Diese Arbeit widme ich
meiner Tochter Venus und
meinen Eltern Gerhard und Gabriele

MODULATION OF FOOD REWARD SYSTEMS BY FASTING AND GHRELIN: HUMAN FUNCTIONAL MRI STUDIES

Christina Prechtl de Hernandez

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Imperial College London
Faculty of Medicine
Department of Investigative Medicine and
MRC Clinical Science Centre
Hammersmith Hospital
London
United Kingdom

Supervisors: Prof. Jimmy Bell
Prof. Gary Frost
Dr. Tony Goldstone

ABSTRACT

Background: To understand the pathophysiology of obesity, the role of hedonic eating needs to be considered. Neuroimaging research has suggested that activity in brain reward systems is modulated by the rewarding properties of food, nutritional state, and individual differences in eating behaviour. The interaction between these factors and the influence of gut hormones, such as the stomach-derived orexigenic hormone ghrelin, on these systems remains unclear.

Methods: In the first study, 20 non-obese subjects were either fasted or ate a filling breakfast of their choice (fed). In the second study, 18 non-obese subjects were either fasted or given a fixed breakfast and received a subcutaneous saline or ghrelin injection. Subjects underwent functional MRI scanning while viewing and rating the appeal of food and object pictures and completed appetite and psychological questionnaires.

Results: The first fMRI study showed that fasting increased activation to pictures of high-calorie over low-calorie foods in the ventral striatum, amygdala, anterior insula, medial and lateral orbitofrontal cortex (OFC). Fasting also biased the appeal towards high-calorie foods. Dietary restraint positively correlated with activation in executive systems when viewing high-calorie compared to low-calorie foods, but negatively correlated with activation in affective systems. Reward drive positively correlated with activation in the ventral striatum to high-calorie foods.

In the second fMRI study activation to high-calorie foods in the insula and/or OFC when fasted and fed was shown to correlate with hunger and predict subsequent food intake. Administration of ghrelin was shown to mimic fasting to increase the appeal of high-calorie, especially sweet foods, and OFC activation to high-calorie foods.

Conclusion: The results suggest an interaction between homeostatic and hedonic aspects of eating behaviour, with fasting and ghrelin biasing brain reward systems towards high-calorie foods.

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STATEMENT OF WORK BY CANDIDATE

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- Dr. Adam Waldman (radiologist, reporting MRI scans)
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Fasting Biases Brain Reward Systems Towards High-Calorie Foods
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2011 Publications

Dietary Restraint Predicts Neural Responses in Executive Self-Control and Affective Reward Systems to Foods of Different Caloric Value
C. G. Prechtl, J. D. Beaver, C. Croese, K. Muhammed, G. Bell, G. Durighel, E. Hughes, A. D. Waldman, S. Scholtz, S. Delirian, G. Frost, J. D. Bell, and A. P. Goldstone
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ABSTRACTS RELATED TO WORK IN THESIS

2008 Oral Communications

Increased desire for food when fasted is associated with increased fMRI activation in the ventral striatum, insula and amygdala
A.P.Goldstone, **C.G. Prechtl de Hernandez**, J.D. Beaver, K.Muhammed, G.Bell, G.Durighel, E.Hughes, A.D.Waldman & J.D.Bell
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2008 Posters

Increased desire for food when fasted is associated with increased activation of the ventral striatum, insula and amygdala in humans
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British Endocrine Society, Harrogate April 2008
Endocrine Abstracts 15:204, 2008

Skipping breakfast biases brain reward systems towards high-calorie foods
C.G. Prechtl de Hernandez, J.D. Beaver, K. Muhammed, G.Bell, G.Durighel, E.Hughes, A.D.Waldman, J.D.Bell & A.P.Goldstone
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C.G. Prechtl de Hernandez, J.D. Beaver, C. Croese, K. Muhammed, G. Bell, G. Durighel, E. Hughes, A.D. Waldman, G. Frost, J.D. Bell, and A.P. Goldstone
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C.G. Prechtl de Hernandez, J.D. Beaver, C. Croese, K. Muhammed, G. Bell, G. Durighel, E. Hughes, A.D. Waldman, G. Frost, J.D. Bell1 & A.P. Goldstone
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Fasting Biases Brain Reward Systems Towards High-Calorie Foods
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Dietary Restraint Influence Brain Activation to Food Pictures of Different Caloric Value
C.G. Prechtl de Hernandez, J.D. Beaver, C. Croese, K. Muhammed, G. Bell, G. Durighel, E. Hughes, A.D. Waldman, G. Frost, J.D. Bell, and A.P. Goldstone
MRC CSC Retreat and Neuroscience Technology symposium South Kensington, London, UK. 7th October 2009

2010 Oral Communications

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American Endocrine Scociety, San Diego, USA, 19-22 June 2010

Ghrelin mimics fasting in preferentially increasing reward responses to high-calorie foods
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2010 Posters

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Insula and orbitofrontal cortex activation to food stimuli predicts human food intake.
C.G. Prechtl, S. Scholtz, N.S. Chhina, G. Durighel, G. Frost, J.D. Bell A.P. Goldstone
European Congress of Obesity, Istanbul, Turkey, 25-28 May 2011

ABBREVIATIONS

a-MSH	a-Melanocyte Stimulating Hormone
ACC	Anterior Cingulate Cortex
AC-PC	Anterior and Posterior commissure
AGRP	Agouti-Related Peptide
ANOVA	Analysis of Variance
ARC	Arcuate Nucleus
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BIS/BAS	Behavioural Inhibition and Activation System
BMI	Body Mass Index
BOLD	Blood Oxygen Level Depend
CART	Cocaine and Amphetamine Regulated Transcript
CCK	Cholecystokinin
CNS	Central Nervous System
CRH	Corticotropin-Releasing Hormone
DEBQ	Dutch Eating Behaviour Questionnaire
DMH	Dorso-Medial Hypothalamus
DMN	Dorso-Medial Nucleus
DLPFC	Dorso-Lateral Prefrontal Cortex
DTI	Diffusion Tensor Imaging
EPI	Echo Planar Imaging
FLAIR	Fluid Attenuation Inversion Recovery
fMRI	functional Magnetic Resonance Imaging
GLP-1	Glucagon-Like Peptide-1
GLP-1R	GLP-1 Receptor
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
GHS-R	Growth Hormone Secretagogue Receptor
GOAT	Ghrelin O-Acyltransferase
IHD	Ischaemic Heart Disease
iv	Intravenous
Kcal	Kilocalories
LBM	Lean Body Mass
LH	Lateral Hypothalamus
M	Molar
mg	Milligram
min	Minutes
ml	Millilitres
MCH	Melanin Concentrating Hormone
MEMRI	Manganese-Enhances MRI
MRI	Magnetic Resonance Imaging
MC4-R	Melanocortin Receptor 4
n	number
NHS	National Health Service
NMR	Nuclear Magnetic Resonance
NPY	Neuropeptide Y
NTS	Nucleus of the Tractus Solitarius
Ob-R	Leptin Receptor
OFC	Orbitofrontal Cortex
OXM	Oxytomodulin
PANAS	Positive and Negative Affect Schedule
PET	Positron Emission Tomography
PFA	Perifornical Area
PFC	Prefrontal Cortex
POMC	Proopiomelanocortin
PP	Pancreas Polypeptide

PVN	Paraventricular Nucleus
PYY3-36	Polypeptide YY 3-36
rCBF	regional Cerebral Blood Flow
RF	Radio Frequency
RIA	Radio-immunoassay
ROI	Region of Interest
SCOFF	Sick Control One Fat Food
SEM	Standard Error of the Mean
sc	Subcutaneous
sec	Seconds
SD	Standard Deviation
TE	Echo Time
TRH	Thyroid Hormone Receptor
TR	Repetition Time
UK	United Kingdom
µg	micrograms
µl	microlitres
VAS	Visual Analogue Scale
VMH	Ventro-Medial Hypothalamus
VTA	Ventral tegemental area
vmPFC	ventro-medial Prefrontal Cortex
WHO	World Health Organisation
Y1-Y5R	Receptors for polypeptide-fold family

Chapter 1

INTRODUCTION

1.1. Epidemiology of obesity

"We must do nothing less than transform the environment in which we all live" promoted the former prime minister Gordon Brown in the "Healthy Weight, Healthy Lives" report for England (Cross-Government Obesity Unit, 2008). Paradoxically, the Department of Health reports that in 2006, 24% of adults (aged over 16 years) and 16% of the children in England were classified as obese (NHS-Information-Centre, 2006). The World Health Organisation now estimates that 1.6 billion people are overweight (Body Mass Index, BMI, between 25 and 30 kg/m^2) and 400 million are obese (BMI above 30 kg/m^2) (WHO, 2006). Overweight and obesity are major contributors to cardiovascular disease, type II diabetes mellitus, musculoskeletal disorders and some cancers (WHO, 2006). In 2003, annual U.S. obesity attributable medical expenditure was estimated at $75 billion (Finkelstein et al., 2004) and surgical treatments such as bariatric surgery are associated with potential significant risks (le Roux CW, 2005). Other non-surgical therapies have proven effective at causing weight loss, but the magnitude and maintenance desired by many patients and physicians cannot yet be delivered, and many drugs have been withdrawn because of significant side effects (Bray, 2005; Christensen et al., 2007; Perrio et al., 2007; Johansson et al., 2009; Sayburn, 2010).

