

The effect of L-cysteine on appetite in humans

A thesis submitted for the degree of
Master of Philosophy in Imperial College
London

Dr. Anjali Amin
2013

Department of Medicine
Division of Diabetes, Endocrinology &
Metabolism
Section of Investigative Medicine

Declaration of Originality

All the work described in this thesis is my own work. Anything outside of my own work has been appropriately referenced.

The human studies were carried out in conjunction with Dr Amy Agahi.

All in-house radioimmunoassays were established and maintained by Professor M. Ghatei (Section of Investigative Medicine).

Copyright declaration

“The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work”

Abstract

High protein diets suppress appetite and facilitate weight loss. However, they are difficult to adhere to. Understanding the mechanisms by which protein suppresses appetite may establish targets for more acceptable interventions to treat or prevent obesity.

Receptor systems that respond to amino acids, the products of protein digestion, have been identified. However, the specific mechanisms regulating protein-induced satiety are unknown. Previous work within our laboratory has investigated the effect of specific amino acids which act as ligands for the following G-protein coupled receptors: CaR, T1R1/T1R3 and GPRC6A on food intake in rodents.

L-cysteine activates the CaR, the T1R1/T1R3 and the GPRC6A. A diet that includes high levels of whey protein, which contains high levels of L-cysteine, has been reported to be more satiating and to suppress circulating levels of the orexigenic hormone ghrelin to a greater extent than other types of protein in humans. Pilot studies suggested that ligands for the GPRC6A receptor can reduce food intake in rodents, and that this effect is at least partly mediated by a reduction in circulating ghrelin levels.

The putative role of L-cysteine in food intake in humans was investigated. The effect of a high protein meal on circulating levels of cysteine was determined. Subsequently, the time course of changes in the circulating levels of L-cysteine following oral consumption of L-cysteine was established. Finally, the effects of different doses of cysteine on subjective assessment of appetite and acute food intake were investigated. These studies suggested that oral administration of L-cysteine at 0.04 or 0.07g/kg resulted in supraphysiological levels of L-cysteine in circulation. Oral administration of L-cysteine significantly influenced subjective markers of appetite compared to vehicle or glycine controls, but did not affect food intake. These data suggest that L-cysteine may be suitable for manipulating appetite in humans.

Acknowledgements

Firstly I would like to thank my supervisor Dr Kevin Murphy for his invaluable support and guidance.

I am also grateful to Professor Stephen Bloom for allowing me to work in his department.

I would also like to thank Dr Amy Agahi for her help with the human studies and Anne McGavigan for her input.

Finally, I would like to thank my family for their constant support.

Abbreviations

AgRP	Agouti-related protein
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AP	Area postrema
ARC	Arcuate nucleus
AST	Aspartate aminotransferase
AUC	Area under the curve
BMI	Body mass index
CaR	Calcium-sensing receptor
CCK	Cholecystokinin
CCK-A	Cholecystokinin-A
CNS	Central nervous system
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acid
GGT	Gamma-glutamyl transpeptidase
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GPCR	G-protein-coupled receptor
GPRC6A	G-protein-coupled receptor family C, group 6, subtype A
I-cell	Enteroendocrine cell
L-cell	Enteroendocrine cell
LCFA	Long-chain fatty acid
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OXM	Oxyntomodulin
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
RP-HPLC	Reverse phase high pressure liquid chromatography
SEM	Standard error of the mean

T1R1/T1R3	Taste receptor type 1 member 1/Taste receptor type 1 member 3
VAS	Visual analogue scale
WHO	World Health Organisation

Table of Contents

Declaration of Originality.....	2
Copyright declaration.....	3
Abstract	4
Acknowledgements.....	5
Abbreviations.....	6
Table of Contents	8
List of figures	10
List of tables	11
CHAPTER 1: INTRODUCTION	12
1.1. Background.....	13
1.2. Energy homeostasis.....	13
1.3. Nutrient sensing	16
1.3.1. The role of the hypothalamus in central nutrient sensing	16
1.3.2. The role of the gut in peripheral nutrient sensing.....	16
1.3.3. Carbohydrate sensing.....	17
1.3.4. Lipid sensing.....	17
1.4. Protein and satiety	18
1.5. Amino acid sensing receptors	20
1.6. Pilot data from our laboratory	21
1.7. Hypotheses	22
1.7.1. Part A:.....	22
1.7.2. Part B:.....	22
1.7.3. Part C:	22
1.8. Aims	22
1.8.1. Part A:.....	22
1.8.2. Part B:.....	23
1.8.3. Part C:	23
CHAPTER 2: MATERIALS & METHODS	24
2.1. Amino acids.....	25
2.2. Subjects	25
2.2.1. Subject recruitment.....	25

2.2.2. Inclusion criteria.....	26
2.2.3. Exclusion criteria	26
2.3. Part A: Effect of a high protein meal on circulating levels of amino acids in healthy human subjects	27
2.4. Part B: Effect of oral amino acid administration on circulating levels of specific amino acids.....	29
2.5. Part C: Effect of specific oral amino acid administration on <i>ad libitum</i> food intake	31
2.6. Amino acid assay	35
2.7. Plasma hormone assays	35
2.7.1. PYY	35
2.7.2. GLP-1	36
2.7.3. PP.....	37
2.7.4. Ghrelin	37
2.8. Statistical analysis.....	38
CHAPTER 3: RESULTS	39
3.1.1. Amino acid analysis.....	40
3.1.2. VAS analysis	41
3.2.1. Amino acid analysis.....	44
3.2.2. VAS analysis	44
3.3.1. VAS analysis	47
3.6.1. Ad libitum meal	54
3.6.2. Food diary analysis.....	54
CHAPTER 4: DISCUSSION	56
APPENDIX I: Plasma concentrations of amino acids following a high protein meal	72

List of figures

Figure 1.1. The pathways by which gut hormones regulate energy homeostasis. ...	14
Figure 2.1. A schematic representation of a study day for Part A of “The effect of L-cysteine on appetite in humans”.....	28
Figure 2.2. A schematic representation of the time-course of Part B of the study “The effect of L-cysteine on appetite in humans”.....	30
Figure 2.3. A schematic representation of a study day for Part B of “The effect of L-cysteine on appetite in humans”.....	31
Figure 2.4. A schematic representation of the time-course of Part C of the study “The effect of L-cysteine on appetite in humans”.....	33
Figure 2.5. A schematic representation of a study day for Part C of the study “The effect of L-cysteine on appetite in humans”.....	35
Figure 3.1. Plasma cysteine concentrations ($\mu\text{mol/L}$) following a high protein meal.	41
Figure 3.2. Visual analogue scale ratings following a high protein meal.	42
Figure 3.3. Visual analogue scale ratings following a high protein meal.	43
Figure 3.4. Plasma cysteine concentrations ($\mu\text{mol/L}$) following administration of cysteine.	44
Figure 3.5. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.....	45
Figure 3.6. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.....	46
Figure 3.7. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.....	47
Figure 3.8. Analysis of VAS ratings of 7 subjects following administration of vehicle, higher dose of glycine or cysteine.	48
Figure 3.9. Analysis of VAS ratings of 7 subjects following administration of vehicle, higher dose of glycine or cysteine.	49
Figure 3.10. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.....	50
Figure 3.11. Analysis of gut hormones of 5-6 subjects following administration of vehicle, glycine or cysteine.....	51
Figure 3.12. Analysis of gut hormones of 5-6 subjects following administration of a higher dose of vehicle, glycine or cysteine.	53
Figure 3.13. Effect of vehicle, glycine and cysteine on <i>ad libitum</i> food intake.	54
Figure 3.14. Effect of vehicle, glycine and cysteine on 3 day food intake.....	55
Figure 4.1. L-Amino acid selectivity profiles at CaR, GPRC6A, and the T1R1/T1R3 heterodimer.	61

List of tables

Table 2.1. Macronutrient content for study meals used in Part C of the study “The effect of specific amino acids on appetite”.....	14
Table 3.1. Baseline characteristics of the 12 participants recruited for Parts A-C.	36

Chapter 1

Introduction

1.1. Background

Obesity, its complications and the associated mortality are major global public health issues. The World Health Organisation (WHO) has declared that global childhood and adult obesity levels have reached epidemic proportions. Once considered a problem only in developed countries, obesity is now also on the rise in developing countries (WHO, 2012). It is predicted that up to 58% of the world's adult population will be overweight or obese by 2030 (Kelly et al., 2008). Worldwide, 2.8 million people die annually as a result of being overweight and an estimated 35.8 million (2.3%) of global disability-adjusted life years are caused by overweight or obesity (WHO, 2009). Obesity is associated with cardiovascular disease, type 2 diabetes and certain forms of cancer, and the rise in the prevalence of obesity has consequently been paralleled by rises in these chronic diseases. The Foresight report highlighted appetite regulation as a major target in the dietary treatment of obesity (Butland, 2008). Of particular interest is the concept of functional foods or novel products, which aim to potentiate satiety. The goal is to design foods or dietary regimens that cause an increased sense of fullness and encourage the individual to stop eating sooner, thus reducing total energy intake (Hill and Peters, 2002).

1.2. Energy homeostasis

Various homeostatic mechanisms exist to maintain a balance between food intake and energy expenditure. Energy homeostasis is a result of coordination of central neural signals and peripheral neural and hormonal signals, as shown in Figure 1.1. Stability of body weight is achieved by maintaining a balance between food intake and energy expenditure.

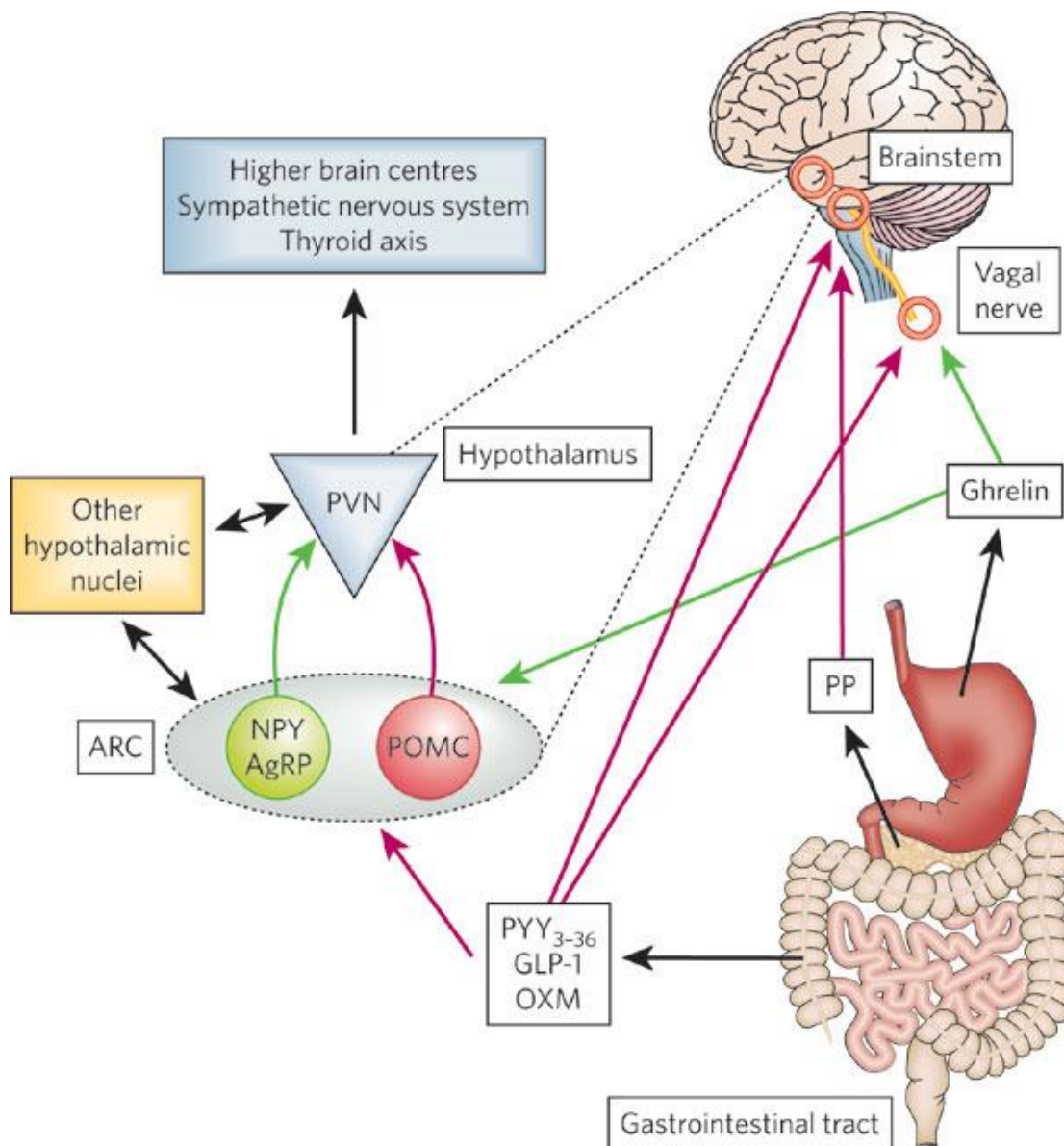


Figure 1.1. The pathways by which gut hormones regulate energy homeostasis.

PVN: paraventricular nucleus; ARC: arcuate nucleus; NPY: neuropeptide Y; AgRP: Agouti-related protein; POMC: pro-opiomelanocortin; PP: pancreatic polypeptide; PYY: peptide tyrosine tyrosine; GLP-1: glucagon-like peptide-1; OXM: oxyntomodulin. From: Murphy KG & Bloom SR (2006) Gut hormones and the regulation of energy homeostasis. *Nature* 444: 854-9 (Murphy and Bloom, 2006)

The hypothalamus and the brain stem are the major brain regions involved in the regulation of food intake and energy expenditure. The hypothalamus is located at the base of the brain just below the thalamus, where it receives afferent signals from the periphery and from other parts of the central nervous system (CNS). The brain stem is at the posterior of the brain, and is structurally continuous with the spinal cord. The brain stem contains the dorsal vagal complex, which receives neural innervations from the gastrointestinal tract, and includes the area postrema (AP), a part of the brainstem which lies outside the blood-brain barrier and hence is a direct target for peripheral satiety signals. Complex neuronal circuitry exists between the brainstem and hypothalamus.

Signalling from the periphery to these brain centres can thus be in the form of neural or hormonal signals. Vagal neural pathways mediate specific chemosensory responses from the gut. Animal and human studies show that inhibiting these pathways via vagotomy or afferent denervation can attenuate the inhibition of gastric emptying and food intake which takes place when nutrients enter the gastrointestinal tract (Wilkinson and Johnston, 1973, Roze et al., 1977, Raybould and Holzer, 1992, Schwartz et al., 1993). Intestinal nutrients initiate signals from gut epithelial cells via vagal afferent endings, with subsequent effects on the central nervous system. These signals activate gastric vagal motor neurons, which cause relaxation of the proximal stomach (Horowitz and Dent, 1991, Azpiroz and Malagelada, 1987), inhibit antral contractions, and stimulate tonic and phasic pyloric pressures.

Hormonal signals are coordinated centrally along with neural signals from the gut (Schwartz et al., 2000). Gut hormones can act via vagal pathways from the gastrointestinal tract to the brain (Schwartz, 2000, Moran, 2000), or can act directly on central neurones (Kalra et al., 1999, Cone et al., 2001). Meal anticipation and the presence of nutrients in the upper part of the gut stimulate gut hormone and neurotransmitter release (Schwartz, 2000).

1.3. Nutrient sensing

Recent research has highlighted the importance of the gut-brain axis and nutrient sensing in the regulation of food intake and metabolism (Stanley et al., 2005, Chaudhri et al., 2006, Badman and Flier, 2005). The body's nutrient sensors have multiple functions, including the detection of nutrient availability and the regulation of post-absorption processes. Sensing of nutrients in the gut and the brain may also play a role in regulating food intake (Blouet and Schwartz, 2010, Dyer et al., 2003, Raybould, 2007, Leyer et al., 1995, Dumoulin et al., 1998).

1.3.1. The role of the hypothalamus in central nutrient sensing

The central nervous system has been suggested to act as a rheostat of acute nutrient availability (Blouet and Schwartz, 2010). The hypothalamus contains a high concentration of nutrient-sensing elements and is thought to be the main centre for coordination of nutrient-related signals. Some of the key hypothalamic nuclei, namely the arcuate, ventromedial and lateral nuclei, are thought to respond specifically to glucose, fatty acids, amino acids and other fuel-related stimuli. Within these neuronal populations, nutrients can act as signalling molecules to directly elicit neurochemical responses which influence energy intake and nutrient utilisation (Blouet and Schwartz, 2010). The arcuate nucleus-median eminence area is incompletely isolated from the circulation by the blood-brain barrier, allowing arcuate neurons to directly respond to circulating nutrients. The arcuate nucleus is therefore thought to represent an important area for central nutrient sensing.

1.3.2. The role of the gut in peripheral nutrient sensing

Peripheral nutrient sensing can occur in the gut, liver or portal circulation, and the resulting signals can be transmitted to the brain via neural signalling and/or the release of gastrointestinal hormones. Specific nutrients are detected peripherally to inhibit food intake in both humans and animal models (Greenberg et al., 1990, Matzinger et al., 2000). Chemosensory cells present in the gut epithelial lining include enterocytes, brush cells and enteroendocrine cells, all of which have direct contact with the intraluminal contents. Enteroendocrine cells play a specialised role

in luminal nutrient sensing, although they represent less than 1% of epithelial cells within the gut. Peptide hormones are released from secretory granules located in the basal cytoplasm of this cell type (Hofer et al., 1999). These cells are often situated deeper within the gut wall than enterocytes, and many have processes which extend to the intestinal lumen via which they can directly sense luminal contents. Those enteroendocrine cells which do not extend to the epithelial surface are controlled indirectly by the luminal content via neural and hormonal signals, and directly by circulating nutrients absorbed from the gut (Sternini et al., 2008).

1.3.3. Carbohydrate sensing

Carbohydrates consumed in the diet are digested to form sugars (glucose, fructose, and galactose) that are absorbed via enterocytes in the gut (Wright et al., 2003). Sugar type and rate of digestion have a significant effect on nutrient sensing pathways. Digestion and absorption of ingested carbohydrate is coordinated by sugar sensing in the gastrointestinal tract, which regulates nutrient absorption, hormone release and gut motility, and which sends satiation signals to the brain (Dyer et al., 2003, Raybould, 2007). Evidence from human studies demonstrates that a centrally detected drop in blood glucose can alter peripheral glucose management. This is mediated by mechanisms including the secretion of counter-regulatory hormones from the pancreas, the release of catecholamines from the adrenal glands (Mitrakou et al., 1991, Taborsky et al., 1998), and the activation of the autonomic nervous system to drive hepatic glucose production (Perseghin et al., 1997). Central infusion of glucose directly into the hypothalamus reduces food intake and body weight (Davis et al., 1981, Panksepp and Rossi, 1981), suggesting that central glucose sensing may also play a role in appetite regulation.

