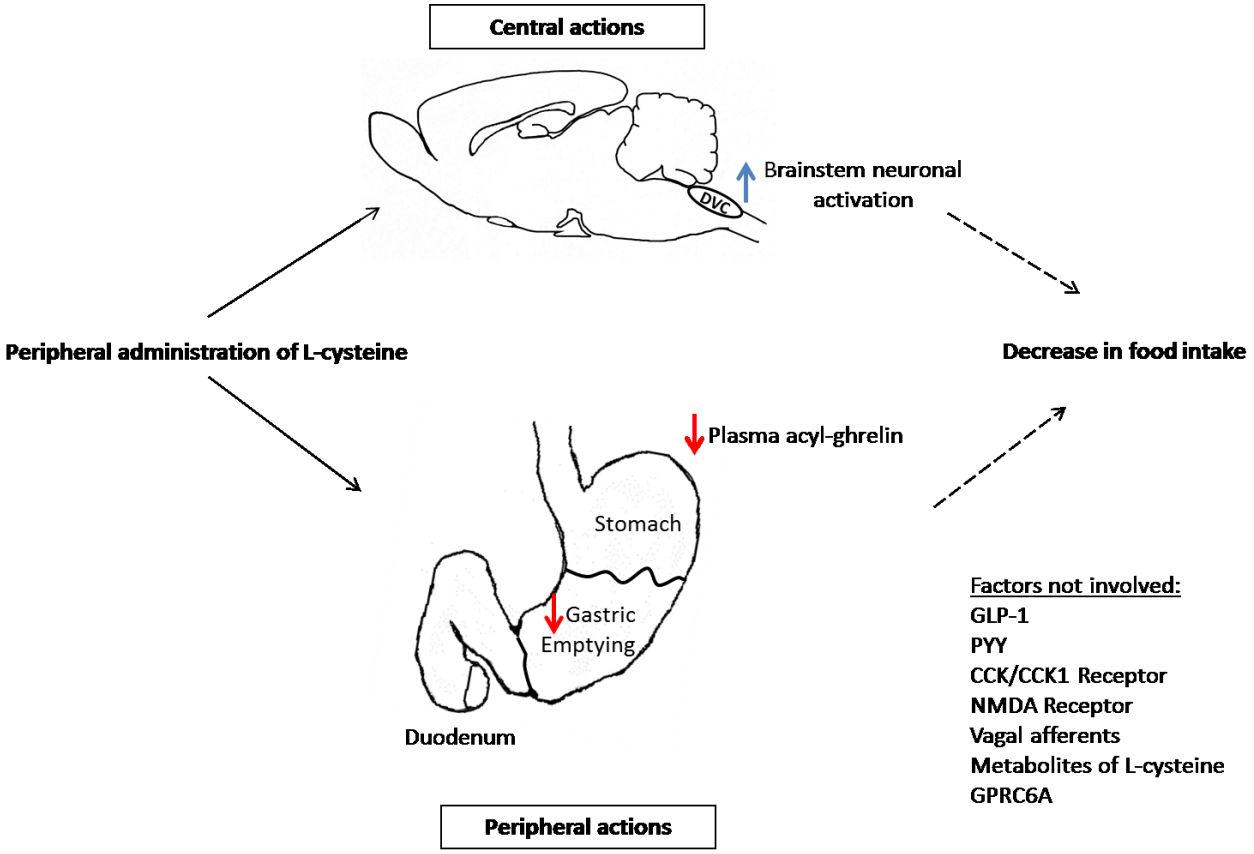


Figure 4.1 – Summary diagram.
Peripheral administration of L-cysteine delays gastric emptying, suppresses plasma acyl-ghrelin levels and increases neuronal activation in the area postrema and nucleus tractus solitarius of the brainstem, and decreases food intake. CCK: Cholecystokinin, DVC: Dorsal vagal complex, GLP-1: Glucagon-like peptide-1, GPRC6A: G-protein coupled receptor group C member 6A, NMDA: N-methyl-D-aspartate, PYY: Peptide YY

The general aim of this thesis was to contribute to our understanding of protein induced satiety by investigating the role of amino acids in appetite regulation. The anorectic effects of amino acids such as L-leucine are well documented, and the studies described in this thesis introduce the anorectic effects of L-cysteine. It is clear that no single amino acid is responsible for mediating protein induced satiety and it is likely that different amino acids exert different effects on appetite through a number of mechanisms, the combination of which contributes towards the success of high protein diets on body weight and appetite. Future work should focus on identifying the amino acids that can suppress appetite either acutely or chronically and the mechanisms by which they mediate their effects. This work could help inform the identification of combinations of amino acids that have the greatest anorectic potential and ultimately a combination that could provide a safe and practical therapeutic treatment for obesity.