The epidemiology of obesity is complex. The fundamental cause of obesity and overweight is an energy imbalance between calories consumed and calories expended, a global shift in diet towards high-calorie, high palatable energy-dense foods that are high in fat and sugars and a trend towards decreased physical activity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization (WHO, 2006). However, for some of the populations it is not as simple as "eating less" and "walking more" in order to lose or maintain body weight. We need to better understand what appetite means, the underlying mechanisms and individual differences in the regulation of human eating behaviour with the aim to develop new strategies to tackle the obesity epidemic.

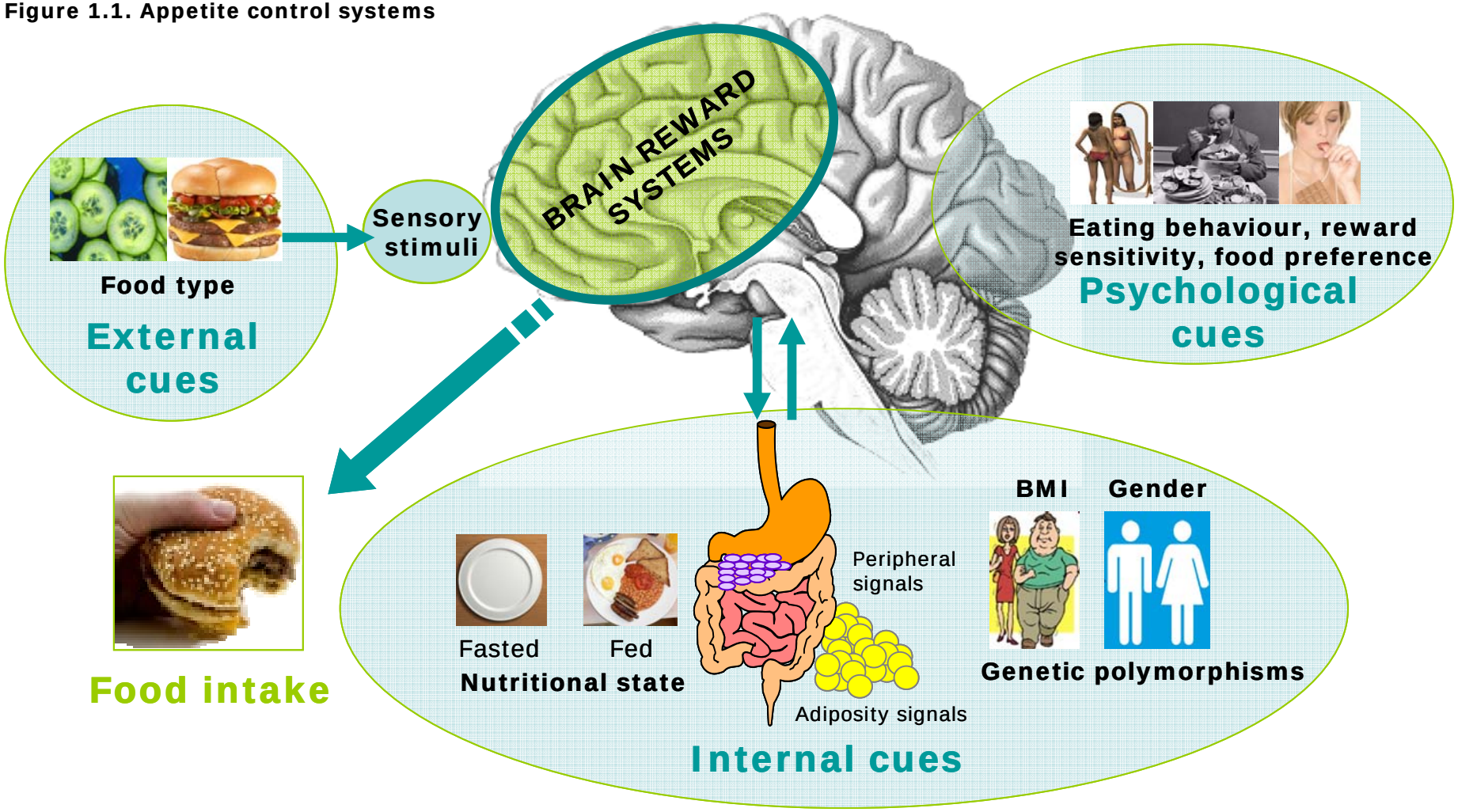
1.2. Appetite and eating behaviour

The etymology of "Appetite" is as follows: Old English appetite, French appétit, Latin appetitus, appetere ("'to strive after, long for'"); ad + petere ("'to seek'"). Appetite is described as an instinctive and physical strong desire; an eagerness or longing of, food or drink; hunger; the desire for some personal gratification, either of the body or of the mind (Davis and Faulds, 1996; FDA, 1997; Bray, 2005; Harper, 2009; Johansson et al., 2009; Sayburn, 2010).

Appetite control seems to be complex (Figure 1.1.) and the evolution of a physiological system that facilitates weight-gain in times of nutritional plenty, with little capacity to lose this again easily, and food seeking behaviour in times of food depriviation challenges the ability of researchers to find therapeutic targets to combat obesity.

Anti-obesity drugs such as Orlistat, Dexfenfluramine, Sibutramine and Rimonabant combined with exercise and diet decrease body weight by 5-15% but a regain of weight is often seen after 4-12 months (Pi-Sunyer et al., 1999, Thearle and Aronne, 2003, Curioni and Andre, 2006). The anorexigenic drugs produce dangerous side-effects and have therefore been withdrawn from the market (FDA, 1997; Bray, 2005; Christensen et al., 2007; Perrio et al., 2007; Sayburn, 2010). As a result of this, just one obesity drug treatment option is left, Orlistat, a drug that reduces intestinal fat absorption (Hartmann et al., 1993). The limited efficacy of pharmacological interventions means it is even more vital for scientists to study the body's own psychological and physiological signals for therapeutic purposes (Figure 1.1.). Furthermore, it is important to consider in addition to brain systems that control our eating behaviour, the role of food hedonics, individual food preference, nutritional state, gender, BMI, genetic factors and psychological cues and traits that influence these systems (Figure 1.1., Figure 1.5.).

Figure 1.1. Appetite control systems



The effect of external cues (smell, vision, taste, and imagination of food), internal cues (nutritional state, hormones, BMI and genetic polymorphisms) and psychological cues (eating behaviour, reward sensitivity and food preference) on brain reward systems.

1.2.1. Peripheral signals

1.2.1.1. Adiposity signals

The discovery of **leptin** (Greek: thin) and its receptor (Ob-R) in 1994 opened a new era in obesity research (Figure 1.2.). This anorexigenic hormone is a 167-amino acid protein encoded by the *ob* gene and released from adipose tissue in proportion to fat stores (Zhang et al., 1994, Campfield et al., 1995, Halaas et al., 1995, Sinha et al., 1996). Crossing the blood-brain barrier into the central nervous system, it acts at the level of the hypothalamus (activates POMC/CART neurons, inhibit NPY/AgRP neurons) by binding to its receptor and activating secondary signals that inhibit food intake and increase energy expenditure in *ob/ob* mice and diet-induced obese mice (Campfield et al., 1995, Golden et al., 1997).