1.3.4. Lipid sensing

Following a meal, fat is digested to form fatty acids and monoglycerides, which are then absorbed. The length of the hydrocarbon chain in a fatty acid determines whether it is described as short (tails of <6 carbons), medium (6-12 carbons) or long (13-21 carbons). Short and medium-chain fatty acids enter the hepatic portal circulation directly through the intestinal capillaries, but long-chain fatty acids

(LCFAs) enter the systemic circulation via the lymphatic system, bypassing the portal circulation, and hence their systemic concentrations are more representative of acute lipid intake. Increasing the concentration of LCFAs within the central nervous system, and particularly within the hypothalamus, inhibits both food intake and hepatic glucose production (Lam et al., 2005, Caspi et al., 2007). The presence of lipids in the gut alters gut hormone release. LCFAs sensed in the gut stimulate the secretion of cholecystokinin (CCK) from duodenal I-cells (Moran and Kinzig, 2004). CCK delays gastric emptying, stimulates contraction of the gall-bladder to release of bile acids into the duodenum, and stimulates pancreatic enzyme production and secretion, allowing the digestion and absorption of dietary lipids (Cheung et al., 2009). CCK also increases satiation via CCK-A receptors on vagal afferents which signal via the nucleus of the solitary tract (NTS) in the brainstem (van de Wall et al., 2005). LCFAs also stimulate the release of the anorectic gut hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) from human enteroendocrine cells (Stoeckel et al., 2008). In addition, oral administration or direct gut administration of free fatty acids (FFAs) causes a rise in GLP-1 and insulin levels in rodent models, a process which may be mediated free fatty acid receptors (Hirasawa et al., 2005). Recent evidence shows duodenal lipid sensing can activate a gut-brain-brown adipose tissue axis to regulate thermogenesis (Blouet and Schwartz, 2012).

1.4. Protein and satiety

Protein has a satiating effect greater than other macronutrients (Porrini et al., 1997, Reid and Hetherington, 1997, Trigazis et al., 1997, Bensaid et al., 2002, Anderson and Moore, 2004, Halton and Hu, 2004). High protein diets inhibit food intake, facilitate weight loss and improve body composition in animal models and humans (Potier et al., 2009, Harper and Peters, 1989, Jean et al., 2001, Morens et al., 2000, Morens et al., 2001, Hannah et al., 1990, Kinzig et al., 2007). These effects are not secondary to changes in palatability; studies have demonstrated that ingestion of high protein diets does not cause conditioned taste aversion and that protein ingestion leads to typical behavioural satiety sequences (Harper and Peters, 1989,

Bensaid et al., 2003). However, high protein diets are difficult to adhere to (Sargrad et al., 2005), and the mechanisms by which a high protein diet regulates food intake are largely unknown.

There is evidence that protein influences gastrointestinal hormones to alter satiety. Protein has been reported to increase levels of anorectic gut hormones (Hall et al., 2003, Calbet and Holst, 2004, Nilsson et al., 2004, Batterham et al., 2006). Following a meal, intestinal L-cells release the gut hormones GLP-1, PYY and oxyntomodulin (OXM) which act on central appetite centres to control eating (Stanley et al., 2004). A high protein meal increases circulating concentrations of both PYY and GLP-1 in normal weight humans (van der Klaauw et al., 2012). High protein meals are associated with significantly higher PYY responses in both lean and obese humans when compared to high-fat and high-carbohydrate diets (Batterham et al., 2006). Ghrelin is an orexigenic peptide hormone primarily synthesised in the stomach (Kojima et al., 1999). There is conflicting evidence regarding the effect of protein on ghrelin. Circulating ghrelin levels increase with fasting, and decrease post-prandially (Cummings et al., 2001, Tschop et al., 2001), and ghrelin administration increases food intake in both animals and humans (Wren et al., 2001, Wren et al., 2000). Some reports have suggested that protein ingestion has a smaller suppressive effect on plasma ghrelin concentrations than ingestion of other macronutrients (Erdmann et al., 2003, Greenman et al., 2004). However, more recent studies provide strong evidence that protein can suppress post-prandial circulating ghrelin levels more potently and/or for longer than carbohydrates (Al Awar et al., 2005, Blom et al., 2006, Tannous dit El Khoury et al., 2006, Bowen et al., 2006a, Bowen et al., 2006b, Leidy et al., 2007, Foster-Schubert et al., 2008). The reasons for these discrepancies remain unclear, but may be related to protein composition.

1.5. Amino acid sensing receptors

There are three members of the G-protein coupled receptor (GPCR) family class C known to promiscuously bind a number of L-amino acid ligands, and thus suited to detecting the varied products of protein digestion. These receptors are the calcium-sensing receptor (CaR), the T1R1/T1R3 heterodimeric receptor and the GPRC6A receptor.

The primary ligands of the CaR are calcium ions, but the CaR can also respond to L-amino acids, in particular the aromatic amino acids. The global CaR knockout mouse has a severe phenotype which includes severely dysfunctional calcium homeostasis, retarded growth and premature death (Kuang et al., 2005), making it difficult to establish whether the CaR has a physiological role in energy homeostasis. More recently, a gut-specific CaR knockout mouse has been developed, which suggests the CaR plays a role in regulating cell growth and proliferation in the gut (Rey et al., 2012)

The functional units of the T1R1 receptor class are dimers comprised of two of three different subunits: T1R1, T1R2, and T1R3. The T1R1/T1R3 heterodimer acts as an L-amino acid taste receptor and is broadly activated by aliphatic amino acids. The T1R1/T1R3 heterodimer has been identified in the gut, where it can act as a luminal sensor for certain L-amino acids, resulting in release of gut hormones (Daly et al., 2012). However, targeted deletion of the T1R1 or the T1R3 receptor in the mouse on a normal diet does not influence body weight or food intake (Zhao et al., 2003).

GPRC6A is a recently cloned receptor expressed in a number of tissues, including the brain, where it is found in the hypothalamus (Wellendorph et al., 2009, Kuang et al., 2005, Wellendorph et al., 2007, Wellendorph and Brauner-Osborne, 2004, Pi et al., 2005). Mice lacking GPRC6A have a complex metabolic phenotype that includes alterations to the reproductive axis, glucose homeostasis, and kidney and bone function (Pi et al., 2008). Interestingly, GPRC6A knockout mice have hepatic steatosis and a significantly higher percentage body fat than wild-type littermates,

suggesting that GPRC6A may regulate energy homeostasis and adiposity. The GPRC6A has been proposed as a nutrient sensing receptor (Wellendorph et al., 2009), and has recently been identified in the gastrointestinal tract (Haid et al., 2011a).

1.6. Pilot data from our laboratory

Receptor systems that respond to amino acids, the products of protein digestion, have been identified. However, the specific mechanisms regulating protein-induced satiety are unknown. Previous work within our laboratory has investigated the effect of specific amino acids which act as ligands for the CaR, T1R1/T1R3 and GPRC6A on food intake in rodents.

The GPRC6A is responsive to between 6 and 8 of the proteinogenic amino acids, including cysteine and lysine (Kuang et al., 2005, Wellendorph et al., 2005, Christiansen et al., 2007, Wellendorph et al., 2007). It does not respond to leucine, which has previously been suggested to play a role in protein induced satiety (Ropelle et al., 2008, Blouet et al., 2008). Cysteine is a non-essential amino acid found in high protein foods, including cereals, whey protein, and meat and dairy products. Cysteine activates the CaR, the T1R1/T1R3 and the GPRC6A (Christiansen et al., 2007, Nelson et al., 2002, Conigrave et al., 2000). A diet that includes high levels of whey protein has been reported to be more satiating and to suppress circulating ghrelin levels to a higher extent than other types of protein in humans (Bowen et al., 2007, Veldhorst et al., 2009).

Pilot studies suggested that ligands for the GPRC6A receptor can reduce food intake in rodents, and that this effect is at least partly mediated by a reduction in circulating ghrelin levels.

Oral administration of cysteine significantly reduced food intake in rats. The effect of cysteine on food intake appeared specific, as behavioural analysis showed that high

doses of cysteine did not alter behaviour, other than to reduce feeding behaviour. Oral administration of cysteine significantly suppressed circulating levels of the gastric hormone ghrelin, but had no effect on circulating levels of insulin, PYY or GLP-1.

The studies described in this thesis investigated the putative role of cysteine in food intake in humans. I wanted first to determine the circulating levels of cysteine achieved following a high protein meal, to better place subsequent cysteine administration studies in a physiological context. These subsequent studies would then establish the effect of cysteine on subjective appetite and food intake.

1.7. Hypotheses

1.7.1. Part A:

A high protein meal will cause a time-dependent increase in circulating levels of specific amino acids.

1.7.2. Part B:

Oral administration of cysteine will cause a time-dependent increase in circulating levels of cysteine.

1.7.3. Part C:

In normal and overweight subjects, increasing doses of cysteine will acutely reduce hunger and food intake in a dose-dependent fashion compared to controls.

1.8. Aims

1.8.1. Part A:

To determine the time course of the increase in circulating levels of cysteine following a high protein meal.

1.8.2. Part B:

To determine the time course of the increase in the circulating levels of cysteine after oral consumption.

To determine the effect of different doses of cysteine on subjective assessment of appetite, using visual analogue scales.

To determine the effect of different doses of cysteine on circulating levels of gut hormones.

1.8.3. Part C:

To determine the effect of different doses of cysteine on acute food intake after an overnight fast compared to a vehicle control.

Chapter 2

Materials & Methods

2.1. Amino acids

Amino acids were purchased from SynPharma International Ltd (Horsham, UK). Amino acid drinks were prepared by mixing 20 mL of Bottlegreen ginger and lemongrass cordial (Gloucester, UK) with 180 mL of water and combining with amino acid. Vehicle control was cordial and water alone.

2.2. Subjects

2.2.1. Subject recruitment

All subjects were recruited by advertisement. Potential subjects were screened to exclude those with disordered eating, as assessed by the SCOFF questionnaire (Morgan et al., 1999) and Eating Attitudes Test (Garner et al., 1982), and those with a high level of restrained eating, as assessed by the standard Dutch Eating Behaviour Questionnaire (Van Strien et al., 1986). During the screening visit for Part C, a sample of the study meal was provided, and an assessment of palatability was made using a nine-point hedonic scale. Subjects who disliked the study meal or rated the study meal as 'like extremely' were also excluded.

Part A:

Five healthy volunteers (2 male, 3 female), with mean age 37.8 years (range 28 - 57) and mean body mass index (BMI) 24.7 kg/m² (range 21.1 – 32.9) were recruited for this study. Each subject attended one study day where they ate a high protein meal. Part A did not require a power calculation, as it was a descriptive pilot study.

Part B:

Seven healthy volunteers (2 male, 5 female) with mean age 33.9 years (range 20 – 57) and mean BMI 24.3 kg/m² (range 20.4 – 32.9) were recruited for this study. Each subject attended four study days where they consumed a dose of L-cysteine (either 0.04 g/kg or 0.07 g/kg), L-glycine (0.04 g/kg or 0.07 g/kg) or vehicle control at each visit. Part B did not require a power calculation, as it was a descriptive pilot study.

Part C:

Twelve normal and overweight or obese but otherwise healthy volunteers (5 male, 7 females), aged 18 - 58 years were recruited for this crossover study. Each subject attended 6 study days (receiving vehicle control and then either 0.04 g/kg or 0.07 g/kg of L-cysteine or L-glycine). It was predicted that a sample size of 17 would have a 90% power to detect a difference in mean calorie intake of 1200kJ (a difference of 20% between treatments) assuming a within-subject standard deviation of 1268kJ, at a significance level of 1%. However, subsequent preclinical data suggested that cysteine would not prove useful as a treatment for obesity, and thus this part of the study was terminated after only 11 subjects had completed the protocol.

The research was approved by the West London Research Ethics Committee (reference number 11/H0707/7). All subjects gave written informed consent, and the research was planned and performed in accordance with the Declaration of Helsinki.

2.2.2. Inclusion criteria

Inclusion criteria were: male or female, aged 18 to 60 years (inclusive), normal and overweight/obese people as classified by a BMI 18.5-35 kg/m² (inclusive) who were otherwise healthy (based on a pre-study assessment) and people willing and able to give written informed consent and to understand, participate and comply with the study requirements.

2.2.3. Exclusion criteria

Exclusion criteria were: treatment with any medication that might have affected the study outcome (e.g. medication affecting appetite regulation and/or blood flow), haemorrhagic disorders and anticoagulant treatment, inborn errors of metabolism, hepatic impairment as defined by screening visit liver function tests (aspartate aminotransferase (AST) or alanine aminotransferase (ALT) or gamma-glutamyl transpeptidase (GGT) of three times the upper normal reference limit), current

pregnancy or breast feeding, delivery within the last year, significant intercurrent disease or history of clinically significant disease of any type, in particular liver, kidney, or heart disease, any form of diabetes mellitus or psychiatric illness, history of cancer (excluding skin cancer), history of severe or multiple allergies, severe adverse drug reaction or leucopaenia, smokers, regular drinkers of more than three units of alcohol daily, subjects who had taken amino acid supplements either continuously or intermittently in the preceding 12 months, history of, or current evidence of, abuse of alcohol or any drug substance, licit or illicit, regular intake of over-the-counter medication (other than the occasional paracetamol/aspirin), poor compliers or subjects unlikely to attend, and blood donation within the 12 week period before the initial study dose.

2.3. Part A: Effect of a high protein meal on circulating levels of amino acids in healthy human subjects

After the screening appointment subjects were asked to attend one study session, during which they were asked to eat a high protein meal (400g of cooked chicken breast consisting of 93.2g protein, 1.6g carbohydrate and 5.2g fat) and have blood samples taken. A schematic representation of a study day for Part A is illustrated in Figure 2.1.

Subjects were asked to attend each study day following an overnight fast. They were asked to refrain from taking strenuous exercise and drinking alcohol for twenty-four hours before the study visit. They were asked not consume any food or drink with the exception of water from 2100h on the evening prior to the study day and to consume identical evening meals at 2000h on the evening before the study visit.

On the study day, the female subjects were asked to provide a urine specimen in order to perform a pregnancy test. The subjects then had an intravenous cannula placed in their arm and serial blood sampling beginning at t=15 minutes and continuing for the remainder of the study day at the time points indicated in figure

2.1. They were also asked to complete visual analogue scales (VAS) that rated hunger, satiety and nausea at these time points (Flint et al., 2000b, Stubbs et al., 2000). The VAS consisted of 100 mm lines with text expressing the most positive and the most negative rating for each variable anchored at either end.

At T=0, subjects were given access to a high protein meal which they were asked to consume within 10 minutes. Baseline plasma and serum were assayed for routine clinical chemistry (liver and kidney function, calcium and electrolytes). Plasma levels of amino acids and the appetite and metabolic function markers insulin, GLP-1, PYY and ghrelin were measured at baseline and subsequent time points. Blood samples were collected into lithium heparin-coated tubes (International Scientific Supplies Ltd, Bradford, UK) containing 2000 kallikrein inhibitor units (0.2 mL) aprotinin (Trasylol, Bayer Schering Pharma, Berlin, Germany). Samples immediately underwent centrifugation for 10 minutes at 4°C (Hettich Zentrifugen Rotina 420R), after which, plasma was separated immediately and stored at -20°C until analysis. Cannulae were flushed with 5 ml 0.9% saline after collecting each blood sample. Immediately prior to collection of the subsequent sample, 1 ml deadspace blood was collected and discarded.

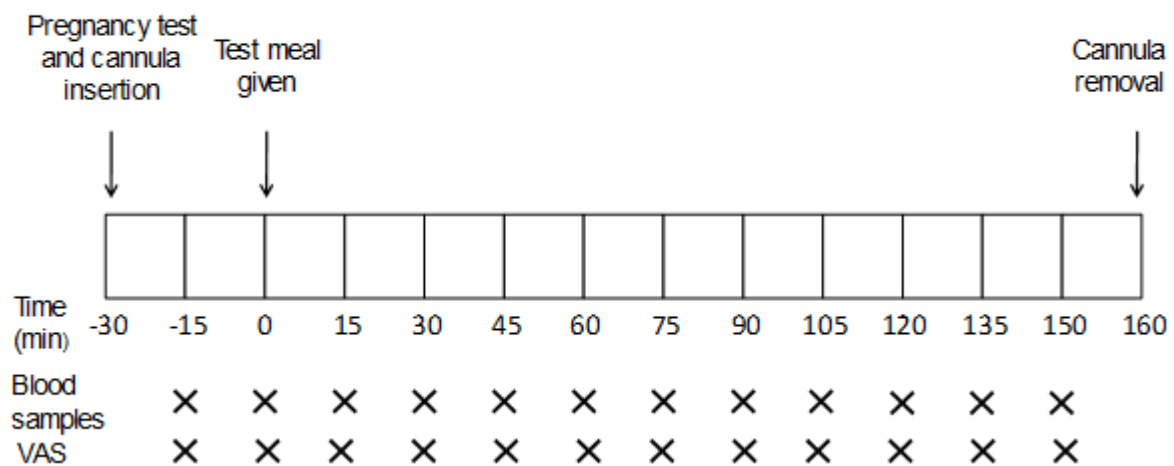


Figure 2.1. A schematic representation of a study day for Part A of “The effect of L-cysteine on appetite in humans”.

x represents time points at which blood samples and visual analogue scales (VAS) were collected.

2.4. Part B: Effect of oral amino acid administration on circulating levels of specific amino acids

After the screening appointment, subjects were then asked to attend 5 study sessions, at least one week apart, during which they received either a vehicle control, a dose of L-cysteine (0.04 g/kg or 0.07 g/kg) or a dose of L-glycine (0.04 g/kg or 0.07 g/kg). A schematic diagram giving an overview of Part B of the study can be seen in figure 2.2.

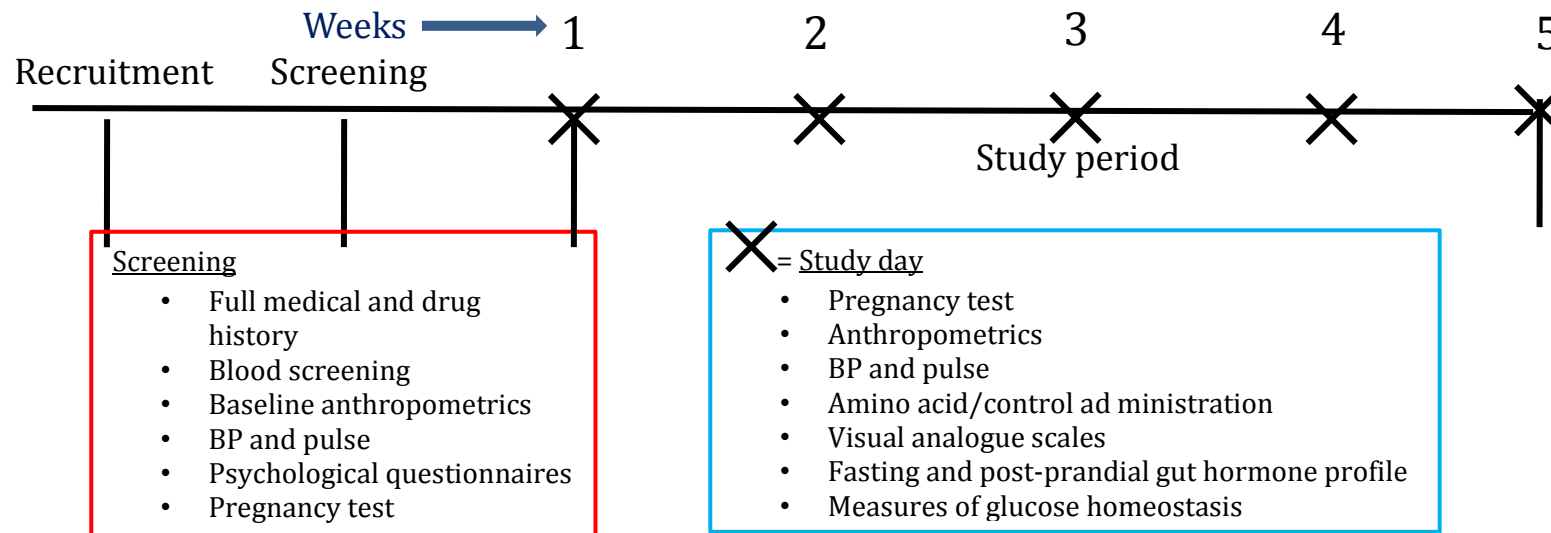


Figure 2.2. A schematic representation of the time-course of Part B of the study “The effect of L-cysteine on appetite in humans”.