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Appendix 1

Solutions

Antifreeze

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

Blocking Solution

3% (v/v) goat serum 0.25% (v/v) Triton X in 0.01M PBS

Dextran coated charcoal

2.4g charcoal and 0.24g dextran dissolved in 100ml phosphate buffer with gelatine.

H_20_2 (0.6%)

0.6% H_20_2 (v/v) in methanol.

Phosphate buffer (0.06M)

48g Di-sodium Hydrogen Phosphate ($Na_2HPO_4.2H_20$), 4.14g Potassium Dihydrogen Phosphate (KH_2PO_4), 18.61g EDTA ($(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$), 2.5g Sodium Azide ($NaN_3$) were dissolved in 5l of cooled boiled GDW, pH 7.29, this was stored at 4°C.

Phosphate buffer with gelatine

As above, with 12.5g gelatine.

Phosphate buffered saline (PBS) (1M)

87g Sodium chloride (NaCl), 14.1g Di-sodium Hydrogen Phosphate (Na_2HPO_4) and 2.72g Potassium Dihydrogen Phosphate KH_2PO_4 in 1L GDW.

Phosphate buffered saline (PBS) (0.01M)

A 1:100 dilution of 1M PBS as prepared above.

Sucrose solution (40%)

400g of sucrose was dissolved in 1L GDW

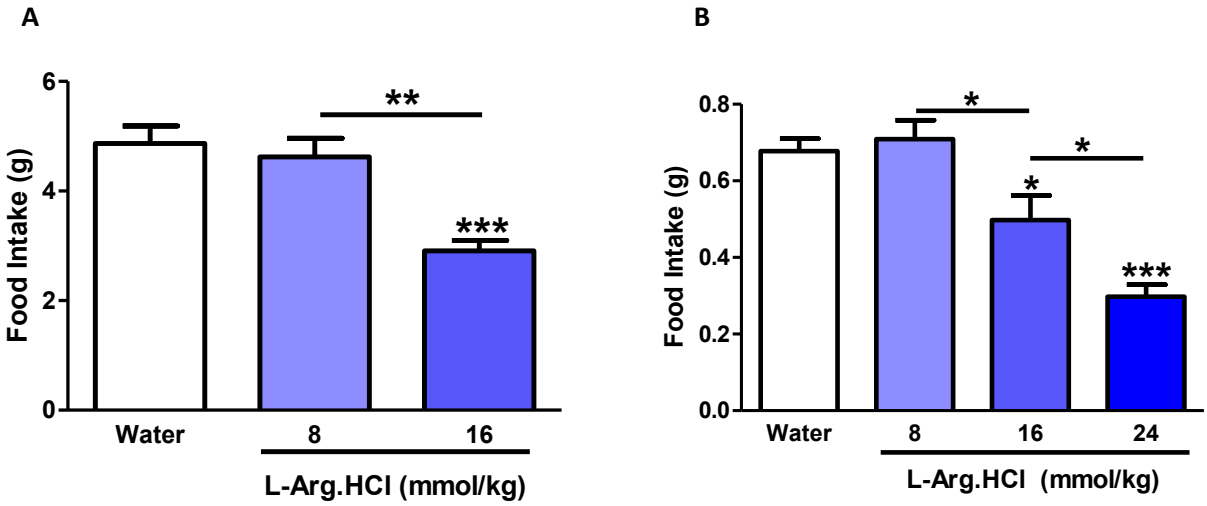
Tris/HCl solution (0.1M) (pH 7.6)

12.1g Trizma base in 1L GDW, pH 7.6 with HCl.

Veronal Buffer

5.15g Sodium Barbitone ($C_8H_{11}N_2NaO_3$) and 0.15g Sodium Azide (NaN_3) dissolved in 0.5L boiled GDW.

Appendix 2



Appendix 2 The effect of OG administration of L-Arginine.HCl on 0-1 hour food intake in rats and mice.

The effect of oral gavage administration of water, 8 or 16mmol/kg L-Arg.HCl on food intake following an overnight fast in rats (n=9-10) (A). The effect of oral gavage administration of water, 8, 16 or 24mmol/kg L-Arg.HCl on food intake following an overnight fast in mice (n=9-10) (B). *p<0.05, **p<0.01, ***p<0.001