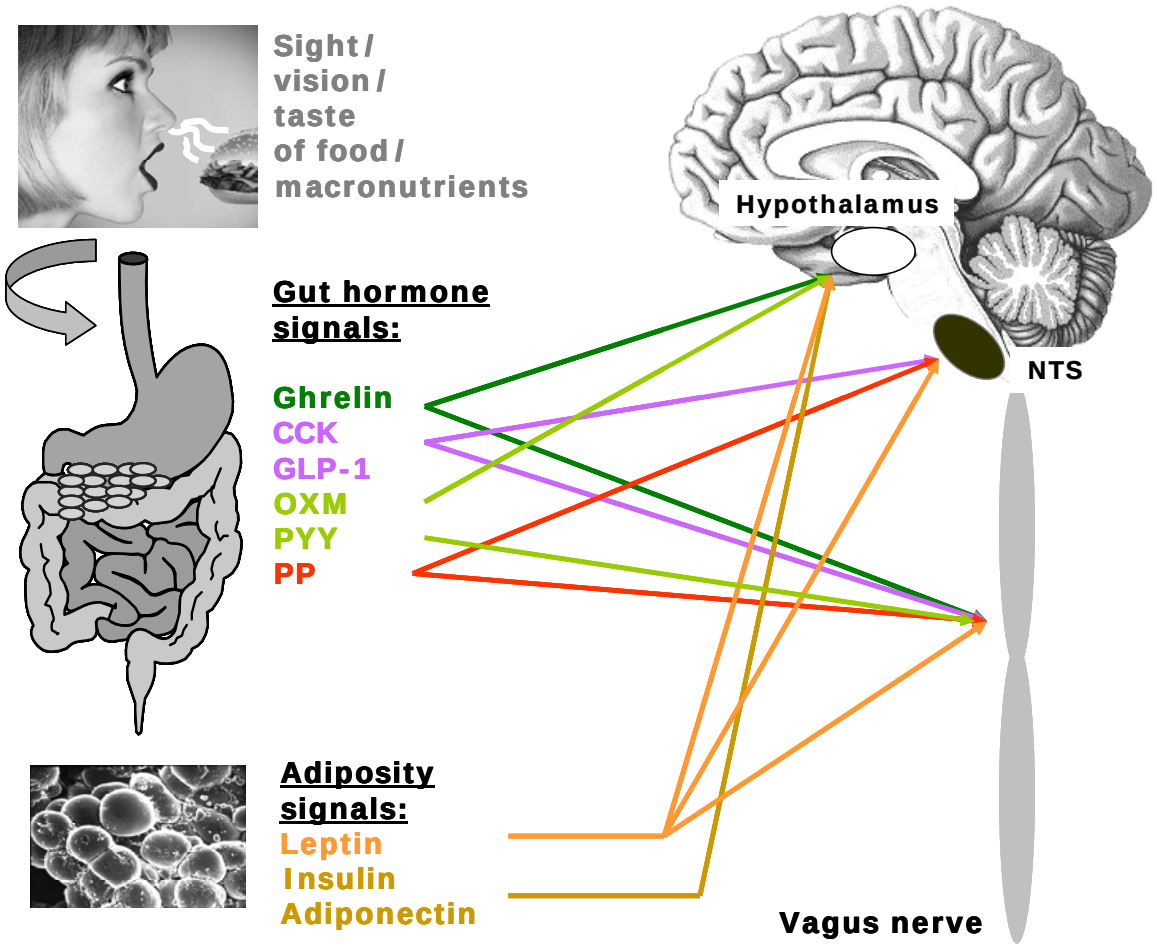
Restriction of food intake, over a period of days, results in a suppression of leptin levels, which can be reversed by refeeding. Exogenous leptin replacement decreases fast-induced hyperphagia, and chronic peripheral administration of leptin to wild-type rodents results in reduced food intake, loss of body weight and fat mass (Wynne et al., 2005).

Although a small subset of obese human subjects have a relative leptin deficiency, the majority of obese animals and humans have a proportionally high circulating leptin (Maffei et al., 1995, Considine et al., 1996), suggesting possible leptin resistance. Leptin administered subcutaneously to obese human subjects has only shown a modest effect on body weight (Heymsfield et al. 1999, Fogteloo et al. 2003).

Plasma **insulin** was first described in 1992 (Schwartz et al., 1992). It is released from the pancreas and increases at times of positive energy balance and decreases at times of negative energy balance (Woods et al., 1974) (Figure 1.2.). Unlike leptin, insulin secretion increases rapidly after a meal (Polonsky et al., 1988). Insulin penetrates the blood–brain barrier and acts in the brain as an anorexigenic signal, decreasing food intake and body weight in rodents (Baura et al., 1993, Wynne et al., 2005b). There are several insulin receptor substrates (IRSs) including IRS-1 and IRS-2, both identified in neurons (Baskin

et al., 1994, Burks et al., 2000). The phenotype of IRS-2-knockout mice is associated with an increase in food intake, increased fat stores and infertility (Burks et al., 2000). Insulin receptors are widely distributed in the brain, with highest concentrations found in the olfactory bulbs, hypothalamus and ventral tegmental area (VTA) (Marks et al., 1990; Iniguez et al., 2008). The mechanisms by which insulin acts as an adiposity signal remain to be fully elucidated (Wynne et al., 2005b).

Figure 1.2. Peripheral control of appetite



Anorexigenic and orexigenic gut hormone signals and adiposity signals influencing homeostatic and hedonic brain systems.

1.2.1.2. Gastrointestinal (GI) signals

The GI tract is the largest endocrine organ in the body. Gut hormones have a key role in signaling nutrient intake. Together with metabolic and neural signals they regulate appetite and energy expenditure. After entering the gut lumen, nutrients trigger the secretion of several peptides which activate the central nervous system via different pathways. Apart from ghrelin (*orexigenic*), which stimulates appetite and food intake, all other gut hormones have anorexigenic effects: they promote satiation (cause meal termination) and/or satiety (postponing the initiation of the next meal) (Moran et al., 2005).

Orexigenic hormones

Ghrelin

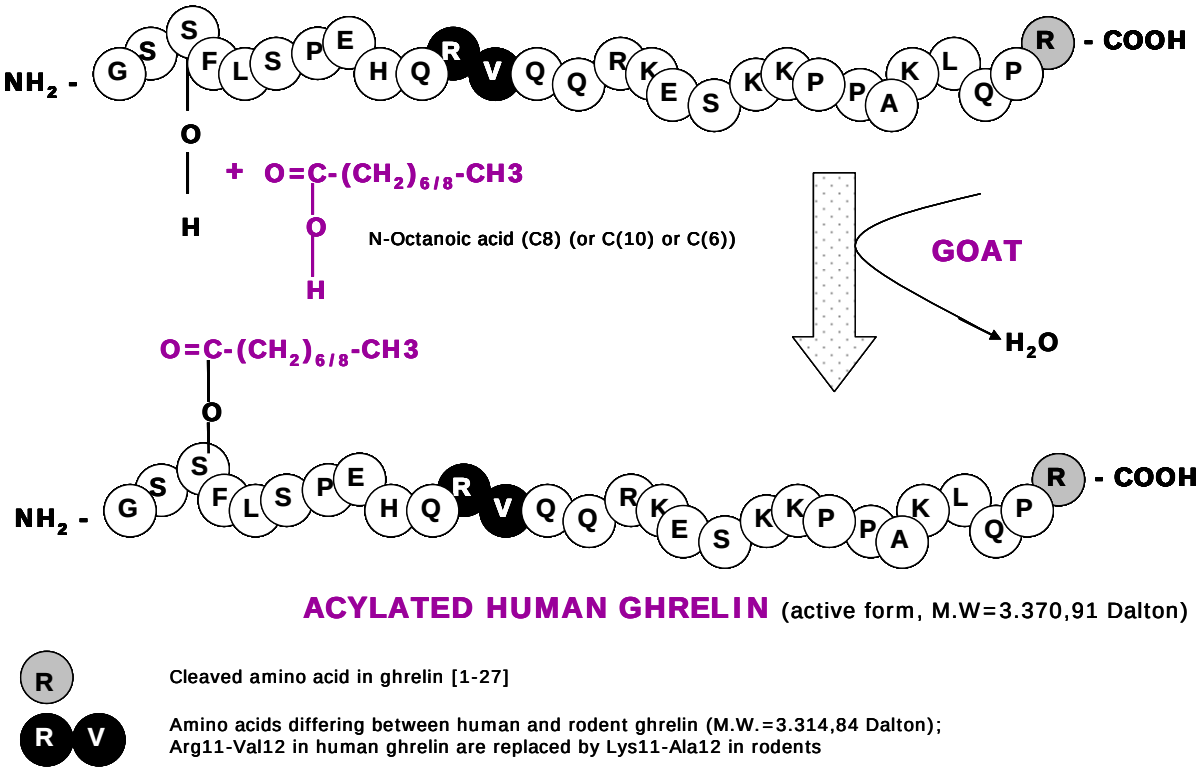
History

Ghrelin was discovered after the identification of its receptor, the growth hormone (GH) secretagogue receptor 1a (GHSR-1a) (Kojima et al., 1999; Howard et al. 1996) (Figure 1.2.). The name is based on its role as a GH-releasing peptide, with reference to the Proto-Indo-European root ghre, meaning to grow.

Synthesis and forms

It is primarily synthesized as a pre-prohormone consisting of 117 amino acids. Cleavage of preproghrelin results in two mature ghrelin molecules, a 28 amino acid form (C-terminal Arg) or 27 amino acid form (C-terminal Pro) (Hosoda et al., 2003). Ghrelin is released from X/A-like cells of the gastric oxyntic glands, with lower levels also expressed in the small intestine and the hypothalamus (Kojima et al., 1999; Tschop et al., 2001). In order to be biologically active, ghrelin requires post-translational modification in which the third amino acid residue (serine) is acylated with n-octanoid acid or n-decanoic acid (Kojima et al., 1999) (Figure 1.3.). The enzyme responsible for this acylation, ghrelin O-acyl transferase (*GOAT*), was recently identified (Yang et al., 2008).

Figure 1.3. Post-translational modification of ghrelin.



Post-translational modification of ghrelin: the third amino acid residue (serine) is acylated with n-octanoid acid or n-decanoic acid via *GOAT* (ghrelin O-acyl transferase).