Following the initial recruitment, subjects attend a screening to deem if they are suitable for the study, followed by 5 study visits for those suitable. x represent each study visit.

The study was conducted as described for part A above, except at T=0, subjects were given a dose of vehicle control, L-cysteine (0.04 g/kg or 0.07 g/kg) or L-glycine (0.04 g/kg or 0.07 g/kg), which they were asked to consume within 10 minutes instead of the high protein meal. A schematic representation of a study day for Part B is illustrated in Figure 2.3.

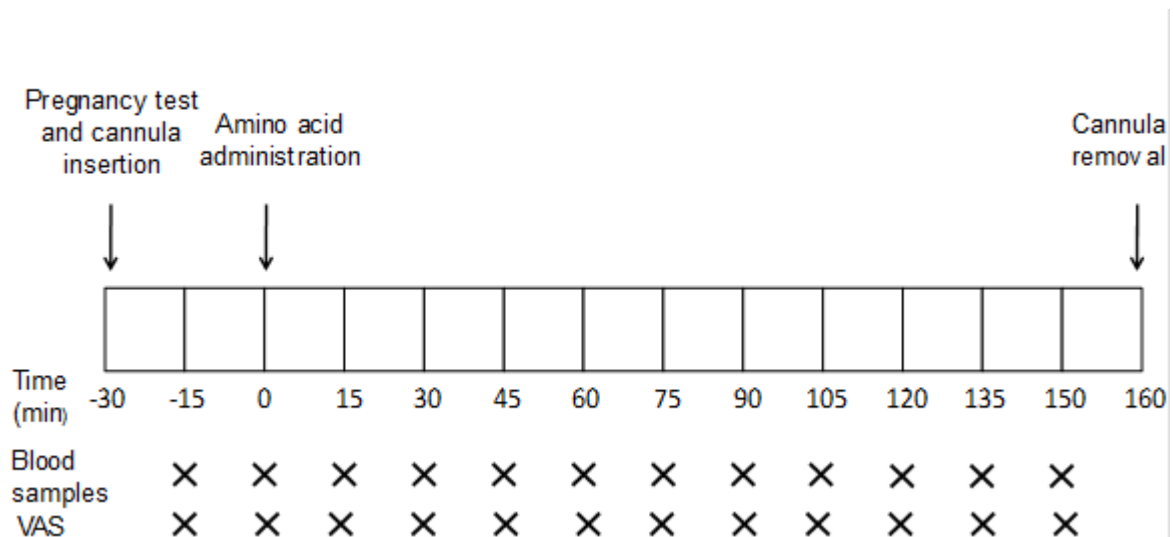


Figure 2.3. A schematic representation of a study day for Part B of “The effect of L-cysteine on appetite in humans”.

× represents time points at which blood samples and visual analogue scales (VAS) were collected.

2.5. Part C: Effect of specific oral amino acid administration on *ad libitum* food intake

After the screening appointment, subjects were asked to attend 6 study sessions, each at least 1 week apart, during which they would receive either vehicle control or a dose of L-glycine (0.04 g/kg or 0.07 g/kg) or L-cysteine (0.04 g/kg or 0.07 g/kg). The study was conducted in a single-blinded fashion. On their first visit, subjects always received vehicle control after an overnight fast. This protocol was an identical protocol to that used for the subsequent fasted visits, and was intended to

acclimatise the subject to the clinical environment and to experimental procedures. Results from the first vehicle control visit were not included in the analysis. Following the first vehicle control visit, subjects were randomly assigned to receive vehicle control, cysteine 0.04 g/kg, cysteine 0.07 g/kg, glycine 0.04g/kg or glycine 0.07g/kg. An overview of Part C can be seen in figure 2.4. Doses were based on the results of Part B. They did not exceed a dose of 7 grams, with a dose of up to 20 grams demonstrated to be safe (Davis et al., 1972, Carlson et al., 1989).

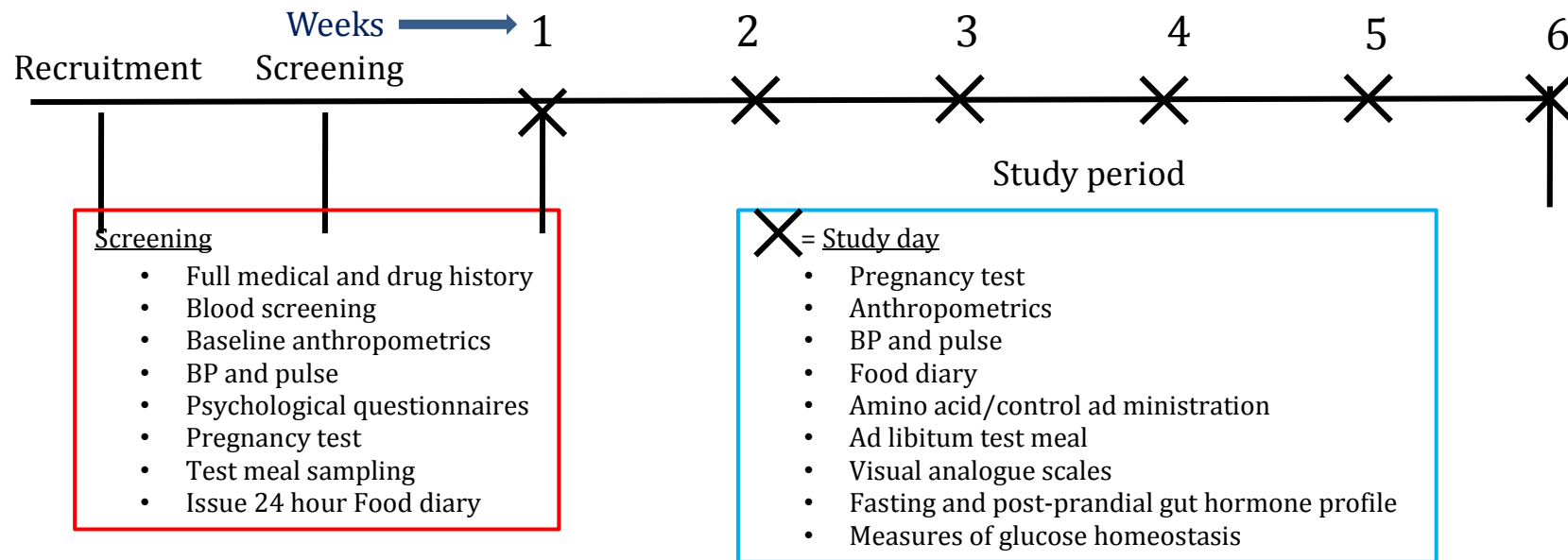


Figure 2.4. A schematic representation of the time-course of Part C of the study “The effect of L-cysteine on appetite in humans”.

Following the initial recruitment, subjects attend a screening to deem if they are suitable for the study, followed by 6 study visits for those suitable. x represent each study visit.

The study was conducted as described for part B above, except at T=60, subjects were given a buffet meal consisting of the preferred test meal decided at the screening. A schematic representation of a study day for Part C is illustrated in Figure 2.5. The timing of the meal was chosen to correspond with the time at which there were optimal circulating amino acid concentrations post-consumption in Part B and based upon hunger and fullness data of the VAS obtained in Part B. Subjects were asked to eat until they were comfortably full. Water was freely available during the mealtime. The meal was served with the curtains drawn around each subject for 30 minutes, and consisted of either a macaroni cheese (631 kJ/100g) or a chicken korma with rice (653 kJ/100g). The macronutrient content for both meals is described in Table 2.1. Each subject had previously decided on their choice of meal during their screening visit; they were subsequently served the same meal during each of their study visits. Food was weighed pre- and post-consumption. Energy intake was calculated from the weight of food consumed. Blood samples and a VAS were taken at the time points indicated in figure 2.5. The time points used were based on the results of Part B. Amino acids and hormones were measured as described above.

Nutrition (per 100g)	Macaroni cheese	Chicken korma & rice
Energy	631kJ 151kcal	653kJ 156kcal
Protein	7.0g	7.2g
Carbohydrate	14.0g	14.4g
Total sugars	1.0g	3.0g
Starch	13.0g	11.4g
Fat	7.4g	7.7g
Salt	0.50g	0.53g

Table 2.1. Macronutrient content for study meals used in Part C of the study “The effect of specific amino acids on appetite”.

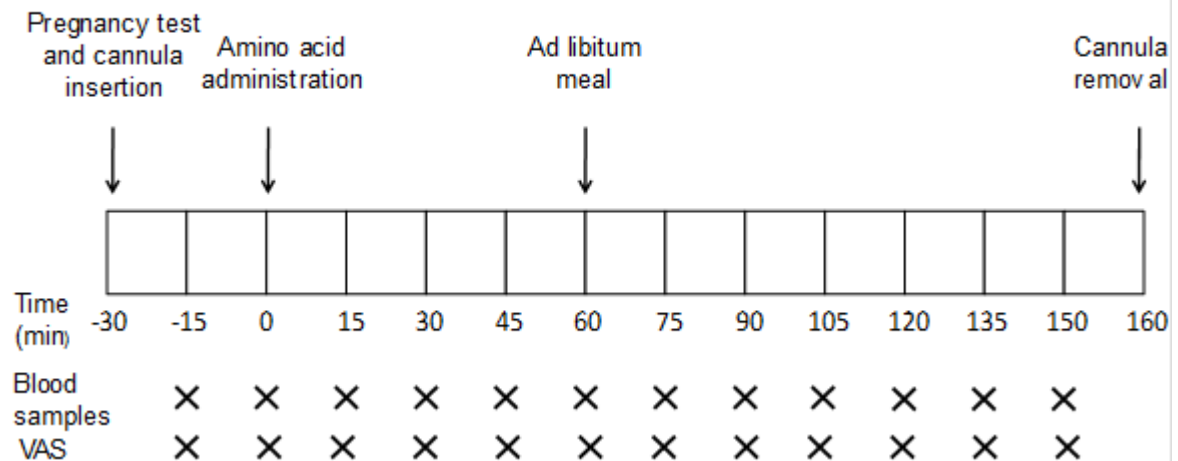


Figure 2.5. A schematic representation of a study day for Part C of the study “The effect of L-cysteine on appetite in humans”.

x represents time points at which blood samples and visual analogue scales (VAS) were collected.

2.6. Amino acid assay

Amino acids were measured by the Department of Chemical Pathology at Imperial College Healthcare NHS Trust using a dedicated amino acid analyser (JEOL AminoTac), which used automated high performance cation exchange analysis. Underivatized amino acids were separated on a cation exchange resin column based upon net charge of particular amino acids.

2.7. Plasma hormone assays

2.7.1. PYY

PYY-like immunoreactivity was measured with a previously established specific and sensitive in-house radioimmunoassay (Adrian et al., 1985). The antiserum (Y21) was produced in rabbits against synthetic porcine PYY coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:50,000. This antibody cross-reacts fully with both PYY₃₋₃₆ and PYY₁₋₃₆, but not with pancreatic polypeptide, neuropeptide Y, or other known

gastrointestinal hormones. The ^{125}I PYY was prepared by the iodogen method (Wood et al., 1981) and purified by reverse phase high pressure liquid chromatography (RP-HPLC). The specific activity of the ^{125}I PYY label was 54 Bq/fmol. The assay was performed in total volume of 0.7 ml of phosphate buffer (0.05M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.006M KH_2PO_4 , 0.01M $\text{C}_{10}\text{H}_{14}\text{H}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$, 0.008M NaN_3) (pH 7.3 ± 0.1) containing 0.3% bovine serum albumin. The assay was incubated for three days at 4°C before separation of the free and antibody bound label by goat anti-rabbit antibody. Samples were pre-incubated for at least 60 minutes with 100 μl of goat anti-rabbit solid-phase secondary antibody (Pharmacia Diagnostics AB, Uppsala, Sweden). Immediately prior to centrifugation, 500 μl of 0.01% Triton-X-100 solution and 100 μl polyethylene glycol (PEG) (1:1) was added to each tube. The samples were then centrifuged at 1,500g at 4°C for 20 minutes. Bound and free label were separated and both the pellet and supernatant counted for 180 seconds in a γ -counter (model NE1600 (Thermo Electron Corporation, San Jose, California, U.S.A.)). PYY concentrations in the samples were calculated using a non-linear plot (RIA software, Thermo Electron Corporation) and the results calculated with reference to the standard. The detection limit of the assay was 2.5 pmol/l, with an intra-assay coefficient of variation of 5.8 %. All samples were assayed in one assay to avoid inter-assay variation.

2.7.2. GLP-1

GLP-1- like immunoreactivity was measured by a specific and sensitive in-house radioimmunoassay, previously established (Kreymann et al., 1987). The antibody was produced in rabbits against GLP-1 coupled to bovine serum albumin. The antibody cross-reacted 100% with all amidated forms of GLP-1 but did not cross react with glycine extended forms [GLP-1₁₋₃₇ and GLP-1₇₋₃₇] or any other known pancreatic or gastrointestinal peptide. ^{125}I -GLP-1 was prepared by the iodogen method (Wood et al., 1981) and purified by RP-HPLC. The specific activity of the ^{125}I GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.7 ml of sodium barbital buffer (0.05M $\text{C}_8\text{H}_{11}\text{N}_2\text{O}_3\text{Na}$, 0.005M NaN_3) (pH 8) containing 0.3 % bovine serum albumin (BSA). The assay was incubated for three days at 4°C before separation of the free and antibody bound label by charcoal absorption. The free fraction

was separated using 4mg of charcoal per tube suspended in phosphate buffer with gelatine (0.0625mM gelatine, 0.05M Na₂HPO₄.2H₂O, 0.006M KH₂PO₄, 0.01M C₁₀H₁₄H₂O₈Na₂.2H₂O, 0.008M NaN₃), added immediately prior to centrifugation. The samples were then centrifuged at 1,500g at 4°C for 20 minutes. Bound and free label were separated, counted and analysed as described above. The limit of detection was 7.5 pmol/l with an intra-assay variation of 5.4%. All samples were assayed in one assay to avoid inter-assay variation.

2.7.3. PP

Human plasma PP concentrations were measured using a specific and sensitive RIA (Adrian et al., 1976). An antiserum against human PP was produced in rabbits and the radiolabelled PP prepared by the iodogen method (Adrian et al., 1976). It detects human PP fully but does not cross-react with any other known gastrointestinal hormone. ¹²⁵I-labelled PP was prepared by the iodogen method (Wood et al., 1981) and purified by HPLC. The specific activity of the ¹²⁵I PP label was 54 Bq/fmol. The assay was performed in a total volume of 0.7ml of phosphate buffer (0.06M) (pH 7.2) containing 0.3 % BSA. The assay was incubated for 4 days at 4°C before being separated, counted and analysed as described above for GLP-1. All samples were assayed in one assay to avoid inter-assay variation.

2.7.4. Ghrelin

Human plasma ghrelin concentrations were measured using a specific and sensitive enzyme-linked immunosorbent assay (ELISA) (Merck Millipore, Billerica, MA, USA). Plasma was separated by centrifugation as described above, acidified with HCl to a final concentration of 1N (Hosoda et al., 2004), frozen and stored at -20C. The assay was carried out as per manufacturer's instructions.

2.8. Statistical analysis

Combined data are shown as the mean \pm standard error of the mean (SEM). Comparisons of energy intake were by one-way analysis of variance (ANOVA), followed by a post hoc Dunnett's test. Comparisons of VAS scores were by one-way ANOVA, followed by a post hoc Dunnett's test. The threshold for statistical significance in each case was set at $p < 0.05$. Analyses were performed using Prism version 5.01 software (Graphpad Software, San Diego, CA, USA).

Chapter 3

Results

The clinical characteristics of the recruited subjects are summarized in Table 3.1. No serious adverse events were experienced by any of the subjects.

Characteristic	All subjects
Age (yr)	35.9 ± 11.7
Female:Male	7:5
Weight (kg)	65.5 ± 14.6
Height (m)	1.7 ± 0.1
BMI (kg/m ²)	23.7 ± 4.8
Blood pressure	116/69 ± 12/3
Ethnic group	
Caucasian	10
South Asian	1
Afro-Caribbean	1

Table 3.1. Baseline characteristics of the 12 participants recruited for Parts A-C. Data represent the mean ± SEM. BMI: body mass index.

3.1. To establish physiological levels of circulating amino acids following a high protein meal (Part A)

3.1.1. Amino acid analysis

Following consumption of the high protein meal, plasma cysteine levels increased from baseline, with peak levels achieved at 45 minutes after administration, and remained raised 150 minutes after administration. For the

5 subjects who consumed the high protein meal, plasma cysteine concentrations are shown in figure 3.1 below.

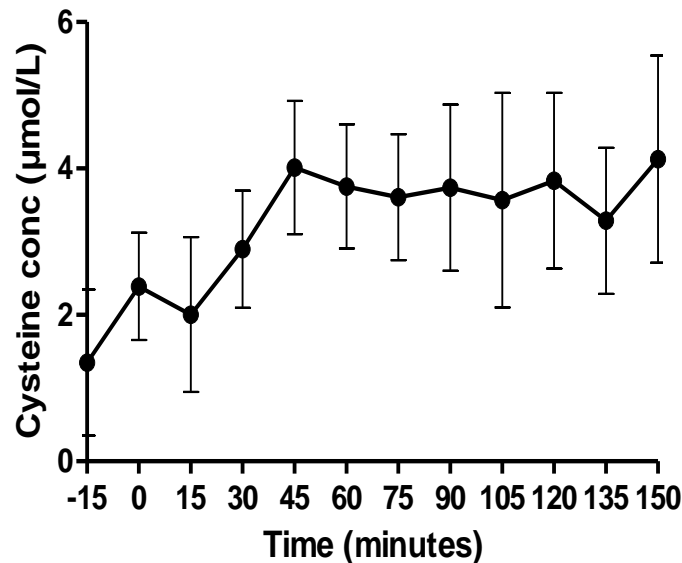


Figure 3.1. Plasma cysteine concentrations (µmol/L) following a high protein meal.