GOAT

The highest expression of GOAT occurs in the pancreas and stomach in humans and stomach and intestine in mice (Yang et al., 2008, Sakata et al., 2009). New research suggests that the ghrelin-GOAT 'lipid-sensor' system informs the central nervous system about availability, rather than absence, of calories (Kirchner et al., 2009). Using murine genetic models with specific loss (GOAT-null) or gain (mice overexpressing both human GOAT and human ghrelin) of GOAT function, they show that GOAT is necessary and sufficient to mediate the impact of certain dietary lipids on body adiposity. Using mass spectrometry and gene expression profiling, they also find that activation of the GOAT-ghrelin system is triggered by a specific lipid-rich environment rather than by caloric deprivation (Kirchner et al., 2009).

Mechanism of ghrelin action

The acylated peptides are widely believed to be the biologically active forms of ghrelin based upon only their capability to bind and activate the only identified ghrelin receptor GHSR-1a and the fact that their administration triggers both GH secretion and food intake (Bednarek et al., 2000, Banks et al., 2002, Hosoda and Kangawa, 2003). Ghrelin is the only orexigenic gut hormone which stimulates acute food intake after peripheral and central administration of ghrelin in rats (Wren et al., 2001) and over a 7-day period, this also results in weight gain (Tschop et al., 2000). Recent data also reported that transgenic mice overexpressing the non-octanylated form (des-acyl ghrelin) have lower body weight and body length when compared with control mice (Ariyasu et al., 2005). Inhoff and colleagues suggested that the biological role of des-acyl ghrelin is paradoxically to decrease food intake (Inhoff et al., 2009).

Neary et al. demonstrated that *only* 30 minutes after intravenous infusion of 5.0pmol/kg/min of ghrelin, energy intake is increased by 28 percent in cancer patients with anorexia compared to infusion of saline (Neary et al., 2004). Druce et al. showed that subcutaneous ghrelin injection (3.6 nmol/kg) increases food intake in obese as well as lean healthy subjects by 20 percent after *only* 30 minutes compared to injection of saline (Druce et al., 2005; Druce et al., 2006). Other studies demonstrated that subcutaneous ghrelin injection (3.6 nmol/kg) to malnourished dialysis patients increases food intake by 25 percent after *only* 30 minutes compared to injection of saline (Wynne et al., 2005b; Ashby et al., 2009). Wren and colleagues showed that an intravenous infusion of 5.0pmol/kg/min of ghrelin to non-obese volunteers increased energy intake by 28% compared to infusion of saline (Wren et al., 2001).

Ghrelin is broken down by various enzymes including butyrylcholinesterase and isophospholipase (De Vriese et al., 2004). Ghrelin plasma levels rise before the start of a meal and fall again rapidly after the meal (Cummings et al., 2001). The suppression is proportional to the caloric load of the meal (Callahan et al., 2004). Studies employing exogenous ghrelin administration show that the half-life of total ghrelin is between 10 and 31 minutes in the bloodstream (Akamizu et al., 2004). A steady-state level of total ghrelin

(acylated and des-acylated ghrelin) was reached within 60-90 minutes after intravenous infusion in humans (Vestergaard et al., 2007). Suppression of acyl-ghrelin and total ghrelin (acylated and des-acylated) is strongly stimulated by ingested proteins and weakly by lipids (Foster-Schubert et al., 2008). However, the timing of ghrelin spikes differ depending on individual eating habits; someone who does not eat breakfast would not exhibit the usual morning ghrelin spike, for example (Drazen et al., 2006). Recent data suggested that acyl-ghrelin levels decline with longterm fasting (61.5 hours), whereas des-acyl ghrelin levels are increased (Liu et al., 2008). Thus, increased appetite occurs during short-term fasting but wanes over time; this may relate to the lowering of acyl-ghrelin (Liu et al., 2008). Acylation may be regulated independently of secretion by nutrient availability in the gut or by esterases that cleave the acyl group. The new 'lipid-sensor' GOAT, which is essential for ghrelin acylation, is regulated by nutrient availability, depending on specific dietary lipids as acylation substrates (Kirchner et al., 2009).

A recent study demonstrated that GOAT KO mice which lack acylated ghrelin and therefore GH decreased blood glucose levels after prolonged fasting (Zhao et al., 2010). Therefore, the body's response to hypoglycemia is to mount a stress response, including the release of corticotropin and GH that act to raise blood glucose. This study suggests that the ghrelin-producing cell senses glucose to regulate ghrelin release. A GOAT antagonist GO-CoA-Tat potently inhibits GOAT in vitro, in cultured cells, and in mice and therefore acylated ghrelin which results in an increase in insulin and reduction in blood glucose. This study further showed that GO-CoA-Tat prevents weight gain in mice through the same mechanism (Barnett et al., 2010).

Ghrelin's action on the homeostatic regulation of metabolism

Studies suggest that the appetite-stimulating effects of ghrelin are centrally mediated, in part by neurons within the arcuate nucleus (ARC) of the hypothalamus, the brainstem and the vagus nerve (Nakazato et al., 2001, Tamura et al., 2002, Williams et al., 2003, le Roux et al., 2005). The brainstem also seems to have separate hyperphagic effects; administration of ghrelin to the hindbrain ventricle induced hyperphagia and *c-fos*

expression in the nucleus solitary tract (NTS), but not hypothalamus (Faulconbridge et al., 2008). Ghrelin is able to increase growth hormone (GH) secretion through the phospholipase C –protein kinase C (PLC-PKC) pathway via activation of the GHSR-1a (Chen et al., 1996), which in turn promotes nutrient incorporation into muscle and other tissue. The highest density of the classic G-protein-coupled receptors GHS-1a in the CNS is found in the hypothalamus and the pituitary gland; with lower expression in the hippocampus, midbrain and hindbrain (Zigman et al., 2006; Cummings et al., 2007; Kuo et al., 2007). Peripheral ghrelin may also act in the gut, relaying on information to the brain via the vagus nerve (Date et al., 2002).

Specifically, GHSR-1a is expressed in the ARC of the hypothalamus predominantly on orexigenic Neuropeptide Y (NPY) and Agouti-related petide (AGRP) neurons (Hahn et al., 1998, Willesen et al., 1999, Nakazato et al., 2001). NPY/AGRP neurons project directly and indirectly via the lateral hypothalamus to the ventral tegmental aera (VTA) and amygdala (Saper et al., 2002, Kelley, 2004). Functional activation of this neuronal NPY/AGRP population by ghrelin has been demonstrated electrophysiologically (Cowley et al., 2003), by induction of early gene expression (Hewson and Dickson, 2000), and by increased expression of the peptides themselves (Kamegai et al., 2001; Nakazato et al., 2001). When GHSR-1a expression is diminished in the ARC, rats exhibit decreased body weight, lower adipose tissue and reduced food intake. Further, administration of ghrelin to rats with lower GHSR-1a expression fails to stimulate either feeding or GH secretion (Shuto et al., 2002; Castaneda et al., 2010).

Kuo and colleagues introduced a new imaging technique in rodents to show the effect of ghrelin on the hypothalamus. Using the manganese ion (Mn2+) as an activity-dependent magnetic resonance imaging (MRI) contrast agent, they showed a dose-dependant increase in signal intensity in the hypothalamus, including the arcuate, paraventricular, and ventromedial nuclei, after peripheral injection of the orexigenic peptide ghrelin (Kuo et al., 2007).

In rodents, intracerebroventricular administration of ghrelin induced *c-fos* expression, a marker of neuronal activation, in orexin-producing neurons in the lateral

hypothalamus. Ghrelin-induced feeding is also suppressed in orexin knockout mice. This study identifies a novel hypothalamic pathway that links ghrelin and orexin in the regulation of feeding behavior and energy homeostasis (Toshinai et al., 2003). Orexin neurons can project to brain reward centres especially the VTA which will be discussed in more detail later (see 1.2.2.1.).

Ghrelin's action on brain reward systems

Circulating ghrelin may also act directly on the corticolimbic reward and memory systems, involving the VTA, ventral striatum, amygdala, orbitofrontal cortex (OFC), insula and hippocampus (Abizaid et al., 2006; Zigman et al., 2006; Cummings et al., 2007; Malik et al., 2008). New research suggests that peripheral ghrelin enhances the intake of rewarding foods in rodents (Egecioglu et al., 2010), especially sweet taste consumption and preference regardless of its caloric content (Disse et al., 2010). Ghrelin has been shown to increase sweet food palatability in humans: in previous ghrelin feeding studies in healthy volunteers, researchers used sweet foods, e.g. Madeira cake, cinnamon and raisin loaf or sweet brioche rolls, as the *ad libitum* meal to demonstrate its ability to increase food intake (Druce et al., 2005; Wynne et al., 2005a). Some of the previous ghrelin studies on food intake in humans, using savoury ham sandwiches, curry, spaghetti bolognese or pasta as test meals were still able to see an increase in food intake in cancer cachexia and renal dialysis patients (Neary et al., 2004; Goldstone et al., 2005; le Roux et al., 2005b; Ashby et al., 2009), though interestingly one using a savoury test meal in vagotomised but otherwise healthy patients did not see any orexigenic effect of ghrelin (le Roux et al., 2005b).

Ghrelin and obesity

Although ghrelin has been reported to increase food intake and body weight in rodents and humans (Tschop et al., 2000; Castaneda et al., 2010), circulating plasma ghrelin levels in obese people, both children and adults, are lower than in matched lean controls (Goldstone et al., 2001; Shiiya et al., 2002; le Roux et al., 2005a; Castaneda et al.,

2010). In binge eating patients (i.e. compulsive overeating) (Tanaka et al., 2003) and in hyperphagic patients with Prader-Willi syndrome (Cummings et al., 2002; Goldstone et al., 2005) plasma ghrelin levels are high, suggesting that peripheral hyperghrelinaemia might be involved in the pathophysiology of compulsive eating and therefore obesity. Interestingly, gastric by-pass surgery patients experience a 72% reduction in 24 hour ghrelin levels and a profound reduction in hunger (Cummings et al., 2004). Furthermore, 12 months following surgery, the average ghrelin levels increase by 62% whereas the BMI decreased by 30% (Holdstock et al., 2003). However, while decreases in circulating ghrelin have been reported after such surgery, in other studies ghrelin levels inappropriately remain unchanged despite weight loss, and additional studies have found appropriate increases after surgery (le Roux et al., 2007, Pournaras and leRoux, 2010). One recent study measured both acylated and deacylated ghrelin at fasting levels after bypass surgery and found that immediately after surgery acylated ghrelin significantly decreased when total ghrelin remained unchanged (Isbell et al., 2010). To date, most research studies measured fasting ghrelin levels after bariatric surgery and and only few studies have measured postprandial ghrelin levels (Pournaras and leRoux, 2010).

Although the role of ghrelin in the aetiology of obesity is not fully understood, it is considered as a vital target because of its capacity to induce a positive energy balance state (Castaneda et al., 2010). Recent studies show that the absence of both ghrelin and its receptor GHSR-1a protects mice against diet induced obesity (Wortley et al., 2005; Zigman et al., 2005). However, ghrelin immunization to reduce body weight has failed (Zorrilla et al., 2006) and some studies with knockout for ghrelin or ghrelin receptor with mice from a pure C57BL/6 background found only small differences in food intake and body weight under caloric restriction or high fat diet compared with wild-type control mice (Sun et al., 2008). Investigators are now looking at GHSR-1a antagonists (BIM-28163, L-NOX-B11) to reduce food intake and body weight but results are controversial and more research, and also long-term studies are needed to determine whether these antagonists could be used in the treatment of obesity (Neary et al., 2009). The antagonist GO-CoA-Tat

potently inhibits GOAT and has been demonstrated to prevent weight gain in mice (Barnett et al., 2010).

More work is required to understand whether surgical or pharmacological manipulations of ghrelin levels will be a useful way to treat obesity (Castaneda et al., 2010). In the last couple of years researchers have also begun to focus not only on traditional homeostatic systems but also the effect of ghrelin and other internal signals on brain reward systems (see Section 1.2.2.2).

Anorexigenic hormones

Cholecystokinin (CCK) was the first gut hormone identified to have satiety effects (Gibbs et al., 1973). CCK is secreted by the I-cells in the proximal part of the small intestine, inhibits gastric emptying, and is thought to play a role in meal termination (Figure 1.2.). Fat and protein-rich foods stimulate CCK release (Polak et al., 1975, Liddle et al., 1985). Previous studies suggest that the CCK-1 receptor is responsible for the ability of CCK to reduce food intake in rats (Gibbs et al., 1973), and humans (Kissileff et al., 1981, Pi-Sunyer et al., 1982, Lieverse et al., 1994, Lieverse et al., 1995). CCK-induced satiation is mediated by binding CCK-1 receptors on the vagus nerve (MacLean, 1985, Ritter and Ladenheim, 1985, Moran et al., 1997, Kopin et al., 1999, Beglinger et al., 2001), brainstem (Edwards et al., 1986) and hypothalamus (Blevins et al., 2000). Morbidly obese individuals showed lower fasting CCK levels than obese and lean individuals (Zwirska-Korczala et al., 2007). CCK gene and CCK-1 receptor gene variations may increase predeposition to obesity (Arya et al., 2004, de Krom et al., 2007).

However, the usefulness of CCK as an obesity therapeutic target may be limited by side effects such as vomiting, nausea and taste aversion (Swerdlow et al., 1983). CCK may also be limited by its short term effect on satiety. CCK does not reduce feeding when injected more than 30 minutes before a meal in rats (Gibbs et al., 1973).

Peptide Tyrosine Tyrosine or Peptide YY *(PYY)* is related to the pancreatic peptide family (Figure 1.2.). PYY1-36 is synthesised by endocrine L cells throughout the distal gut (Adrian et al., 1985). Amino acids tyrosine and proline, are cleaved from the N-terminus of PYY1-36 by the enzyme di-peptidyl peptidase IV (DPP-IV), which produces PYY3-36, the major circulating form of the hormone in the fed and fasted state (Batterham et al., 2006, le Roux et al., 2006). PYY is important in postponing initiation of the next meal rather than acting as a meal terminator. Peak concentrations of PYY correlate with caloric content, macronutrient composition and consistency of the meal (Adrian et al., 1985, Batterham et al., 2006, Helou et al., 2008). The effects of PYY are mediated by the Y2-receptor, found in the central nervous system, nodose ganglion and vagal afferents (Batterham et al., 2002, Abbott et al., 2005a, Koda et al., 2005). Kuo and colleagues showed that the effect of PYY on food intake is regulated via the hypothalamus, including the arcuate, paraventricular, and ventromedial nuclei, after peripheral injection of peptide YY(3-36) caused a reduction in signal intensity in those areas imaged using Maganese Enhanced MRI (Kuo et al., 2007).

Pyy knockout mice display hyperphagia and increased subcutaneous and visceral adiposity with the phenotype reversed after exogenous replacement (Batterham et al., 2006). Researchers demonstrated how combination therapy could increase the potency of PYY3-36 when co-administration of PYY3-36 and GLP-1 (Neary et al., 2005) and PYY3-36 and oxyntomodulin (Field et al., 2010) in humans and rodents resulted in greater reductions of food intake.

Pancreatic polypeptide (*PP*) belongs to the polypeptide-fold family, like NPY and PYY. PP is produced in the pancreatic islets of Langerhans (Adrian et al., 1976) (Figure 1.2.). PP concentrations remain elevated for up to 6 hours after food ingestions and PP concentrations reflect the caloric content of the meal (Adrian et al., 1976). Anorexigenic actions of the hormone are thought to be mediated by the brainstem, parventricular nucleus (PVN) and ARC of the hypothalamus with highest affinity for Y4-receptors, followed by Y1-and Y5-receptors (Berglund et al., 2003). PP reduced food intake after

repeated peripheral administration in rodents (Asakawa et al., 1999, Asakawa et al., 2003). Over-expression of PP in the pancreatic islets of mice resulted in a lean phenotype with reduced food intake (Ueno et al., 1999). Chronic administration of PP to *ob/ob* mice decreases body weight (Asakawa et al., 2003). In humans, intravenous infusion of PP in normal-weight volunteers resulted in a 25% reduction in food intake, lasting 24 hours after the infusion stopped (Batterham et al., 2003). PP also reduces food intake in patients with the genetic obesity Prader-Willi syndrome (Berntson et al., 1993).

Glucagon-like peptide *(GLP-1)* belongs to the family of incretins (gut-derived hormones that increase insulin secretion) (Figure 1.2.). GLP-1 is produced from pre-proglucagon in the pancreas, intestine and CNS. Prohormones convertase 1 and 2 direct the cleavage of pre-proglucagon resulting in a specific balance of hormones (GLP-1 and Oxyntomodulin, see next paragraph). The main stimulus to GLP-1 secretion is food intake. Fast absorbable proteins, such as whey-protein, evoke greater GLP-1 responses than slow proteins, like caseins (Tessari et al., 2007). GLP-1 acts mainly via the vagus nerve and the brainstem (Abbott et al., 2005a). GLP-1 acts on the GLP-1 receptor, which is widely expressed throughout the CNS including hypothalamus and amygdala (Turton et al., 1996), brainstem (area postrema and nucleus of the solitary tract (NTS)) (Tang-Christensen et al., 2001; Baggio et al., 2004) and peripheral tissues such as pancreatic ß-cells (Bullock et al., 1996, Naslund et al., 1999). Administration of GLP-1 reduces food intake in rats when administered peripherally or centrally (Tang-Christensen et al., 1996, Turton et al., 1996). GLP-1 also has an anorectic effect in both lean and obese humans with gastric emptying and dose-dependent reduction in food intake (Verdich et al., 2001). After 5 days of subcutaneous GLP-1 injections, obese subjects lost 0.55 kg in weight (Naslund et al., 2004). The peptide exendin-4 has proved a useful tool in determining the mechanisms by which the actions of GLP-1 on appetite are mediated. Exendin-4 is a 39-amino acid peptide extracted from the saliva of the Gila monster. It is structurally related to GLP-1 and is a potent agonist at GLP-1 receptors. This GLP-1 agonist, called Exenatide, has been recently licensed as a drug for diabetes mellitus type 2 as it effectively controls

blood glucose without producing dangerous side effects (Chaudhri et al., 2006; Chakraborti, 2010). Simultanously, Exenatide significantly reduces weight in patients with type 2 diabetes mellitus (Diamant et al., 2010). Exenatide, like other GLP-1 targeting drugs can have side effects, like nausea. Therefore therapy could be ameliorated by combining lower doses of two or more gut hormones in order to achieve the desired reduction in weight loss (Chaudhri et al., 2006).