Five healthy subjects consumed a high protein meal (400g of cooked chicken breast consisting of 93.2g protein, 1.6g carbohydrate and 5.2g fat) at t=0, following an overnight fast. During the study, subjects had blood samples taken every 15 minutes to measure plasma cysteine concentration. All values represent group mean \pm SEM.

3.1.2. VAS analysis

Following consumption of the high protein meal, subjects felt less hungry and fuller, with a sharp fall in hunger at 15 minutes (Figure 3.2 (A)) and a rise in fullness at 15 minutes (Figure 3.2 (B)). This effect on hunger appeared sustained for the time period of the study (150 minutes). Similarly, subjects did not feel like eating after 15 minutes (Figure 3.2 (C,D)), and again this effect lasted throughout the study period. There was no evidence of any lasting side effects with the high protein meal (Figure 3.3 (A-E)).

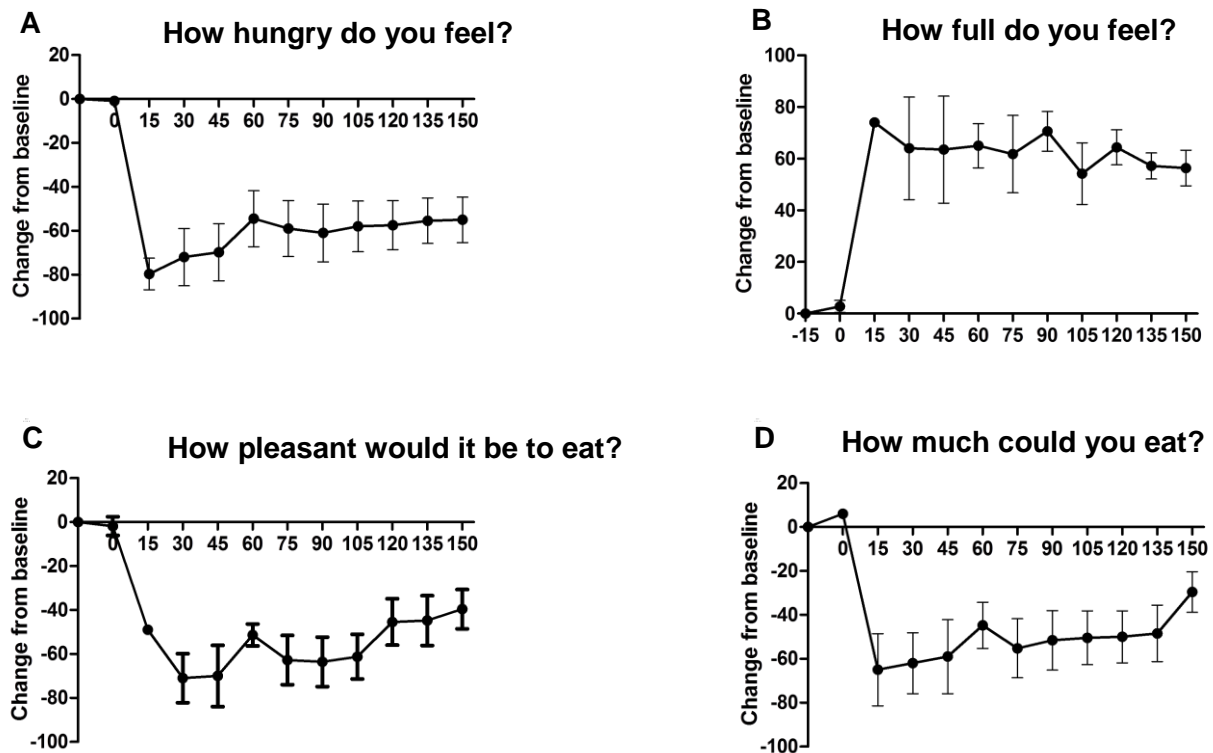


Figure 3.2. Visual analogue scale ratings following a high protein meal.

Five healthy subjects consumed a high protein meal (400g of cooked chicken breast consisting of 93.2g protein, 1.6g carbohydrate and 5.2g fat) following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires every 15 minutes. **A**, How hungry do you feel? **B**, How full do you feel? **C**, How pleasant would it be to eat? **D**, How much could you eat? All values represent group mean \pm SEM.

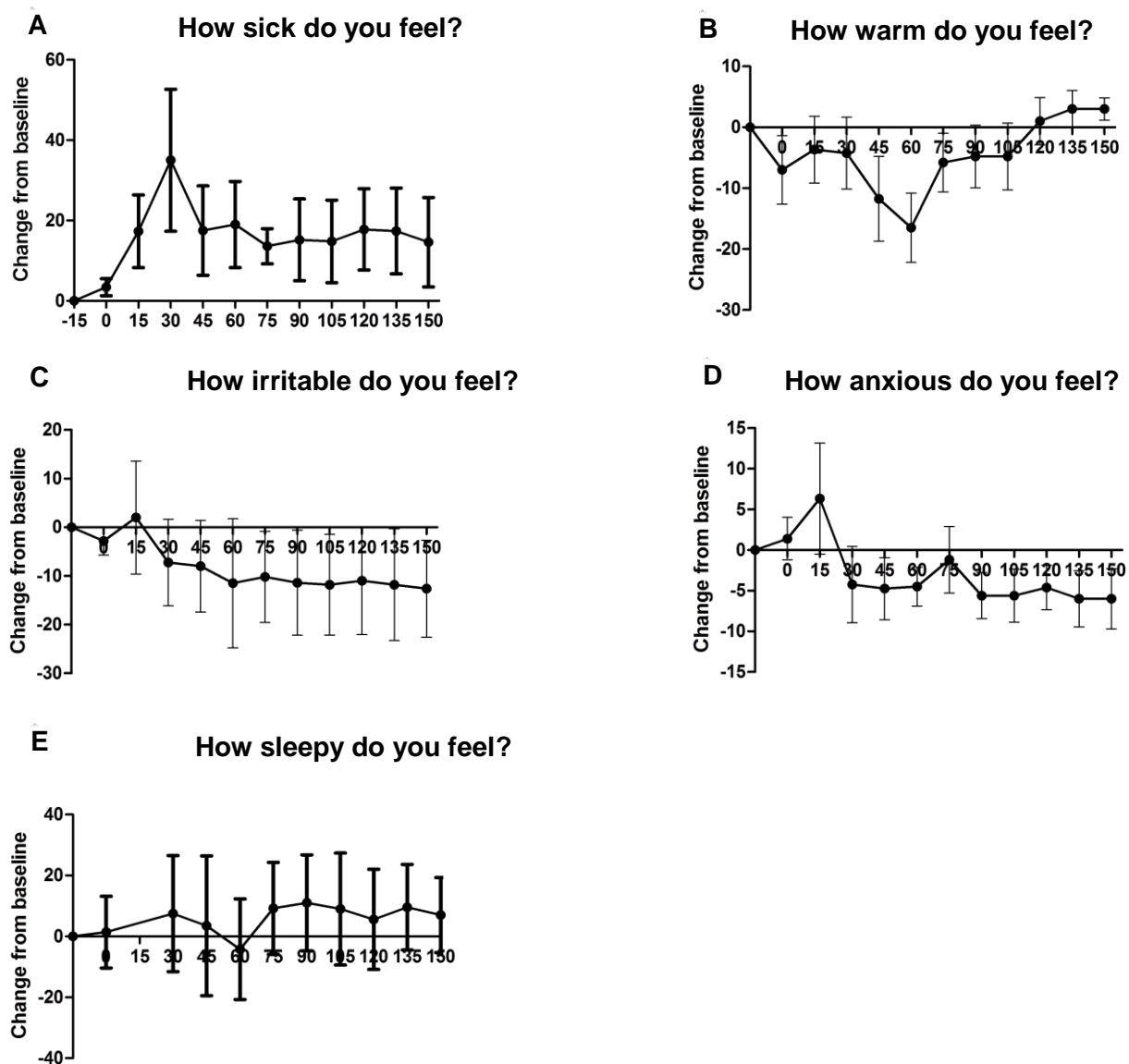


Figure 3.3. Visual analogue scale ratings following a high protein meal.

Five healthy subjects consumed a high protein meal (400g of cooked chicken breast consisting of 93.2g protein, 1.6g carbohydrate and 5.2g fat) following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires every 15 minutes. **A**, How sick do you feel? **B**, How warm do you feel? **C**, How irritable do you feel? **D**, How anxious do you feel? **E**, How sleepy do you feel? All values represent group mean \pm SEM.

3.2. To determine circulating levels of amino acids following oral administration of cysteine, glycine control or vehicle control

3.2.1. Amino acid analysis

Following consumption of oral cysteine (0.04g/kg), plasma cysteine levels increased from baseline, with peak levels achieved at 60 minutes after administration, and remained raised at 150 minutes after administration. For 5 subjects, plasma cysteine concentrations following administration of 0.04g/kg of cysteine are shown in figure 3.4 below.

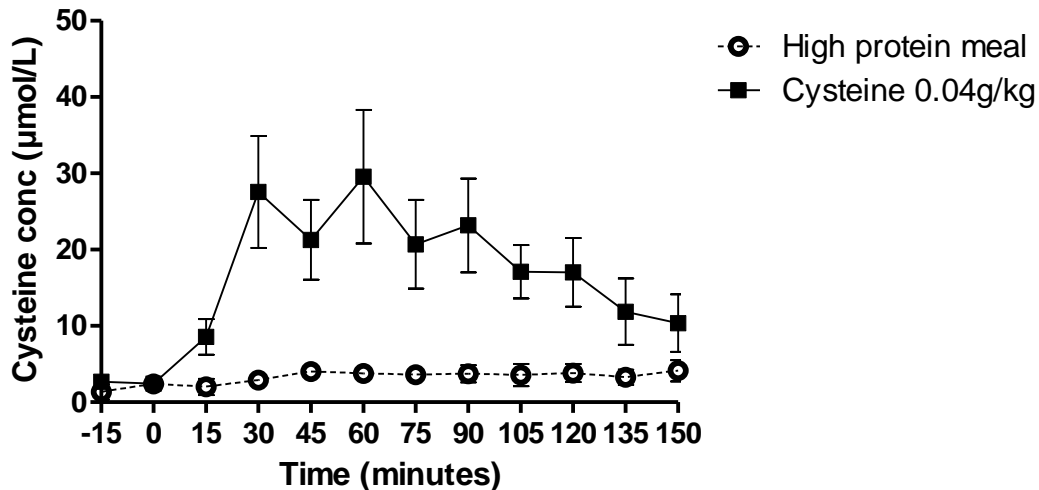


Figure 3.4. Plasma cysteine concentrations (µmol/L) following administration of cysteine.

Five healthy subjects consumed 0.04g/kg of cysteine at t=0, following an overnight fast. During the study, subjects had blood samples taken every 15 minutes to measure plasma cysteine concentration. These levels are compared to those found following administration of the high protein meal. All values represent group mean \pm SEM.

3.2.2. VAS analysis

Following oral consumption of vehicle, glycine (0.04g/kg) and cysteine (0.04g/kg), VAS were measured as shown in figure 3.5 (A-H) below. There was no difference by ANOVA or area under the curve (AUC) in hunger, fullness and a need to eat between the groups. In addition, there was no difference in any ill-effects caused by the amino acids (Figure 3.6 and 3.7) except for a small increase in anxiety with cysteine (figure 3.7 (A-B)).

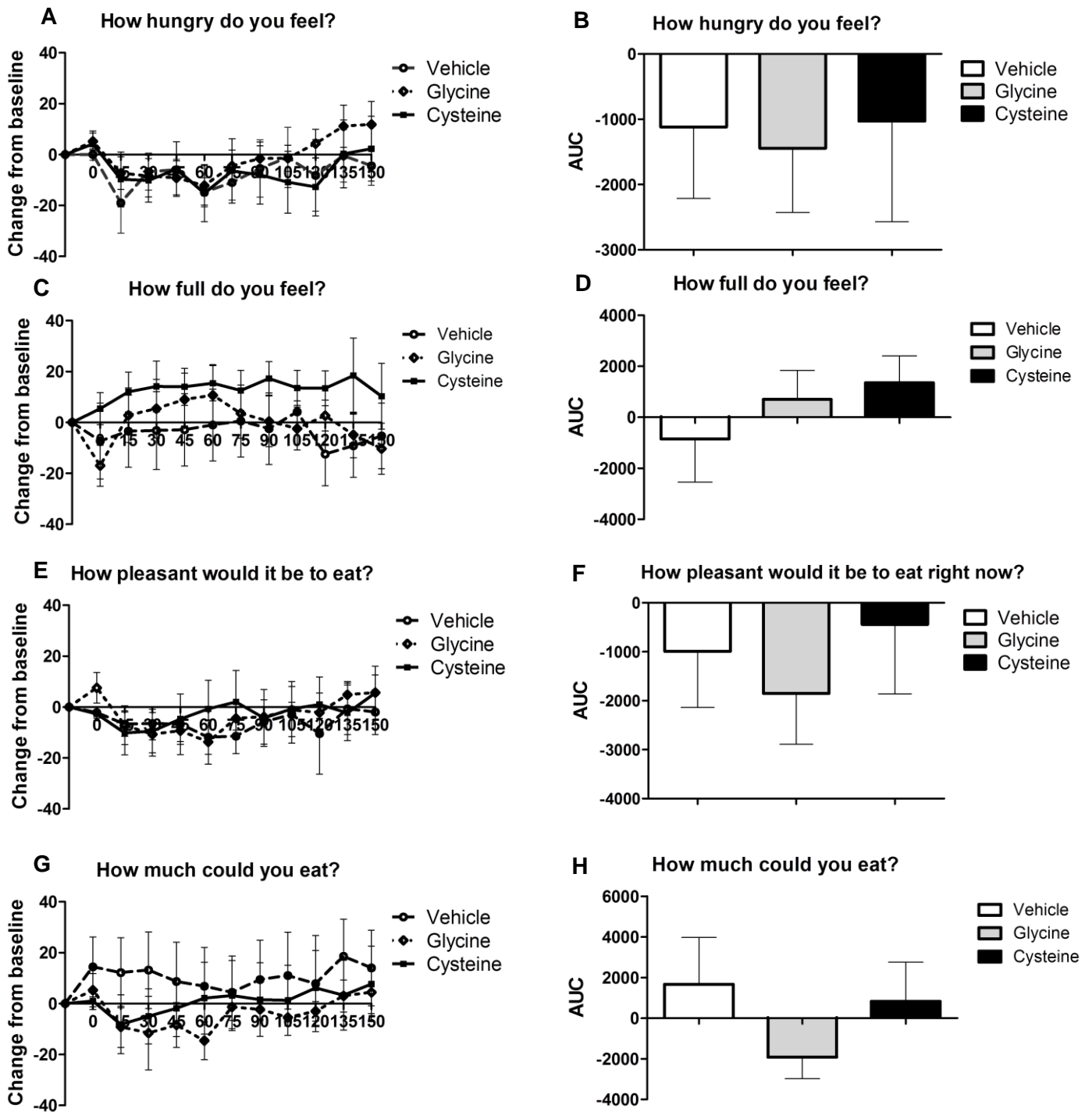


Figure 3.5. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.

Subjects consumed vehicle, glycine (0.04g/kg) or cysteine (0.04g/kg) at t=0, following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger, nausea and fullness. **A**, How hungry do you feel? **B**, AUC for How hungry do you feel? **C**, How full do you feel? **D**, AUC for How full do you feel? **E**, How pleasant would it be to eat? **F**, AUC for How pleasant would it be to eat? **G**, How much could you eat? **H**, AUC for How much could you eat? No differences by ANOVA. All values represent group mean \pm SEM.

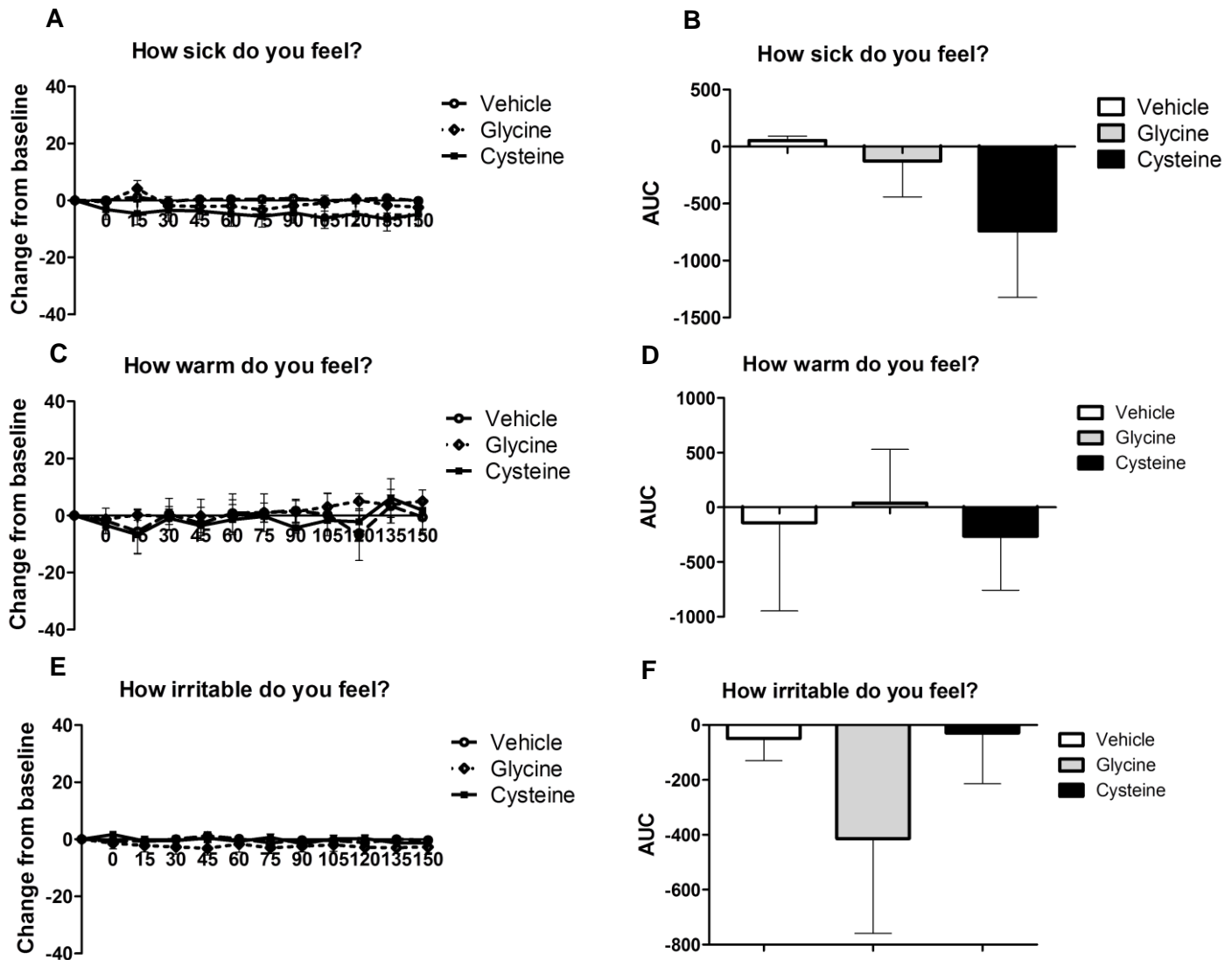


Figure 3.6. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.