Oxyntomodulin *(OXM)* also belongs to the family of incretins (Figure 1.2.). OXM is released from the intestinal L-cells in proportion to caloric intake, remaining elevated for several hours (Ghatei et al., 1983, Le Quellec et al., 1992). The downstream anorectic effects of OXM appear to be mediated by GLP-1 receptor since these effects were abolished in GLP-1 receptor null mice (Cohen et al., 2003; Baggio et al., 2004; Dakin et al., 2004). Compared to GLP-1, OXM has an equal potency for inhibiting food intake and reducing body weight in rodents and humans (Fehmann et al., 1994; Dakin et al., 2001; Dakin et al., 2002; Cohen et al., 2003; Baggio et al., 2004; Wynne et al., 2006). Peripheral OXM administration altered activity in the hypothalamic ARC, PVN and supra-optic nuclei using manganese-enhanced magnetic resonance imaging (MEMRI) in fasted mice. In contrast, peripheral GLP-1 injection modulated activity in the PVN and VMN suggesting that they may have differential effects (Chaudhri et al., 2006, Parkinson et al., 2009).

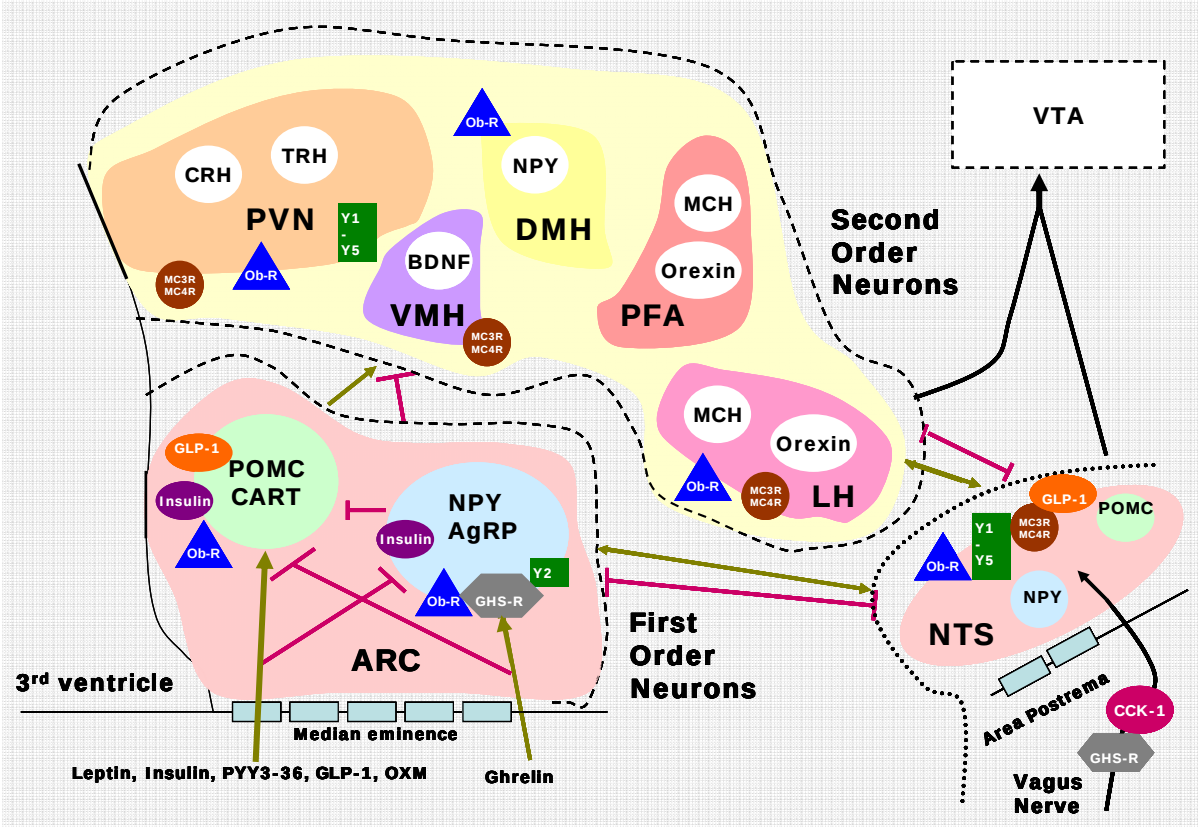
There are more than 50 other gut hormones and peptides, but only a few have been investigated for their potential as anti-obesity agents.

1.2.2. Appetite control systems

1.2.2.1. Homeostatic brain systems

The hypothalamus regulates many aspects of energy homeostatis, is implicated in the process of balancing food intake and maintaining body weight (Figure 1.4.). The ARC in particular, is thought to play a key role in the integration of signals regulating appetite. The ARC encloses the third ventricle and is located near the median eminence, a region with an incomplete blood brain barrier (Broadwell and Brightman, 1976).

Figure 1.4. Central control of appetite.



Complex homeostatic control of feeding: Internal signals enter the hypothalamus via the median eminence or the brainstem via the vagus nerve. First order neurons in the arcuate nucleus (ARC) project to second order neurons such as the lateral hypothalamus (LH) which then sends orexigenic signals to brain reward systems (ventral tegemental area – VTA). All abbreviations can be found in the "Abbreviations" – list on page 7.

Primary neurons within ARC

There are two primary populations of neurons within the ARC which influence energy homeostasis (Cone et al., 2001). One neuronal circuit reduces food intake, via the expression of the neuropeptides **pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transccript (CART)** (Elias et al., 1998a, Kristensen et al., 1998, Wynne et al., 2005b). Another neuronal circuit stimulates food intake, via the expression of **NPY** and **AGRP** (Broberger et al., 1998, Hahn et al., 1998) (Figure 1.4.).

NPY and AgRP neurons are inhibited by leptin, insulin, PYY3-36 and GLP-1 which inhibit food intake while they are stimulated by ghrelin which stimulates food (Mercer et al., 1996, Christophe, 1998, Nakazato et al., 2001, Flier, 2004, Zigman et al., 2006). POMC/CART neurons are stimulated by leptin, PYY3-36, GLP-1 and insulin but inhibited by neighbouring NPY/AgRP neurons and ghrelin (Mercer et al., 1996, Christophe, 1998, Cowley et al., 2001, Nakazato et al., 2001, Zigman et al., 2006) (Figure 1.4.).

Secondary neurons within ARC

Hypothalamic second order nuclei receive neuronal projections from the axons of NPY/AgRP and POMC/CART neurons in the ARC (Elias et al., 1998b, Elmquist et al., 1998, Kalra et al., 1999) (Figure 1.4.). NPY/AgRP and POMC/CART can project to the **paraventricular nucleus (PVN)**, where the anorexigenic neuropeptides thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin are synthesised (Sawchenko and Swanson, 1983, Verbalis et al., 1993, Richard et al., 2000).

Projections from first order neurons to the **lateral hypothalamic area (LHA) and perifornical area (PFA)**, where the orexigenic molecules melanin-concentrating hormone (MCH) and orexins A and B are produced, can lead to increased food intake and body weight (Qu et al., 1996, Elias et al., 1998b, Sweet et al., 1999, Nahon, 2006).

Sakurai et al. characterised two individual receptors for the **orexin system**, OxR1 and OxR2 (Sakurai et al., 1998). Orexin neurons can project to brain reward centres especially the VTA; and in circumstances of starvation, the orexin peptide may mediate both arousal and feeding responses in order to initiate food-seeking behaviour (Chemelli

et al., 1999). Administration of orexin has been shown to stimulate food consumption, and orexin signaling in VTA has been implicated in intake of high-fat food.

Recent studies have shown that orexin is involved in motivated behavior for drugs of abuse as well as natural rewards. In self-administration studies, the OxR1 antagonist SB-334867 (SB) reduced the reponse to high-fat pellets, sucrose pellets and ethanol, but not cocaine, demonstrating that signalling at orexin receptors is necessary for reinforcement of specific rewards (Cason et al., 2010).

First order axons NPY/AgRP and POMC/CART can also project to the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH). The **DMH** expresses NPY cells whose activation leads to hyperphagia in rats (Kalra et al., 1999, Chen et al., 2004). Lesions in the **VMH** produce hyperphagia and obesity (King et al., 1978).

Brainstem feeding pathways

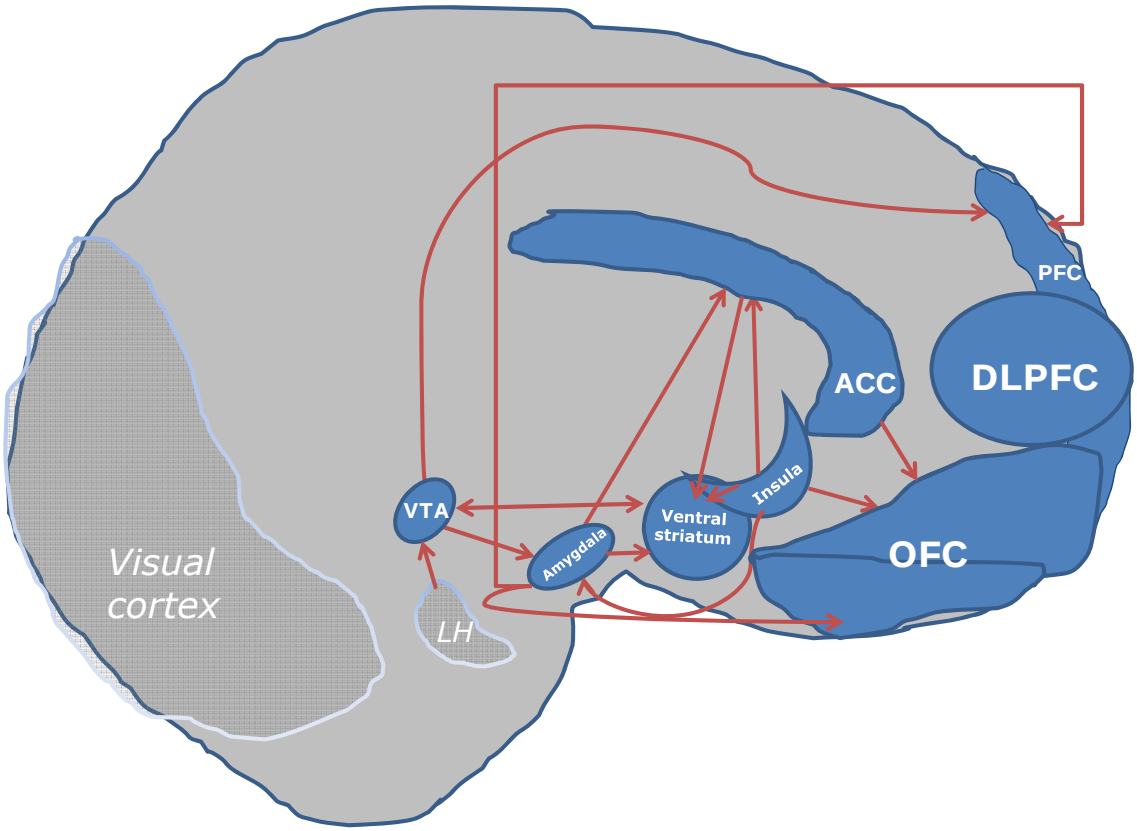
In addition to hypothalamic circuits, the brainstem, in particular the **NTS**, also plays a vital role in the regulation of energy homeostasis (Ricardo and Koh, 1978) (Figure 1.4.). It can respond to peripheral signaling and also receives vagal afferents from the gastrointestinal tract, and from here information can be transferred to the ARC (Kalia and Sullivan, 1982, Sawchenko and Swanson, 1983).

The architecture and functioning of the opposing hypothalamic circuits enables rapid control over energy homeostasis because the brain can simultaneously turn up and down anabolic or catabolic systems in order to control feeding. Yet, it is important to understand that the pleasure of eating derives from the taste, smell and texture of food and that each individual shows different eating behaviour and food perception. We therefore need to include study of the links between homeostatic and hedonic control of appetite. Hedonic eating is thought to be able to override the satiety effect of positive energy balance signals (Saper et al., 2002, Harrold and Williams, 2003, Kringelbach, 2004, Mela, 2006, Lowe et al., 2009), but the hedonic systems may also be responsive to negative energy balance states.

1.2.2.2. Hedonic brain reward systems

Mesocorticolimbic brain regions are involved in reward and emotional processing, learning and memory, and have been implicated in hedonic eating. These include the prefrontal cortex (dorsolateral PFC), orbitofrontal cortex (OFC), anterior cingulate cortex (ACC), insula, amygdala, nucleus accumbens (NAc, ventral striatum) and VTA (midbrain) (Van Vugt, 2009) (Figure 1.5., Table 1.1.).

Figure 1.5. Hedonic brain reward systems



Sagittal view of the brain showing those areas involved in the hedonic control of eating behaviour: VTA (ventral tegmental area) – dopaminergic projections to corticolimbic brain areas; ventral striatum – motivation, reward expectancy, novelty processing; insular cortex – taste processing; amygdala – emotional processing; ACC (anterior cingulate cortex) – descision making; PFC (prefrontal cortex) – translation of external and internal cues into behavioural responses; DLPFC (dorsolateral prefrontal cortex) – self-control; OFC (orbitofrontal cortex) – encoding of reward value.

Table 1.1. Brain reward areas involved in eating behaviour

Brain reward area	Function
Midbrain / VTA	Dopaminergic projections to limbic areas (NAc (ventral striatum), PFC, amygdala, LH and hippocampus), implicated in motivation and positive reinforcement, expression of GHS-R receptors and receives input from the hypotahalmus (e.g. orexin)
NAc / ventral stritum	Expression of D1-, D2- and D3 receptors and endogenous opioids, projection to the midbrain (VTA, substantia nigra), incentive motivational role in food intake and activation of this reward system enables behavioural responses necessary for obtaining a goal object, aimed at anticipated food receipt
Insula	Primary gustatory cortex involved in taste aversion as well as in sensory and rewarding aspects of taste processing, sends efferent projections to the anterior cingulate cortex (ACC), OFC, LH and NAc, and innervates the amygdala
Amygdala	Emotional processing particularly positive and negative affect, reward processing, and behaviour, projections to the OFC, PFC, NAc and ACC
OFC	Role in encoding taste, flavour, and food reward; medial OFC is related to the monitoring, learning and memory of the incentive salience of reward value of reinforcers, whereas lateral OFC is related to encoding reward value supported by the decrease in activation seen during taste of a food repeatedly consumed to a state of aversion ('sensory-specific satiety'), close contact with the amygdala, insula and hippocamous
ACC	The ventral affective subdivision: assessment of the salience of emotional and motivational information, connected to the amygdala, NAc, hypothalamus, anterior insula, hippocampus and OFC The dorsal cognitive subdivision: Modulation of attention or executive function, influence on response selection, motivation, conflict monitoring and cost/benefit or reward/risk assessment, influenced by anxiety and fearful situations, reciprocal interconnected with the lateral PFC and motor areas
PFC / DLPFC	PFC: Executive control, translates homeostatic and environmental information into adaptive behavioural responses making choices, decisions DLPFC: goal-directed decisions and exercising self-control, reduce conflict

The **VTA** is located medial to the substantia nigra in the **midbrain** (Fields et al., 2007) (Figure 1.5.). Although the VTA is positioned strategically between the caudal hypothalamus and brainstem its physiological function was largely ignored until the discovery that it includes dopaminergic neurons (Dahlstroem and Fuxe, 1964) and that these neurons project widely to limbic areas implicated in motivation and positive reinforcement (Fallon and Moore, 1978, Swanson, 1982). This conclusion was supported by the observation that destruction of midbrain dopamine system produced profound motivational deficits (Ungerstedt, 1971). Previous studies have showed that VTA neurons fire when animals receive an unexpected reward (Schultz et al., 1993). Approximately 60% of VTA neurons in the rat are dopaminergic (Swanson, 1982) and a large number are indentified as GABAergic neurons (Van Bockstaele and Pickel, 1995, Fields et al., 2007). The VTA receives input from many brain areas. It receives glutamatergic projections from the prefrontal cortex (PFC) (Sesack and Pickel, 1992), amygdala (Semba and Fibiger, 1992) and lateral hypothalamus (Rosin et al., 2003). The input from the LH also includes afferents that contain the peptides orexin (Fadel and Deutch, 2002) or MCH (Semba and Fibiger, 1992, Fields et al., 2007). The VTA sends dopaminergic projections to the NAc (ventral striatum), PFC, amygdala, LH and hippocampus (Fields et al., 2007).

The **NAc** comprises the **ventral** components of the **striatum** (Figure 1.5.). There are two major fuctional components of the NAc, the core and the shell, which are characterized by differential inputs and outputs (Carlezon and Thomas, 2009). The NAc contains GABAergic neurons, cholinergic neurons and GABA-containing medium spine neurons (Meredith, 1999). These neurons express D1-, D2- and D3-like receptors and endogenous opioids and project back to the midbrain (VTA, subtantia nigra) (Carlezon and Thomas, 2009).