Subjects consumed vehicle, glycine (0.04g/kg) or cysteine (0.04g/kg) at t=0, following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger, nausea and fullness. **A**, How sick do you feel? **B**, AUC for How sick do you feel? **C**, How warm do you feel? **D**, AUC for How warm do you feel? **E**, How irritable do you feel? **F**, AUC for How irritable do you feel? No differences by ANOVA. All values represent group mean \pm SEM

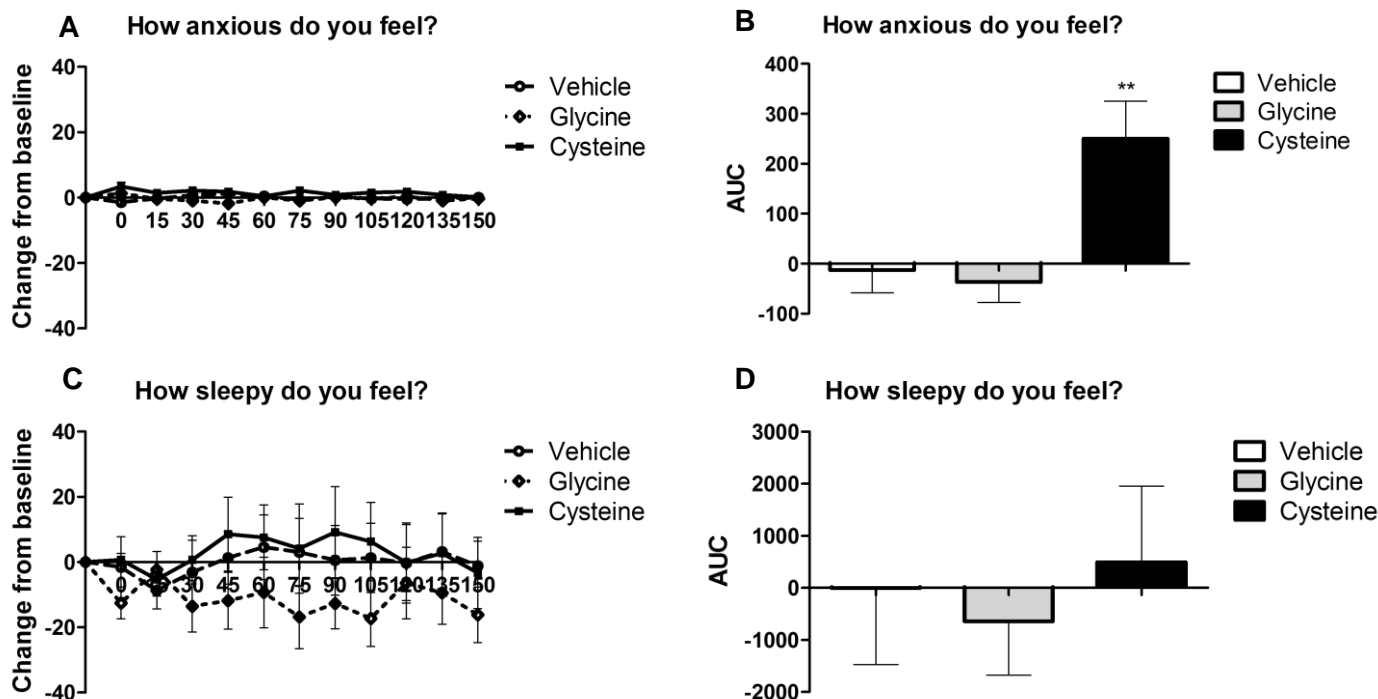


Figure 3.7. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.

Seven healthy subjects consumed vehicle, glycine (0.04g/kg) or cysteine (0.04g/kg) at t=0, following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger, nausea and fullness. **A**, How anxious do you feel? **B**, AUC for anxious do you feel? **C**, How sleepy do you feel? **D**, AUC for How sleepy do you feel? ** p<0.01. All values represent group mean \pm SEM

3.3. To determine circulating levels of amino acids following oral administration of a higher dose of cysteine, glycine control or vehicle control

3.3.1. VAS analysis

Following oral consumption of vehicle, glycine (0.07g/kg) and cysteine (0.07g/kg), VAS were measured as shown in figure 3.8 below. There was a reduction in the AUC for “How hungry do you feel?” (p<0.05) (B), “How pleasant would it be to eat?” (D), “How much could you eat?” (p<0.05) (F). There was no difference by ANOVA or AUC in fullness (Figure 3.8(G,H)) between the groups. There was no difference in ill effects caused by the amino acids (Figure 3.9 and 3.10).

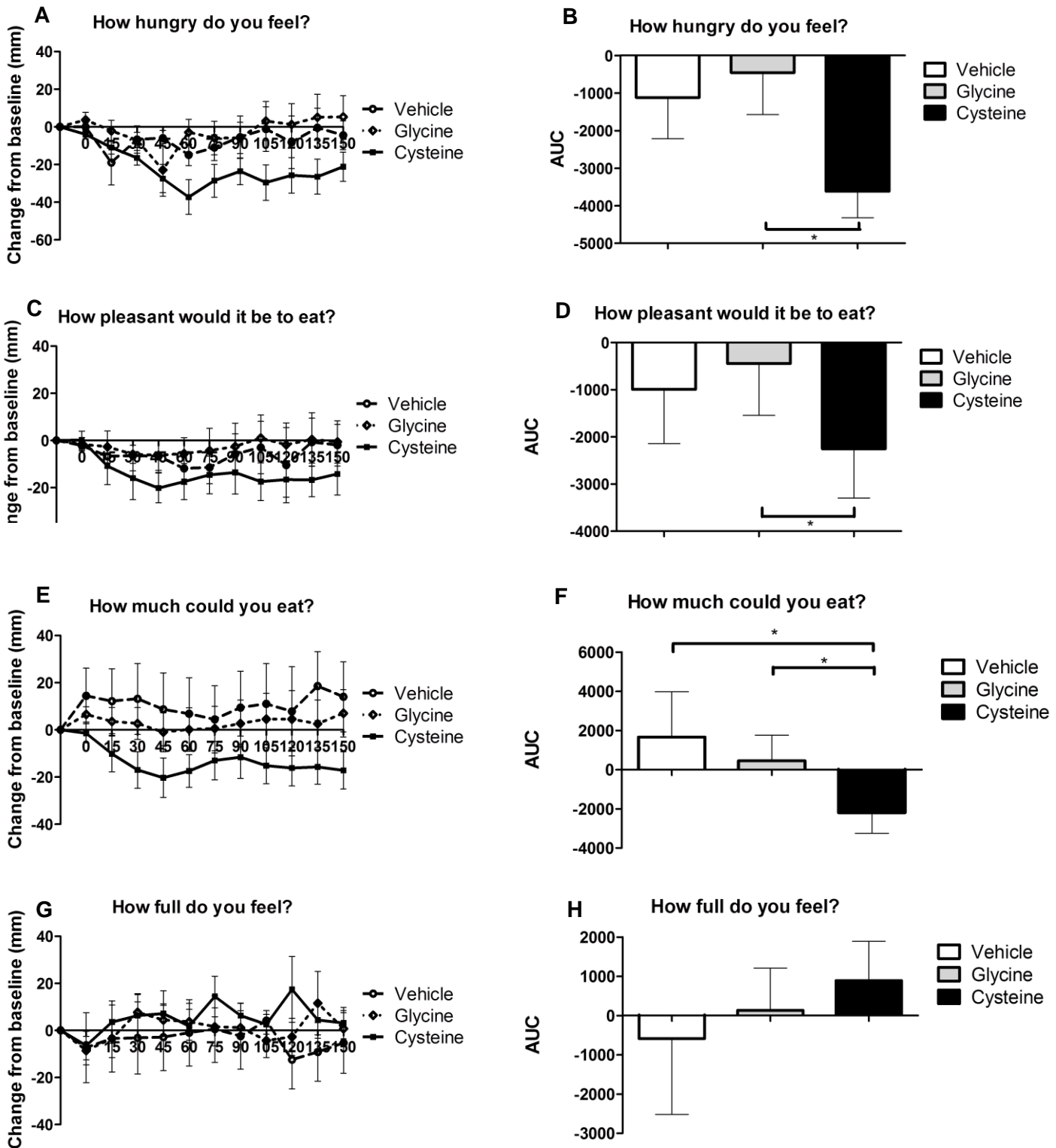


Figure 3.8. Analysis of VAS ratings of 7 subjects following administration of vehicle, higher dose of glycine or cysteine.

Seven healthy subjects consumed vehicle, glycine (0.07g/kg) or cysteine (0.07g/kg) at t=0, following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger, nausea and fullness. **A**, How hungry do you feel? **B**, AUC for How hungry do you feel? **C**, How full do you feel? **D**, AUC for How full do you feel? **E**, How pleasant would it be to eat? **F**, AUC for How pleasant would it be to eat? **G**, How much could you eat? **H**, AUC for How much could you eat? * $p < 0.05$. All values represent group mean \pm SEM.

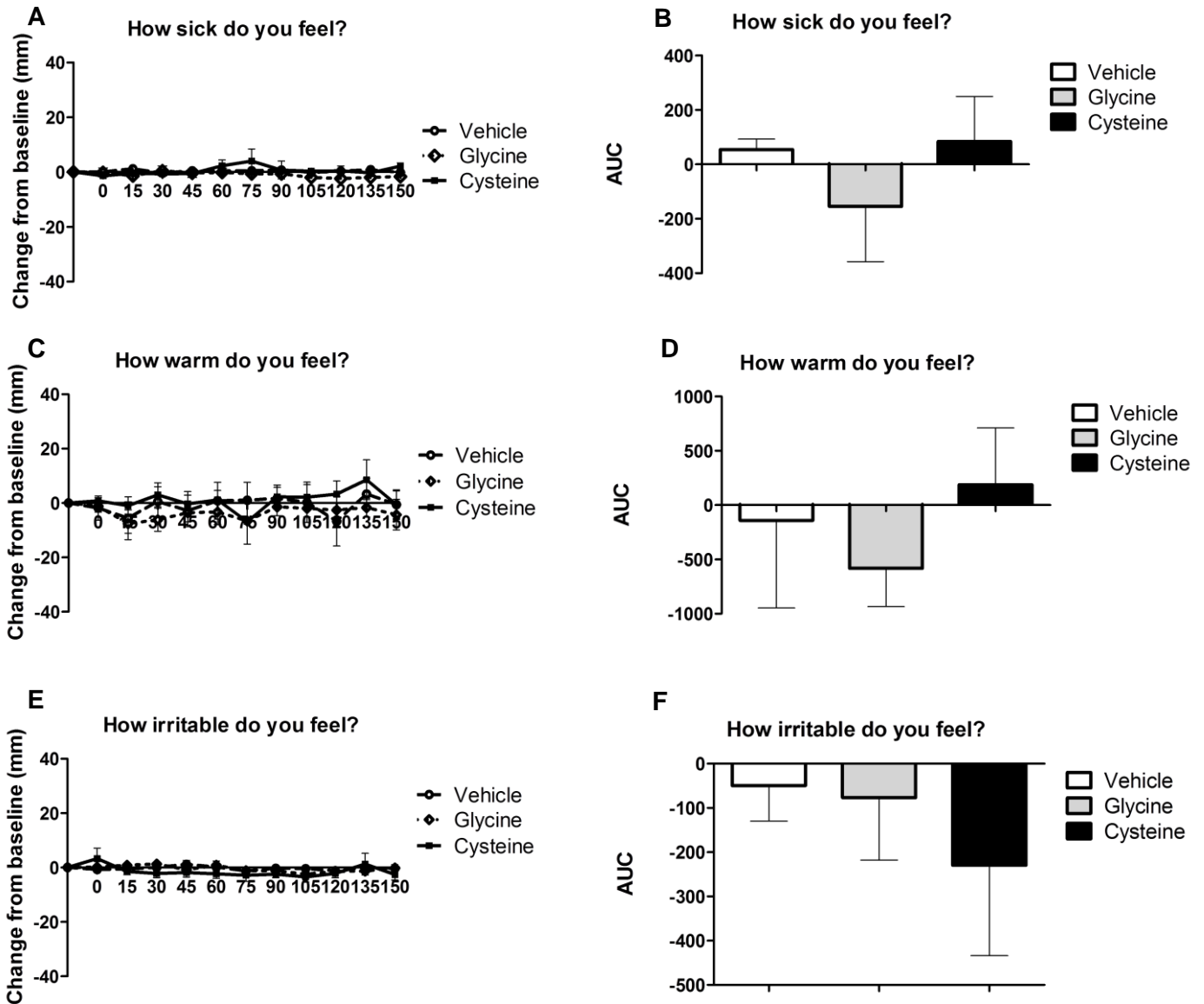


Figure 3.9. Analysis of VAS ratings of 7 subjects following administration of vehicle, higher dose of glycine or cysteine.

Seven healthy subjects consumed vehicle, glycine (0.07g/kg) or cysteine (0.07g/kg) at t=0, following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger, nausea and fullness. **A**, How sick do you feel? **B**, AUC for How sick do you feel? **C**, How warm do you feel? **D**, AUC for How warm do you feel? **E**, How irritable do you feel? **F**, AUC for How irritable do you feel? No differences by ANOVA. All values represent group mean \pm SEM

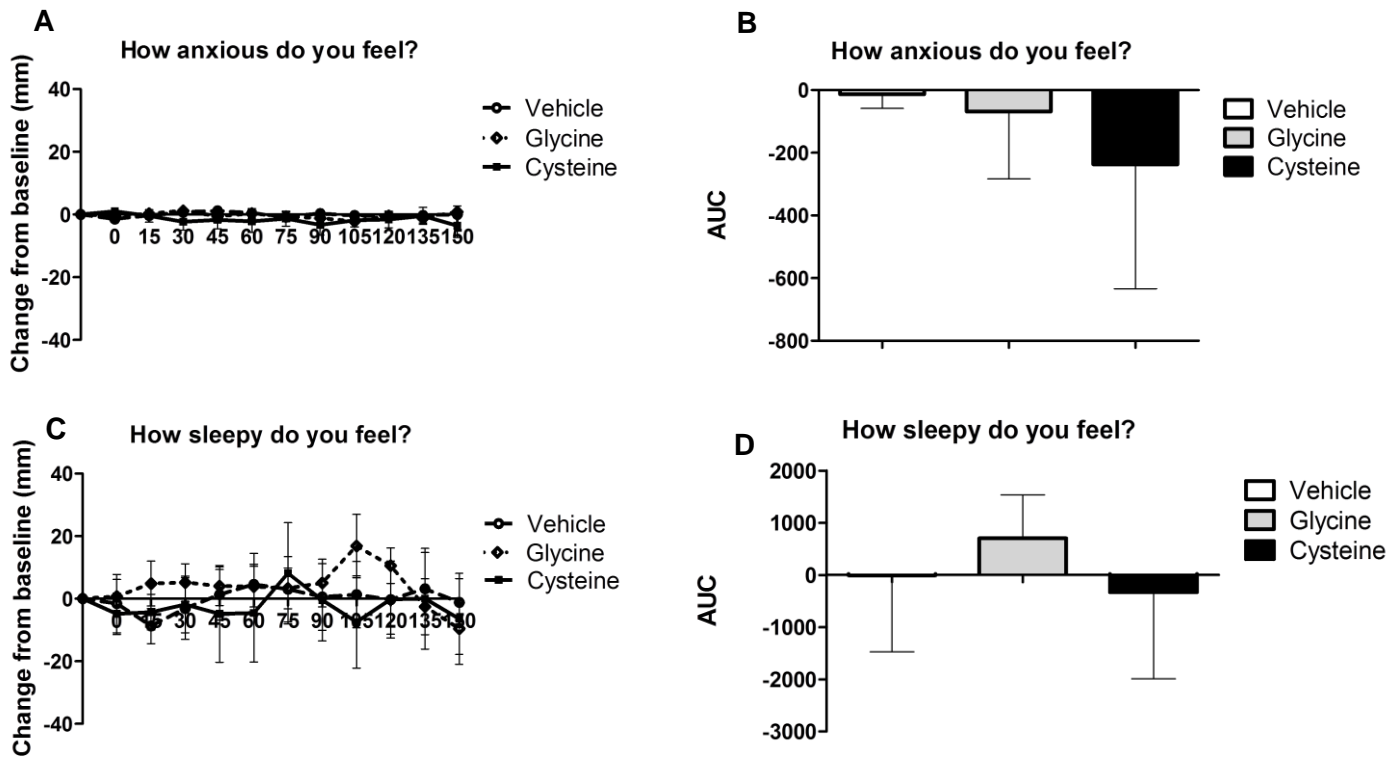


Figure 3.10. Analysis of VAS ratings of 7 subjects following administration of vehicle, glycine or cysteine.

Seven healthy subjects consumed vehicle, glycine (0.07g/kg) or cysteine (0.07g/kg) at t=0, following an overnight fast. During the study, subjects completed 100 mm visual analogue scale questionnaires relating to hunger, nausea and fullness. **A**, How anxious do you feel? **B**, AUC for anxious do you feel? **C**, How sleepy do you feel? **D**, AUC for How sleepy do you feel? No differences by ANOVA. All values represent group mean \pm SEM.

3.4. To determine gut hormone levels following oral administration of cysteine, glycine control or vehicle control

Following oral consumption of vehicle, glycine (0.04g/kg) and cysteine (0.04g/kg), GLP-1, PYY, PP and ghrelin were measured. There was no difference by ANOVA or AUC in any of the hormones measured (Figure 3.11 (A-H)).

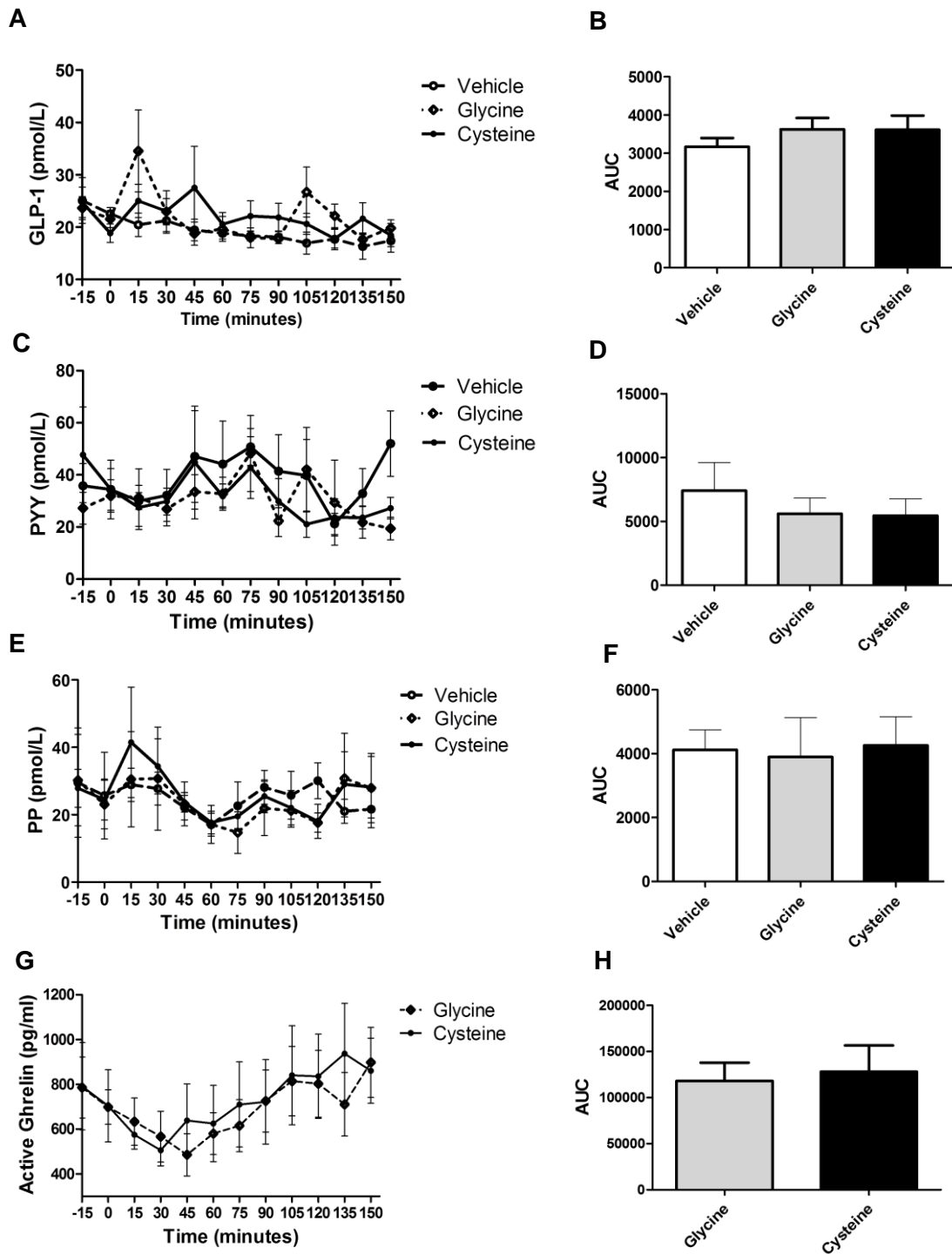


Figure 3.11. Analysis of gut hormones of 5-6 subjects following administration of vehicle, glycine or cysteine.

Five to six healthy subjects consumed vehicle, glycine (0.04g/kg) or cysteine (0.04g/kg) at t=0, following an overnight fast. During the study, blood samples were taken serially to determine gut hormone levels. No differences by ANOVA. All values represent group mean \pm SEM.

3.5. To determine gut hormone levels following oral administration of a higher dose of cysteine, glycine control or vehicle control

Following oral consumption of vehicle, glycine (0.07g/kg) and cysteine (0.07g/kg), GLP-1, PYY, PP and ghrelin were measured. There was a significant reduction in AUC for PP ($p < 0.05$) when compared to both vehicle and glycine (Figure 3.12 (F)) but no difference by ANOVA (Figure 3.12 (E)). There was no difference by ANOVA or AUC in GLP-1 or PYY (Figure 3.12 (A-D)).

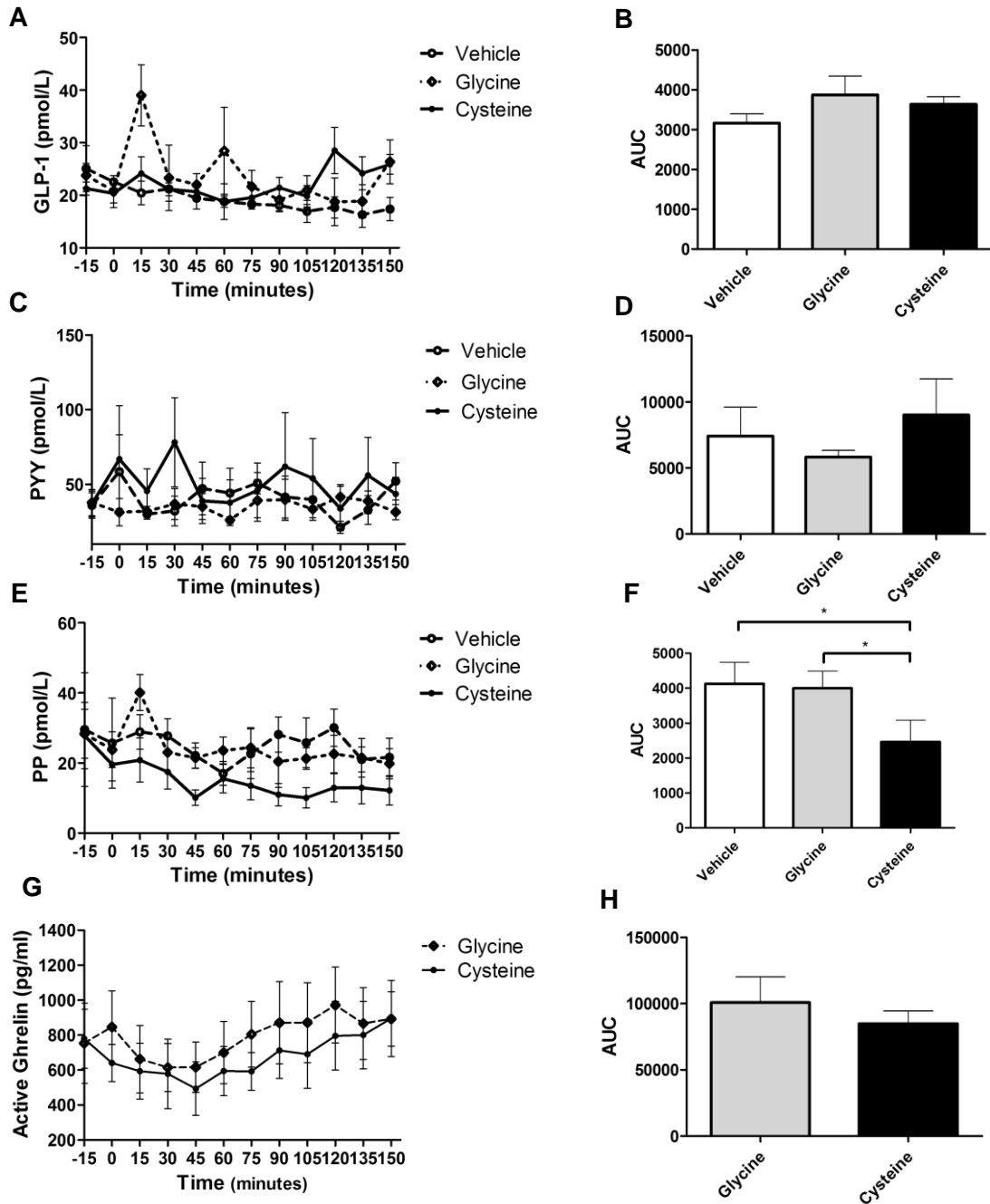


Figure 3.12. Analysis of gut hormones of 5-6 subjects following administration of a higher dose of vehicle, glycine or cysteine.

Five to six healthy subjects consumed vehicle, glycine (0.07g/kg) or cysteine (0.07g/kg) at t=0, following an overnight fast. During the study, blood samples were taken serially to determine gut hormone levels. * p<0.05. All values represent group mean \pm SEM.

3.6. To determine the effect of cysteine on food intake

3.6.1. Ad libitum meal

There was no significant difference between food intake at an *ad libitum* buffet lunch following consumption of vehicle, glycine or cysteine. For the 11 subjects who participated, the mean energy intake was 3796.0 ± 518.9 kJ when they received vehicle, 4057.5 ± 555.0 kJ when they received glycine 0.04g/kg and 3680.0 ± 638.0 kJ when they received cysteine 0.04g/kg . This is illustrated in figure 3.13 below.

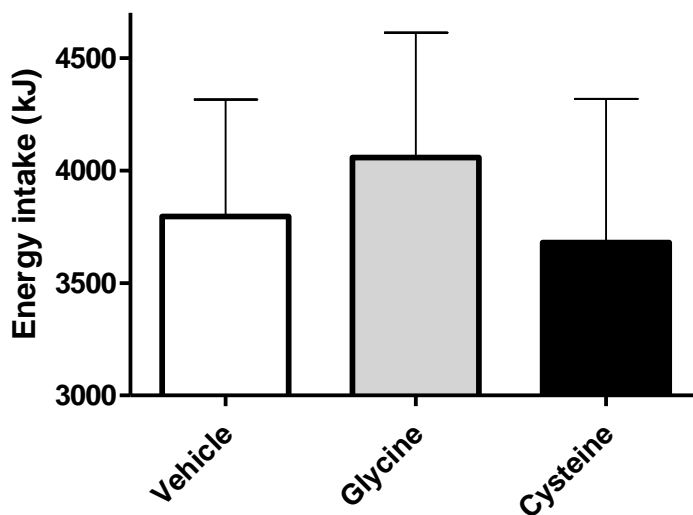


Figure 3.13. Effect of vehicle, glycine and cysteine on *ad libitum* food intake.

On each study day, vehicle glycine (0.04g/kg) or cysteine (0.04g/kg) was administered at $t=0$ min. An *ad libitum* buffet meal was given at $t=60$ min in order to measure energy intake on all study days. The energy intake during the *ad libitum* meal following each amino acid administration is shown as mean \pm SEM for 11 subjects.

3.6.2. Food diary analysis

There was no significant difference between food intake following the study visit based on food diary analysis following consumption of vehicle, glycine or cysteine. For the 9 subjects whose food diaries were complete and analysed, the mean energy intake on day 1 was 8221.9 ± 836.2 kJ when they received

vehicle, 8184.6 ± 735.2 kJ when they received glycine 0.04g/kg and 7651.4 ± 933.2 kJ when they received cysteine 0.04g/kg, on day 2 was 8394.2 ± 1090.4 kJ when they received vehicle, 9264.4 ± 1010.2 kJ when they received glycine 0.04g/kg and 8407.7 ± 1155.3 kJ when they received cysteine 0.04g/kg. This is illustrated in figure 3.14 below.

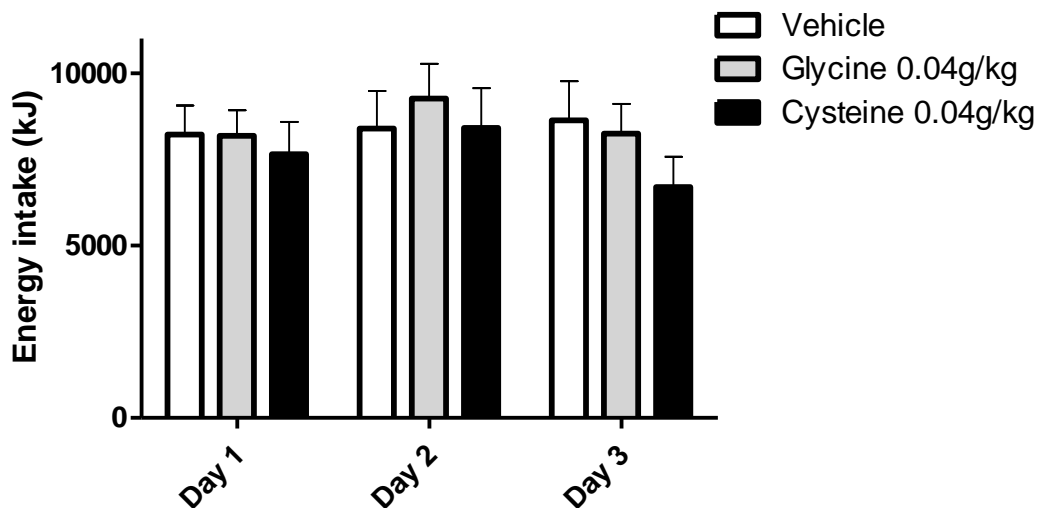


Figure 3.14. Effect of vehicle, glycine and cysteine on 3 day food intake.

Day 1 represents the day prior to the study visit. Day 2 represents the day of the study visit. Day 3 represents the day after the study visit. A 3 day food diary was given prospectively to all subjects prior to each study visit to complete in order to estimate energy intake prior to, during and after the study. On each study day vehicle, glycine (0.04g/kg) or cysteine (0.04g/kg) was administered at t=0 min. An *ad libitum* meal was given during the study visit (day 2) and this data is included. The energy intake for days 1-3 is shown as mean \pm SEM for 9 subjects.

3.7. To determine the effect of a higher dose of cysteine on food intake

Unfortunately the 0.07g/kg dose of cysteine induced minor adverse effects (dizziness) in one subject who took part in the study and hence this dose was not investigated further.

Chapter 4

Discussion

The studies described in this thesis investigated the effect of L-cysteine on appetite in humans. Studies from my laboratory had suggested L-cysteine had the largest anorectic effect of all of the proteinogenic amino acids in rodents. The results of my studies suggest that the higher dose of L-cysteine administered (0.07g/kg) may influence subjective measures of appetite. However, it was also associated with side effects in one subject.

The time course of the increase in circulating plasma cysteine was determined following administration of the high protein meal (consisting of grilled chicken). This meal resulted in a two-fold increase in mean plasma cysteine concentration from 1.9 μ mol/L (mean of baseline samples at t=-15 and t=0) to 4.0 μ mol/L (at t=60). The time course of the increase in circulating plasma cysteine was also determined following administration of oral cysteine. Oral administration of cysteine (0.04g/kg) resulted in a much higher increase in mean plasma cysteine concentration (from 2.6 μ mol/L (mean of baseline samples at t=-15 and t=0) to 29.5 μ mol/L (at t=60)) than the high protein meal. These results demonstrate that any effects that cysteine may have on appetite are unlikely to reflect a physiological effect achieved by circulating cysteine. However, it is possible that following a meal that cysteine acts in combination with other amino acid ligands of the GPRC6A receptor to mediate satiety physiologically.

Peak plasma cysteine levels are achieved at t=60 minutes with both the high protein meal and with administration of 0.04g/kg of cysteine. This was in contrast to plasma levels of all other amino acids measured (taurine, aspartate, threonine, serine, asparagine, glutamate, glutamine, glycine, alanine, valine, methionine, isoleucine, leucine, tyramine, phenylalanine, histidine, lysine, tryptophan, arginine, proline) following the high protein meal, which peaked between t=90 and t=105 (Appendix I). Plasma amino acid concentrations generally start to rise at t=30 for all amino acids except cysteine, alanine and glutamine where there is an increase from baseline by t=15 (Appendix I). By t=30, plasma cysteine levels following the high protein meal have already more than doubled. This rapid rise in plasma levels appears to be unique to cysteine, and does not occur with the other amino acids measured. From the visual analogue scales, the satiating effects of the high protein meal occur by t=30 minutes. This suggests that these particular amino acids may be responsible for the satiating effects of the high protein meal. Alanine and cysteine are both amino acid ligands of the GPRC6A receptor.

The high protein meal reduced appetite, as demonstrated by the results of the visual analogue scales. There was a sustained effect over the time course of the study (ending at t=150). The satiating effect of protein on appetite has previously been demonstrated in numerous other studies (Bertenshaw et al., 2008, Crovetti et al., 1998, Porrini et al., 1997, Latner and Schwartz, 1999). In addition, the satiating effect of protein has been demonstrated to be greater than that resulting from the ingestion of other macronutrients (Porrini et al., 1997, Reid and Hetherington, 1997, Trigazis et al., 1997, Bensaid et al., 2002, Anderson and Moore, 2004, Halton and Hu, 2004).

At the lower doses of amino acids (0.04g/kg), there were no significant effects on subjective measures of appetite between treatments. There was a trend towards significance ($p=0.0875$) in reduction of mean AUC when subjects were questioned on "How much could you eat?" with cysteine when compared to glycine. Interestingly, at the higher dose of cysteine (0.07g/kg), there was a significant reduction in mean AUC when subjects were questioned on "How hungry do you feel?" ($p<0.05$), "How pleasant would it be to eat?" ($p<0.05$) and "How much could you eat?" ($p<0.05$). This suggests that this dose of cysteine can reduce appetite.

Visual analogue scales have been shown to be a valid and reproducible measure of appetite (Flint et al., 2000a) and are used extensively in appetite research. The questions on the VAS used in these studies correlate with hunger ("How hungry do you feel?"), fullness ("How full do you feel?"), prospective food consumption ("How much could you eat?") and desire to eat ("How pleasant would it be to eat?"). The predictive validity of subjective motivation to eat (as determined by desire to eat, hunger, fullness and prospective consumption) on VAS has been studied, with all of these above reported to predict subsequent energy intake (Flint et al., 2000a). Another study has suggested that the responses to the scales for desire to eat and prospective consumption both accurately predict forthcoming food intake (Barkeling et al., 1995). This suggests these parameters in the VAS are the most useful subjective measures of appetite.

The VAS also serve to elucidate any ill effects related to the study treatment. Macronutrients can cause nausea at high doses (Chapman et al., 1999, Williamson et al., 2005) and such feelings could potentially contribute to any effects that cysteine has on appetite. However, the VAS results suggest that cysteine (at both doses) did not induce nausea. There was a significant increase in anxiety with the lower dose of cysteine when compared to glycine (0.04g/kg). However the absolute difference

was small, and may reflect that most subjects received cysteine at their very first study visit, and hence were perhaps more anxious as a result of this. This finding is unlikely to be of clinical relevance, particularly as there was no difference with the higher dose of cysteine.

Although energy intake following administration of cysteine (0.04g/kg) was lower compared to glycine, the difference was very small, and was not statistically significant. Unfortunately the higher dose of cysteine was not investigated in the energy intake study as one subject experienced adverse effects (dizziness). Cysteine is actively transported across the blood brain barrier (Hawkins et al., 2006) and hence the adverse effect may be related to a central effect of cysteine at the higher dose (0.07g/kg). Previous studies on oral cysteine where such side-effects have been reported have used higher doses (up to 20g) (Davis et al., 1972) compared to the doses administered here (maximum 6.5g).

Thus my studies have been unable to demonstrate an effect of cysteine on energy intake, but suggest that cysteine may influence subjective measures of appetite. The mechanism for this effect is unknown. Cysteine is a ligand at the CaR, T1R1/T1R3 receptor and the GPRC6A receptor; promiscuous L-amino acid sensing receptors present in the gut (Haid et al., 2011b, Daly et al., 2013, Chattopadhyay et al., 1998). *In vitro* work has shown that these receptors have a preference for particular classes of amino acids (Figure 4.1), thus in combination potentially responding to all 20 proteinogenic L-amino acids (Wellendorph et al., 2009, Conigrave and Hampson, 2006).

The primary ligands of the CaR are calcium ions (Brown et al., 1993), although the receptor has also been shown to respond to L-amino acids, in particular aromatic amino acids such as L-tryptophan and L-phenylalanine (Conigrave et al., 2000). The CaR is insensitive to, or only weakly activated by, acidic, basic and branched-chain amino acids (Conigrave et al., 2000). In a rat *ex vivo* small intestine preparation, the amino acid-stimulated release of GLP-1, PYY and gastric inhibitory polypeptide (GIP) was blocked by the CaR inhibitor Calhex 231 and augmented by the CaR agonist NPS-R568 (Mace et al., 2012), suggesting a role for the CaR in the release of gut hormones that can influence food intake. However, the CaR knockout mouse phenotype suggests the primary role of CaR is in calcium homeostasis, rather than appetite (Ho et al., 1995).

The T1R1/T1R3 receptor has also been identified in the gut (Bezencon et al., 2007, Dyer et al., 2005). The mouse and human T1R1/T1R3 receptor differ in their relative affinities. The mouse receptor has a particular affinity for aliphatic (including branched chain), amidic, charged, sulphur- and hydroxyl-containing L-amino acids, but not the aromatic amino acids (Nelson et al., 2002, Zhao et al., 2003). The human T1R1/T1R3 receptor has been shown to be more sensitive to L-glutamate and L-aspartate than to other L-amino acids (Li et al., 2002). It is worth noting that the T1R3 receptor also heterodimerises with the T1R2 receptor to form a sweet-sensing receptor (Nelson et al., 2001, Li et al., 2002). The T1R2/T1R3 receptor and members of its transduction pathway have been shown to be present in the gut and to be involved in the glucose-stimulated release of GLP-1 (Jang et al., 2007, Margolskee et al., 2007). The T1R2/T1R3 receptor can also increase glucose-stimulated insulin secretion from pancreatic β -cells (Kyriazis et al., 2012). These data suggest that the taste receptor family is involved in hormone release. However, little is known regarding the role of T1R1/T1R3 in gut hormone release or in satiety.

The GPRC6A receptor is an amino acid sensing receptor, which has been shown to induce GLP-1 secretion from an intestinal L cell line (Oya et al., 2013), thus presenting a mechanism by which this amino acid sensing receptor may mediate satiety. Studies within the research groups on rodent models suggest that amino acid ligands of the GPRC6A receptor do not stimulate the release of GLP-1 and PYY. The rat GPRC6A receptor is most responsive to L-ornithine, glycine and L-citrulline (Wellendorph et al., 2007), the mouse GPRC6A receptor is most responsive to L-ornithine, L-arginine, L-lysine, L-serine, L-alanine, glycine, L-histidine and L-cysteine, whilst the most active agonists at the human GPRC6A receptor are the basic L-amino acids, L-arginine, L-lysine, and L-ornithine (Wellendorph et al., 2005). Any effects that have been seen are unlikely to be occurring via the GPRC6A receptor, as glycine strongly binds to this receptor, yet has little effect on appetite. It is possible that one of the other amino acid sensing receptors may be mediating the effects e.g. the CaR which strongly binds L-cysteine compared to glycine.

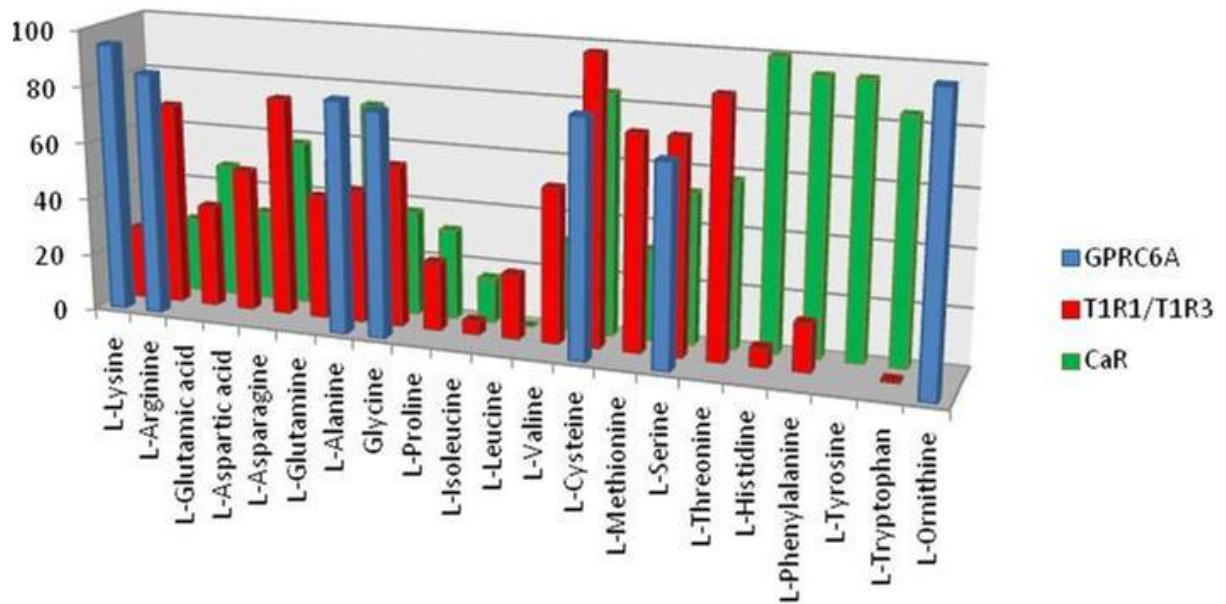


Figure 4.1. L-Amino acid selectivity profiles at CaR, GPRC6A, and the T1R1/T1R3 heterodimer.

Amino acids are grouped according to side-chain charge and polarity. Data have been normalized to allow for comparison of relative amino acid preferences. The profile for CaR was generated by calculating the potentiating effect of each of the 20 amino acids (at 10 mM concentrations) on the Ca^{2+} response. Numbers represent percentage response relative to that of L-histidine (100%) (Conigrave et al., 2000). The profile for GPRC6A is based on reported EC50 values of all 20 L-amino acids from mouse GPRC6A measured in the presence of $1\text{mM}\text{Ca}^{2+}$ and $1\text{mM}\text{Mg}^{2+}$ (Christiansen et al., 2007), here normalized to the L-lysine response (set to 100%). Mouse T1R1/T1R3 data originally in the form of “number of responsive cells” measured in the presence of $2.5\text{mM}\text{MIMP}$ (Nelson et al., 2002) and converted to percentage normalized response by calculating the response relative to that of L-cysteine (set to 100%) (taken from Wellendorph et al, 2009).

These studies suggest that cysteine may exert a pharmacological effect in reducing appetite. It is possible that this pharmacological effect reflects a physiological role; at physiological concentrations, cysteine, may act in concert with other L-amino acids, to mediate part of protein-induced satiety. The mechanism by which cysteine reduces appetite remains unknown. One possible explanation is the satiety effects are mediated by gut hormone release, however my results do not support that. There may be direct effects of cysteine on gut motility, which influence appetite. Cysteine may act directly on appetite centres in the brain to induce satiety. Clinical studies are unlikely to elucidate the mechanism by which amino acids mediate

satiety and, if amino acid sensing receptors are involved, to determine the particular receptor responsible for these effects. Pre-clinical studies using knockout mice or pharmacological antagonists are required to determine the receptors involved in protein and amino acid-induced satiety, and the mechanisms by which this process occurs.

FUTURE WORK

As cysteine may induce side effects as observed in one of my study participants, it is unlikely to be a good target for an anti-obesity agent. However, it may be possible to therapeutically exploit the mechanisms by which it influences appetite. I intend to look at better amino acid ligands of the GPRC6A receptor, including L-arginine and L-lysine (Wellendorph et al., 2009) which animal data from within the laboratory strongly suggests may have anorectic effects.

References

- ADRIAN, T. E., BLOOM, S. R., BRYANT, M. G., POLAK, J. M., HEITZ, P. H. & BARNES, A. J. 1976. Distribution and release of human pancreatic polypeptide. *Gut*, 17, 940-44.
- ADRIAN, T. E., FERRI, G. L., BACARESE-HAMILTON, A. J., FUESSL, H. S., POLAK, J. M. & BLOOM, S. R. 1985. Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology*, 89, 1070-7.
- AL AWAR, R., OBEID, O., HWALLA, N. & AZAR, S. 2005. Postprandial acylated ghrelin status following fat and protein manipulation of meals in healthy young women. *Clin Sci (Lond)*, 109, 405-11.
- ANDERSON, G. H. & MOORE, S. E. 2004. Dietary proteins in the regulation of food intake and body weight in humans. *J Nutr*, 134, 974S-9S.
- AZPIROZ, F. & MALAGELADA, J. R. 1987. Gastric tone measured by an electronic barostat in health and postsurgical gastroparesis. *Gastroenterology*, 92, 934-43.
- BADMAN, M. K. & FLIER, J. S. 2005. The gut and energy balance: visceral allies in the obesity wars. *Science*, 307, 1909-14.
- BARKELING, B., ROSSNER, S. & SJOBERG, A. 1995. Methodological studies on single meal food intake characteristics in normal weight and obese men and women. *Int J Obes Relat Metab Disord*, 19, 284-90.
- BATTERHAM, R. L., HEFFRON, H., KAPOOR, S., CHIVERS, J. E., CHANDARANA, K., HERZOG, H., LE ROUX, C. W., THOMAS, E. L., BELL, J. D. & WITHERS, D. J. 2006. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell Metab*, 4, 223-33.
- BENSAID, A., TOME, D., GIETZEN, D., EVEN, P., MORENS, C., GAUSSERES, N. & FROMENTIN, G. 2002. Protein is more potent than carbohydrate for reducing appetite in rats. *Physiol Behav*, 75, 577-82.
- BENSAID, A., TOME, D., L'HEUREUX-BOURDON, D., EVEN, P., GIETZEN, D., MORENS, C., GAUDICHON, C., LARUE-ACHAGIOTIS, C. & FROMENTIN, G. 2003. A high-protein diet enhances satiety without conditioned taste aversion in the rat. *Physiol Behav*, 78, 311-20.
- BERTENSHAW, E. J., LLUCH, A. & YEOMANS, M. R. 2008. Satiating effects of protein but not carbohydrate consumed in a between-meal beverage context. *Physiol Behav*, 93, 427-36.
- BEZENCON, C., LE COUTRE, J. & DAMAK, S. 2007. Taste-signaling proteins are coexpressed in solitary intestinal epithelial cells. *Chem Senses*, 32, 41-9.
- BLOM, W. A., LLUCH, A., STAFLEU, A., VINOY, S., HOLST, J. J., SCHAAFSMA, G. & HENDRIKS, H. F. 2006. Effect of a high-protein breakfast on the postprandial ghrelin response. *Am J Clin Nutr*, 83, 211-20.
- BLOUET, C., ONO, H. & SCHWARTZ, G. J. 2008. Mediobasal hypothalamic p70 S6 kinase 1 modulates the control of energy homeostasis. *Cell Metab*, 8, 459-67.

- BLOUET, C. & SCHWARTZ, G. J. 2010. Hypothalamic nutrient sensing in the control of energy homeostasis. *Behav Brain Res*, 209, 1-12.
- BLOUET, C. & SCHWARTZ, G. J. 2012. Duodenal lipid sensing activates vagal afferents to regulate non-shivering brown fat thermogenesis in rats. *PLoS one*, 7, e51898.
- BOWEN, J., NOAKES, M. & CLIFTON, P. M. 2006a. Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake. *J Clin Endocrinol Metab*, 91, 2913-9.
- BOWEN, J., NOAKES, M. & CLIFTON, P. M. 2007. Appetite hormones and energy intake in obese men after consumption of fructose, glucose and whey protein beverages. *Int J Obes (Lond)*, 31, 1696-703.
- BOWEN, J., NOAKES, M., TRENERRY, C. & CLIFTON, P. M. 2006b. Energy intake, ghrelin, and cholecystokinin after different carbohydrate and protein preloads in overweight men. *J Clin Endocrinol Metab*, 91, 1477-83.
- BROWN, E. M., GAMBA, G., RICCARDI, D., LOMBARDI, M., BUTTERS, R., KIFOR, O., SUN, A., HEDIGER, M. A., LYTTON, J. & HEBERT, S. C. 1993. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature*, 366, 575-80.
- BUTLAND, B. J., S; KOPELMAN, P; MCPHERSON, K; THOMAS, S; MARDELL, J; PARRY, V. 2008. *Foresight Tackling Obesity: Future Choices - Project report* [Online]. [Accessed].
- CALBET, J. A. & HOLST, J. J. 2004. Gastric emptying, gastric secretion and enterogastrone response after administration of milk proteins or their peptide hydrolysates in humans. *Eur J Nutr*, 43, 127-39.
- CARLSON, H. E., MIGLIETTA, J. T., ROGINSKY, M. S. & STEGINK, L. D. 1989. Stimulation of pituitary hormone secretion by neurotransmitter amino acids in humans. *Metabolism*, 38, 1179-82.
- CASPI, L., WANG, P. Y. & LAM, T. K. 2007. A balance of lipid-sensing mechanisms in the brain and liver. *Cell Metab*, 6, 99-104.
- CHAPMAN, I. M., GOBLE, E. A., WITTERT, G. A. & HOROWITZ, M. 1999. Effects of small-intestinal fat and carbohydrate infusions on appetite and food intake in obese and nonobese men. *The American journal of clinical nutrition*, 69, 6-12.
- CHATTOPADHYAY, N., CHENG, I., ROGERS, K., RICCARDI, D., HALL, A., DIAZ, R., HEBERT, S. C., SOYBEL, D. I. & BROWN, E. M. 1998. Identification and localization of extracellular Ca(2+)-sensing receptor in rat intestine. *Am J Physiol*, 274, G122-30.
- CHAUDHRI, O., SMALL, C. & BLOOM, S. 2006. Gastrointestinal hormones regulating appetite. *Philos Trans R Soc Lond B Biol Sci*, 361, 1187-209.
- CHEUNG, G. W., KOKOROVIC, A. & LAM, T. K. 2009. Upper intestinal lipids regulate energy and glucose homeostasis. *Cell Mol Life Sci*, 66, 3023-7.
- CHRISTIANSEN, B., HANSEN, K. B., WELLENDORPH, P. & BRAUNER-OSBORNE, H. 2007. Pharmacological characterization of mouse GPRC6A, an L-alpha-amino-acid receptor modulated by divalent cations. *Br J Pharmacol*, 150, 798-807.

- CONE, R. D., COWLEY, M. A., BUTLER, A. A., FAN, W., MARKS, D. L. & LOW, M. J. 2001. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*, 25 Suppl 5, S63-7.
- CONIGRAVE, A. D. & HAMPSON, D. R. 2006. Broad-spectrum L-amino acid sensing by class 3 G-protein-coupled receptors. *Trends Endocrinol Metab*, 17, 398-407.
- CONIGRAVE, A. D., QUINN, S. J. & BROWN, E. M. 2000. L-amino acid sensing by the extracellular Ca²⁺-sensing receptor. *Proc Natl Acad Sci U S A*, 97, 4814-9.
- CROVETTI, R., PORRINI, M., SANTANGELO, A. & TESTOLIN, G. 1998. The influence of thermic effect of food on satiety. *Eur J Clin Nutr*, 52, 482-8.
- CUMMINGS, D. E., PURNELL, J. Q., FRAYO, R. S., SCHMIDOVA, K., WISSE, B. E. & WEIGLE, D. S. 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes*, 50, 1714-9.
- DALY, K., AL-RAMMAHI, M., MORAN, A., MARCELLO, M., NINOMIYA, Y. & SHIRAZI-BEECHEY, S. P. 2013. Sensing of amino acids by the gut-expressed taste receptor T1R1-T1R3 stimulates CCK secretion. *Am J Physiol Gastrointest Liver Physiol*, 304, G271-82.
- DALY, K., AL-RAMMAHI, M., MORAN, A. W., MARCELLO, M., NINOMIYA, Y. & SHIRAZI-BEECHEY, S. P. 2012. Sensing of amino acids by the gut-expressed taste receptor, T1R1-T1R3, stimulates CCK secretion. *American journal of physiology. Gastrointestinal and liver physiology*.
- DAVIS, J. D., WIRTSHAFTER, D., ASIN, K. E. & BRIEF, D. 1981. Sustained intracerebroventricular infusion of brain fuels reduces body weight and food intake in rats. *Science*, 212, 81-3.
- DAVIS, J. M., SPAIDE, J. K. & HIMWICH, H. E. 1972. Effects of tranlycypromine and L-cysteine on plasma amino acids in controls and schizophrenic patients. *The American journal of clinical nutrition*, 25, 302-10.
- DUMOULIN, V., MORO, F., BARCELO, A., DAKKA, T. & CUBER, J. C. 1998. Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. *Endocrinology*, 139, 3780-6.
- DYER, J., SALMON, K. S., ZIBRIK, L. & SHIRAZI-BEECHEY, S. P. 2005. Expression of sweet taste receptors of the T1R family in the intestinal tract and enteroendocrine cells. *Biochem Soc Trans*, 33, 302-5.
- DYER, J., VAYRO, S., KING, T. P. & SHIRAZI-BEECHEY, S. P. 2003. Glucose sensing in the intestinal epithelium. *European journal of biochemistry / FEBS*, 270, 3377-88.
- ERDMANN, J., LIPPL, F. & SCHUSDZIARRA, V. 2003. Differential effect of protein and fat on plasma ghrelin levels in man. *Regul Pept*, 116, 101-7.
- FLINT, A., RABEN, A., BLUNDELL, J. E. & ASTRUP, A. 2000a. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. *Int J Obes Relat Metab Disord*, 24, 38-48.

- FLINT, A., RABEN, A., BLUNDELL, J. E. & ASTRUP, A. 2000b. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*, 24, 38-48.
- FOSTER-SCHUBERT, K. E., OVERDUIN, J., PRUDOM, C. E., LIU, J., CALLAHAN, H. S., GAYLINN, B. D., THORNER, M. O. & CUMMINGS, D. E. 2008. Acyl and total ghrelin are suppressed strongly by ingested proteins, weakly by lipids, and biphasically by carbohydrates. *J Clin Endocrinol Metab*, 93, 1971-9.
- GARNER, D. M., OLMSTED, M. P., BOHR, Y. & GARFINKEL, P. E. 1982. The eating attitudes test: psychometric features and clinical correlates. *Psychological medicine*, 12, 871-8.
- GREENBERG, D., SMITH, G. P. & GIBBS, J. 1990. Intraduodenal infusions of fats elicit satiety in sham-feeding rats. *Am J Physiol*, 259, R110-8.
- GREENMAN, Y., GOLANI, N., GILAD, S., YARON, M., LIMOR, R. & STERN, N. 2004. Ghrelin secretion is modulated in a nutrient- and gender-specific manner. *Clin Endocrinol (Oxf)*, 60, 382-8.
- HAID, D., WIDMAYER, P. & BREER, H. 2011a. Nutrient sensing receptors in gastric endocrine cells. *Journal of molecular histology*, 42, 355-64.
- HAID, D., WIDMAYER, P. & BREER, H. 2011b. Nutrient sensing receptors in gastric endocrine cells. *J Mol Histol*, 42, 355-64.
- HALL, W. L., MILLWARD, D. J., LONG, S. J. & MORGAN, L. M. 2003. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *Br J Nutr*, 89, 239-48.
- HALTON, T. L. & HU, F. B. 2004. The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. *J Am Coll Nutr*, 23, 373-85.
- HANNAH, J. S., DUBEY, A. K. & HANSEN, B. C. 1990. Postingestional effects of a high-protein diet on the regulation of food intake in monkeys. *Am J Clin Nutr*, 52, 320-5.
- HARPER, A. E. & PETERS, J. C. 1989. Protein intake, brain amino acid and serotonin concentrations and protein self-selection. *J Nutr*, 119, 677-89.
- HAWKINS, R. A., O'KANE, R. L., SIMPSON, I. A. & VINA, J. R. 2006. Structure of the blood-brain barrier and its role in the transport of amino acids. *J Nutr*, 136, 218S-26S.
- HILL, J. O. & PETERS, J. C. 2002. Biomarkers and functional foods for obesity and diabetes. *Br J Nutr*, 88 Suppl 2, S213-8.
- HIRASAWA, A., TSUMAYA, K., AWAJI, T., KATSUMA, S., ADACHI, T., YAMADA, M., SUGIMOTO, Y., MIYAZAKI, S. & TSUJIMOTO, G. 2005. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med*, 11, 90-4.
- HO, C., CONNER, D. A., POLLAK, M. R., LADD, D. J., KIFOR, O., WARREN, H. B., BROWN, E. M., SEIDMAN, J. G. & SEIDMAN, C. E. 1995. A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Nat Genet*, 11, 389-94.
- HOFER, D., ASAN, E. & DRENCKHAHN, D. 1999. Chemosensory Perception in the Gut. *News Physiol Sci*, 14, 18-23.

- HOROWITZ, M. & DENT, J. 1991. Disordered gastric emptying: mechanical basis, assessment and treatment. *Baillieres Clin Gastroenterol*, 5, 371-407.
- HOSODA, H., DOI, K., NAGAYA, N., OKUMURA, H., NAKAGAWA, E., ENOMOTO, M., ONO, F. & KANGAWA, K. 2004. Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. *Clin Chem*, 50, 1077-80.
- JANG, H. J., KOKRASHVILI, Z., THEODORAKIS, M. J., CARLSON, O. D., KIM, B. J., ZHOU, J., KIM, H. H., XU, X., CHAN, S. L., JUHASZOVA, M., BERNIER, M., MOSINGER, B., MARGOLSKEE, R. F. & EGAN, J. M. 2007. Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. *Proc Natl Acad Sci U S A*, 104, 15069-74.
- JEAN, C., ROME, S., MATHE, V., HUNEAU, J. F., AATTOURI, N., FROMENTIN, G., ACHAGIOTIS, C. L. & TOME, D. 2001. Metabolic evidence for adaptation to a high protein diet in rats. *J Nutr*, 131, 91-8.
- KALRA, S. P., DUBE, M. G., PU, S., XU, B., HORVATH, T. L. & KALRA, P. S. 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine reviews*, 20, 68-100.
- KELLY, T., YANG, W., CHEN, C. S., REYNOLDS, K. & HE, J. 2008. Global burden of obesity in 2005 and projections to 2030. *Int J Obes (Lond)*, 32, 1431-7.
- KINZIG, K. P., HARGRAVE, S. L., HYUN, J. & MORAN, T. H. 2007. Energy balance and hypothalamic effects of a high-protein/low-carbohydrate diet. *Physiol Behav*, 92, 454-60.
- KOJIMA, M., HOSODA, H., DATE, Y., NAKAZATO, M., MATSUO, H. & KANGAWA, K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, 402, 656-60.
- KREYMANN, B., WILLIAMS, G., GHATEI, M. A. & BLOOM, S. R. 1987. Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet*, 2, 1300-4.
- KUANG, D., YAO, Y., LAM, J., TSUSHIMA, R. G. & HAMPSON, D. R. 2005. Cloning and characterization of a Family C orphan G-protein coupled receptor. *Journal of Neurochemistry*, 93, 383-391.
- KYRIAZIS, G. A., SOUNDARAPANDIAN, M. M. & TYRBERG, B. 2012. Sweet taste receptor signaling in beta cells mediates fructose-induced potentiation of glucose-stimulated insulin secretion. *Proc Natl Acad Sci U S A*, 109, E524-32.
- LAM, T. K., SCHWARTZ, G. J. & ROSSETTI, L. 2005. Hypothalamic sensing of fatty acids. *Nat Neurosci*, 8, 579-84.
- LATNER, J. D. & SCHWARTZ, M. 1999. The effects of a high-carbohydrate, high-protein or balanced lunch upon later food intake and hunger ratings. *Appetite*, 33, 119-28.
- LAYER, P., HOLST, J. J., GRANDT, D. & GOEBELL, H. 1995. Ileal release of glucagon-like peptide-1 (GLP-1). Association with inhibition of gastric acid secretion in humans. *Digestive diseases and sciences*, 40, 1074-82.

- LEIDY, H. J., MATTES, R. D. & CAMPBELL, W. W. 2007. Effects of acute and chronic protein intake on metabolism, appetite, and ghrelin during weight loss. *Obesity (Silver Spring)*, 15, 1215-25.
- LI, X., STASZEWSKI, L., XU, H., DURICK, K., ZOLLER, M. & ADLER, E. 2002. Human receptors for sweet and umami taste. *Proc Natl Acad Sci U S A*, 99, 4692-6.
- MARGOLSKEE, R. F., DYER, J., KOKRASHVILI, Z., SALMON, K. S., ILEGEMS, E., DALY, K., MAILLET, E. L., NINOMIYA, Y., MOSINGER, B. & SHIRAZI-BEECHEY, S. P. 2007. T1R3 and gustducin in gut sense sugars to regulate expression of Na⁺-glucose cotransporter 1. *Proc Natl Acad Sci U S A*, 104, 15075-80.
- MATZINGER, D., DEGEN, L., DREWE, J., MEULI, J., DUEBENDORFER, R., RUCKSTUHL, N., D'AMATO, M., ROVATI, L. & BEGLINGER, C. 2000. The role of long chain fatty acids in regulating food intake and cholecystokinin release in humans. *Gut*, 46, 688-693.
- MITRAKOU, A., RYAN, C., VENEMAN, T., MOKAN, M., JENSSEN, T., KISS, I., DURRANT, J., CRYER, P. & GERICH, J. 1991. Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction. *Am J Physiol*, 260, E67-74.
- MORAN, T. H. 2000. Cholecystokinin and satiety: current perspectives. *Nutrition*, 16, 858-65.
- MORAN, T. H. & KINZIG, K. P. 2004. Gastrointestinal satiety signals II. Cholecystokinin. *Am J Physiol Gastrointest Liver Physiol*, 286, G183-8.
- MORENS, C., GAUDICHON, C., FROMENTIN, G., MARSSET-BAGLIERI, A., BENSALD, A., LARUE-ACHAGIOTIS, C., LUENGO, C. & TOME, D. 2001. Daily delivery of dietary nitrogen to the periphery is stable in rats adapted to increased protein intake. *Am J Physiol Endocrinol Metab*, 281, E826-36.
- MORENS, C., GAUDICHON, C., METGES, C. C., FROMENTIN, G., BAGLIERI, A., EVEN, P. C., HUNEAU, J. F. & TOME, D. 2000. A high-protein meal exceeds anabolic and catabolic capacities in rats adapted to a normal protein diet. *J Nutr*, 130, 2312-21.
- MORGAN, J. F., REID, F. & LACEY, J. H. 1999. The SCOFF questionnaire: assessment of a new screening tool for eating disorders. *BMJ*, 319, 1467-8.
- MURPHY, K. G. & BLOOM, S. R. 2006. Gut hormones and the regulation of energy homeostasis. *Nature*, 444, 854-9.
- NELSON, G., CHANDRASHEKAR, J., HOON, M. A., FENG, L., ZHAO, G., RYBA, N. J. & ZUKER, C. S. 2002. An amino-acid taste receptor. *Nature*, 416, 199-202.
- NELSON, G., HOON, M. A., CHANDRASHEKAR, J., ZHANG, Y., RYBA, N. J. & ZUKER, C. S. 2001. Mammalian sweet taste receptors. *Cell*, 106, 381-90.
- NILSSON, M., STENBERG, M., FRID, A. H., HOLST, J. J. & BJORCK, I. M. 2004. Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. *Am J Clin Nutr*, 80, 1246-53.
- OYA, M., KITAGUCHI, T., PAIS, R., REIMANN, F., GRIBBLE, F. & TSUBOI, T. 2013. The G protein-coupled receptor family C group 6 subtype A

- (GPRC6A) receptor is involved in amino acid-induced glucagon-like peptide-1 secretion from GLUTag cells. *J Biol Chem*, 288, 4513-21.
- PANKSEPP, J. & ROSSI, J., 3RD 1981. D-glucose infusions into the basal ventromedial hypothalamus and feeding. *Behav Brain Res*, 3, 381-92.
- PERSEGHIN, G., REGALIA, E., BATTEZZATI, A., VERGANI, S., PULVIRENTI, A., TERRUZZI, I., BARATTI, D., BOZZETTI, F., MAZZAFERRO, V. & LUZI, L. 1997. Regulation of glucose homeostasis in humans with denervated livers. *J Clin Invest*, 100, 931-41.
- PI, M., CHEN, L., HUANG, M. Z., ZHU, W., RINGHOFER, B., LUO, J., CHRISTENSON, L., LI, B., ZHANG, J., JACKSON, P. D., FABER, P., BRUNDEN, K. R., HARRINGTON, J. J. & QUARLES, L. D. 2008. GPRC6A null mice exhibit osteopenia, feminization and metabolic syndrome. *PLoS One*, 3, e3858.
- PI, M., FABER, P., EKEMA, G., JACKSON, P. D., TING, A., WANG, N., FONTILLA-POOLE, M., MAYS, R. W., BRUNDEN, K. R., HARRINGTON, J. J. & QUARLES, L. D. 2005. Identification of a novel extracellular cation-sensing G-protein-coupled receptor. *J Biol Chem*, 280, 40201-9.
- PORRINI, M., SANTANGELO, A., CROVETTI, R., RISO, P., TESTOLIN, G. & BLUNDELL, J. E. 1997. Weight, protein, fat, and timing of preloads affect food intake. *Physiol Behav*, 62, 563-70.
- POTIER, M., DARCEL, N. & TOME, D. 2009. Protein, amino acids and the control of food intake. *Curr Opin Clin Nutr Metab Care*, 12, 54-8.
- RAYBOULD, H. E. 2007. Sensing of glucose in the gastrointestinal tract. *Autonomic neuroscience : basic & clinical*, 133, 86-90.
- RAYBOULD, H. E. & HOLZER, H. 1992. Dual capsaicin-sensitive afferent pathways mediate inhibition of gastric emptying in rat induced by intestinal carbohydrate. *Neurosci Lett*, 141, 236-8.
- REID, M. & HETHERINGTON, M. 1997. Relative effects of carbohydrates and protein on satiety -- a review of methodology. *Neurosci Biobehav Rev*, 21, 295-308.
- REY, O., CHANG, W., BIKLE, D., ROZENGURT, N., YOUNG, S. H. & ROZENGURT, E. 2012. Negative cross-talk between calcium-sensing receptor and beta-catenin signaling systems in colonic epithelium. *The Journal of biological chemistry*, 287, 1158-67.
- ROPELLE, E. R., PAULI, J. R., FERNANDES, M. F., ROCCO, S. A., MARIN, R. M., MORARI, J., SOUZA, K. K., DIAS, M. M., GOMES-MARCONDES, M. C., GONTIJO, J. A., FRANCHINI, K. G., VELLOSO, L. A., SAAD, M. J. & CARVALHEIRA, J. B. 2008. A central role for neuronal AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) in high-protein diet-induced weight loss. *Diabetes*, 57, 594-605.
- ROZE, C., COUTURIER, D., CHARLOT, J. & DEBRAY, C. 1977. Inhibition of gastric electrical and mechanical activity by intraduodenal agents in pigs and the effects of vagotomy. *Digestion*, 15, 526-39.
- SARGRAD, K. R., HOMKO, C., MOZZOLI, M. & BODEN, G. 2005. Effect of high protein vs high carbohydrate intake on insulin sensitivity, body weight, hemoglobin A1c, and blood pressure in patients with type 2

- diabetes mellitus. *Journal of the American Dietetic Association*, 105, 573-80.
- SCHWARTZ, G. J. 2000. The role of gastrointestinal vagal afferents in the control of food intake: current prospects. *Nutrition*, 16, 866-73.
- SCHWARTZ, G. J., BERKOW, G., MCHUGH, P. R. & MORAN, T. H. 1993. Gastric branch vagotomy blocks nutrient and cholecystokinin-induced suppression of gastric emptying. *Am J Physiol*, 264, R630-7.
- SCHWARTZ, M. W., WOODS, S. C., PORTE, D., JR., SEELEY, R. J. & BASKIN, D. G. 2000. Central nervous system control of food intake. *Nature*, 404, 661-71.
- STANLEY, S., WYNNE, K. & BLOOM, S. 2004. Gastrointestinal satiety signals III. Glucagon-like peptide 1, oxyntomodulin, peptide YY, and pancreatic polypeptide. *American journal of physiology. Gastrointestinal and liver physiology*, 286, G693-7.
- STANLEY, S., WYNNE, K., MCGOWAN, B. & BLOOM, S. 2005. Hormonal regulation of food intake. *Physiol Rev*, 85, 1131-58.
- STERNINI, C., ANSELMINI, L. & ROZENGURT, E. 2008. Enteroendocrine cells: a site of 'taste' in gastrointestinal chemosensing. *Current opinion in endocrinology, diabetes, and obesity*, 15, 73-8.
- STOECKEL, L. E., WELLER, R. E., GIDDINGS, M. & COX, J. E. 2008. Peptide YY levels are associated with appetite suppression in response to long-chain fatty acids. *Physiol Behav*, 93, 289-95.
- STUBBS, R. J., HUGHES, D. A., JOHNSTONE, A. M., ROWLEY, E., REID, C., ELIA, M., STRATTON, R., DELARGY, H., KING, N. & BLUNDELL, J. E. 2000. The use of visual analogue scales to assess motivation to eat in human subjects: a review of their reliability and validity with an evaluation of new hand-held computerized systems for temporal tracking of appetite ratings. *The British journal of nutrition*, 84, 405-15.
- TABORSKY, G. J., JR., AHREN, B. & HAVEL, P. J. 1998. Autonomic mediation of glucagon secretion during hypoglycemia: implications for impaired alpha-cell responses in type 1 diabetes. *Diabetes*, 47, 995-1005.
- TANNOUS DIT EL KHOURY, D., OBEID, O., AZAR, S. T. & HWALLA, N. 2006. Variations in postprandial ghrelin status following ingestion of high-carbohydrate, high-fat, and high-protein meals in males. *Ann Nutr Metab*, 50, 260-9.
- TRIGAZIS, L., ORTTMANN, A. & ANDERSON, G. H. 1997. Effect of a cholecystokinin-A receptor blocker on protein-induced food intake suppression in rats. *Am J Physiol*, 272, R1826-33.
- TSCHOP, M., WAWARTA, R., RIEPL, R. L., FRIEDRICH, S., BIDLINGMAIER, M., LANDGRAF, R. & FOLWACZNY, C. 2001. Postprandial decrease of circulating human ghrelin levels. *J Endocrinol Invest*, 24, RC19-21.
- VAN DE WALL, E. H., DUFFY, P. & RITTER, R. C. 2005. CCK enhances response to gastric distension by acting on capsaicin-insensitive vagal afferents. *Am J Physiol Regul Integr Comp Physiol*, 289, R695-703.
- VAN DER KLAUW, A. A., KEOGH, J. M., HENNING, E., TROWSE, V. M., DHILLO, W. S., GHATEI, M. A. & FAROOQI, I. S. 2012. High protein intake stimulates postprandial GLP1 and PYY release. *Obesity*.

- VAN STRIEN, T., ROOKUS, M. A., BERGERS, G. P., FRIJTERS, J. E. & DEFARES, P. B. 1986. Life events, emotional eating and change in body mass index. *International journal of obesity*, 10, 29-35.
- VELDHORST, M. A., NIEUWENHUIZEN, A. G., HOCHSTENBACH-WAELEN, A., VAN VUGHT, A. J., WESTERTERP, K. R., ENGELEN, M. P., BRUMMER, R. J., DEUTZ, N. E. & WESTERTERP-PLANTENGA, M. S. 2009. Dose-dependent satiating effect of whey relative to casein or soy. *Physiol Behav*, 96, 675-82.
- WELLENDORPH, P. & BRAUNER-OSBORNE, H. 2004. Molecular cloning, expression, and sequence analysis of GPRC6A, a novel family C G-protein-coupled receptor. *Gene*, 335, 37-46.
- WELLENDORPH, P., BURHENNE, N., CHRISTIANSEN, B., WALTER, B., SCHMALE, H. & BRAUNER-OSBORNE, H. 2007. The rat GPRC6A: cloning and characterization. *Gene*, 396, 257-67.
- WELLENDORPH, P., HANSEN, K. B., BALSGAARD, A., GREENWOOD, J. R., EGEBJERG, J. & BRAUNER-OSBORNE, H. 2005. Deorphanization of GPRC6A: a promiscuous L-alpha-amino acid receptor with preference for basic amino acids. *Mol Pharmacol*, 67, 589-97.
- WELLENDORPH, P., JOHANSEN, L. D. & BRAUNER-OSBORNE, H. 2009. Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients. *Mol Pharmacol*, 76, 453-65.
- WHO 2012. World Health Statistics.
- WILKINSON, A. R. & JOHNSTON, D. 1973. Effect of truncal, selective and highly selective vagotomy on gastric emptying and intestinal transit of a food-barium meal in man. *Ann Surg*, 178, 190-3.
- WILLIAMSON, M. J., LEVINE, M. E. & STERN, R. M. 2005. The effect of meals of varying nutritional composition on subjective and physiological markers of nausea in response to optokinetic motion. *Digestion*, 72, 254-60.
- WOOD, W. G., WACHTER, C. & SCRIBA, P. C. 1981. Experiences using chloramine-T and 1, 3, 4, 6-tetrachloro-3 alpha, 6 alpha-diphenylglycoluril (Iodogen) for radioiodination of materials for radioimmunoassay. *J Clin Chem Clin Biochem*, 19, 1051-6.
- WREN, A. M., SEAL, L. J., COHEN, M. A., BRYNES, A. E., FROST, G. S., MURPHY, K. G., DHILLO, W. S., GHATEI, M. A. & BLOOM, S. R. 2001. Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab*, 86, 5992.
- WREN, A. M., SMALL, C. J., WARD, H. L., MURPHY, K. G., DAKIN, C. L., TAHERI, S., KENNEDY, A. R., ROBERTS, G. H., MORGAN, D. G. A., GHATEI, M. A. & BLOOM, S. R. 2000. The Novel Hypothalamic Peptide Ghrelin Stimulates Food Intake and Growth Hormone Secretion. *Endocrinology*, 141, 4325-4328.
- WRIGHT, E. M., MARTIN, M. G. & TURK, E. 2003. Intestinal absorption in health and disease--sugars. *Best practice & research. Clinical gastroenterology*, 17, 943-56.
- ZHAO, G. Q., ZHANG, Y., HOON, M. A., CHANDRASHEKAR, J., ERLNBACH, I., RYBA, N. J. & ZUKER, C. S. 2003. The receptors for mammalian sweet and umami taste. *Cell*, 115, 255-66.

APPENDIX I: Plasma concentrations of amino acids following a high protein meal