It is well established that the NAc plays a key role in reward. The ventral striatum plays an incentive motivational role in food intake and activation of this reward system enables behavioural responses necessary for obtaining a goal object, aimed at anticipated food receipt (Berridge, 1996, Kelley, 2004). Imaging work in humans has shown that fasting increases the activation of the ventral striatum when viewing visual food stimuli

(Hinton et al., 2004, Beaver et al., 2006, Farooqi et al., 2007, Fuhrer et al., 2008, Stoeckel et al., 2008, Goldstone et al., 2009, Passamonti et al., 2009, Schur et al., 2009, Wang et al., 2009). Drugs of abuse and natural rewards have the common action of elevating extracellular concentrations of dopamine in the NAc (Kelley, 2004). Moreover, lesions of the NAc reduce the rewarding effects of stimulants (i.e. cocaine) and opiates (Kelsey et al., 1989). Pharmacological studies in the rat and monkeys suggest that D2- and D3 receptors play a critical role in reward (Caine et al., 1999, Caine et al., 2000). Mice lacking dopamine D2-receptors have reduced sensitivity to the rewarding effects of cocaine (Welter et al., 2007) and morphine (Maldonado et al., 1997). D3 receptor antagonists have been shown to reduce cocaine-seeking behaviour and nicotine self-administration in rats (Heidebreder et al., 2005). These data suggests that the D2/D3 receptors and therefore the NAc/ventral striatum play an essential role in reward expectations (Volkow et al., 2007).

The **insula cortex** is a cerebral cortex structure within the temporal lobe and the frontal lobe, and is divided into two parts: the larger anterior insula and the smaller posterior insula (Figure 1.5.). The insula is considered to be the primary gustatory cortex involved in taste aversion as well as in sensory and rewarding aspects of taste processing (Welzl et al., 2001, James et al., 2009). Previous studies suggest that representations of experience with food (colour, taste, flavour, reward value) are generated in the insula (de Araujo et al., 2003). Functional MRI studies in humans show a role for the insula in taste processing but also increased insula activation after smell or sight of food stimuli (Gordon et al., 2000, Small et al., 2001, Wang et al., 2004, DelParigi et al., 2005, Holsen et al., 2005, Simmons et al., 2005, St-Onge et al., 2005, Porubska et al., 2006, Fuhrer et al., 2008, Stice et al., 2008, Cornier et al., 2009, James et al., 2009, Schienle et al., 2009, Schur et al., 2009, Siep et al., 2009, Wang et al., 2009). The insula receives visceral information by way of afferent inputs from the NTS and lateral parabrachial nucleus, and sends efferent projections to the ACC, OFC, LH, NAc, and amygdala (Van Vugt, 2009).

The **amygdala** is a part of the telencephalon, appears almond shaped and lies in the anterior temporal lobe (Murray, 2007) (Figure 1.5.). The amygdala contains an enormous diversity of nuclei and cell types. According to current thinking, the amygdala contributes to emotion, reward, motivation, learning, memory and attention (Murray, 2007). The amygdala is involved in emotional processing and particularly responds to positive and negative affect (Pessoa and Ungerleider, 2004). Bilateral damage of the amygdala disrupts emotional responses (Murray, 2007) and monkeys with amygdala lesions are fearless, have impairments in emotional learning, deficits in the perception of emotion, and impaired memory for emotional events (Cardinal et al., 2002). Recent functional imaging research has shown that the amygdala is activated when viewing or tasting a food (LaBar et al., 2001; Gottfried et al., 2003; Killgore et al., 2003; Hinton et al., 2004; Holsen et al., 2005; Fuhrer et al., 2008; Stoeckel et al., 2008; Goldstone et al., 2009; James et al., 2009; Passamonti et al., 2009; Schur et al., 2009; Wang et al., 2009). The amygdala receives projections from the insula, fusiforum (visual cortex) and VTA (Van Vugt, 2009) and usually returns them to the OFC, PFC, NAc and ACC (Amaral and Price, 1984, Morgane et al., 2005). Some amygdala functions are carried out in concert with the OFC, such as updating the values of expected outcomes stored in the OFC (Holland and Gallagher, 2004).

The **OFC** occupies the ventral surface of the frontal part of the brain (Kringelbach, 2005) (Figure 1.5.). The human OFC plays an important role in encoding taste, flavour, and food reward (Small et al., 2007). Olfactory, visual, gustatory, and somatosensory information (e.g crunchiness) are determinants for the palatability of food and such signals reach the OFC through different sensory brain pathways (de Araujo et al., 2003, Rolls et al., 2003, Verhagen and Engelen, 2006, Verhagen, 2007). It is important to distinguish between the lateral and the medial OFC (Kringelbach and Rolls, 2004). A large meta-analysis of the existing neuroimaging data was used to show that activity in the medial OFC is related to the monitoring, learning and memory of the reward value of reinforcers, whereas activity in the lateral OFC is related to the evaluation of punishers that can lead to a change in

behaviour (Kringelbach & Rolls, 2005). This meta-analysis further showed that a posterior–anterior OFC distinction was shown, with more complex or abstract reinforcers (such as monetary gain and loss) being represented more anteriorly in the OFC than less complex reinforcers such as taste.

A large number of functional imaging studies have implicated the OFC in processing of reward value when fasting and viewing visual food stimuli (O'Doherty et al., 2000, Small et al., 2001, Gottfried et al., 2003, Hinton et al., 2004, Wang et al., 2004, DelParigi et al., 2005, Holsen et al., 2005, Simmons et al., 2005, Killgore and Yurgelun-Todd, 2006, Porubska et al., 2006, DelParigi et al., 2007, Fuhrer et al., 2008, Stoeckel et al., 2008, Schienle et al., 2009, Schur et al., 2009, Wang et al., 2009). The proposed primary role for the OFC in encoding reward value is further supported by the decrease in activation seen during taste of a food repeatedly consumed to a state of aversion ('sensory-specific satiety') (Kringelbach & Rolls, 2004). Small and colleagues further showed that the OFC also encodes retronasal olfaction and oral somatosensation as during eating, distinct sensory inputs fuse into a unitary flavor percept, and there is evidence that this percept is encoded in the OFC (Small et al., 2007). One fMRI study found that activation in the mid-anterior parts of the OFC correlate with subjective pleasantness ratings of liquid foods when eaten to satiety providing a neural correlate of sensory-specific satiety to a liquid whole food (Kringelbach et al., 2003). The posterior parts of the OFC process the sensory information for further multimodal intergration (Kringelbach, 2005). The OFC is in close contact with the amygdala, insula and hippocampus (Verhagen and Engelen, 2006).

The cingulate cortex is one of the largest parts of the limbic system. The **anterior cingulated cortex (ACC)** can be differentiated from the posterior cingulate cortex (PCC) on the basis of cytoarchitecture and patterns of projections, as well as function (Figure 1.5.). The ACC has been characterized as 'executive' in function, whereas the PCC is characterized as 'evaluative' (Devinsky et al., 1995). The two major subdivisions of the ACC include the dorsal cognitive ACC and the ventral affective ACC (Bush et al., 2000). Functional imaging techniques have shown activation of the ACC when presented with

food stimuli (Bohon et al., 2009, Martin et al., 2009, Passamonti et al., 2009, Schienle et al., 2009). Activity in the ACC is seen during the delay period before the outcome of reward-related decisions, and is modulated by both outcome risk and anticipatory arousal (Critchley et al., 2001). The affective ventral ACC subdivision is primarly involved in assessing the salience of emotional and motivational information and the regulation of emotional responses (Devinsky et al., 1995, Whalen et al., 1998, Bush et al., 2000, Passamonti et al., 2008). It is connected to the amygdala, NAc, hypothalamus, anterior insula, hippocampus and OFC (Devinsky et al., 1995). The dorsal ACC, by contrast, is able to modulate attention or executive function, influencing response selection, motivation, conflict monitoring and cost/benefit or reward/risk assessment, and is influenced by anxiety and fearful situations (Devinsky et al., 1995, Carter et al., 1999, Bush et al., 2000, Carter and van Veen, 2007). It is reciprocally interconnected with the lateral PFC and motor areas (Devinsky et al., 1995).

Cortico-limbic reward systems of reward appear to be under executive control of the **prefrontal cortex (PFC)** (Figure 1.5.). The PFC receives internal and external sensory information, emotional and cognitive information from the limbic system, and motor and executive information from the cortical areas. It is thus able to translate homeostatic and environmental information into adaptive behavioural responses making choices and decisions (Kringelbach, 2005; Lenard and Berthoud, 2008; Piech et al., 2010). The **dorsolateral PFC (DLPFC)** is especially crucial for cognitive regulation of behaviour, goal-directed decisions and exercising self-control (Heekeren et al., 2004; Rorie and Newsome, 2005; Hare et al., 2009; Kable and Glimcher, 2009; Piech et al., 2010) (Figure 1.5.). Self-reported dieters show greater activation in the left DLPFC during a food picture choice paradigm using fMRI (Hare et al., 2009). Activation of the DLPFC has been reported when subjects view high-calorie compared to low-calorie foods especially when fasted (Davids et al. 2010, Killgore et al., 2003, Goldstone et al., 2009, Schur et al., 2009). Interestingly, compared with lean men, obese men have less postprandial activation in the left DLPFC, irrespective of meal size (Le et al., 2006). Since top-down control appears to

be mostly implemented in the DLPFC, it is suggested that following detection of conflict by the ACC, the lateral areas of the dorsal PFC are engaged to reduce conflict (Carter & van Veen, 2007).

In the following section techniques on how to image brain reward systems will be discussed.

1.3. Neuroimaging techniques

Imaging studies have shown that peripheral signals, central brain systems, and individual differences in food preference, nutritional state, gender, BMI, genetic polymorphisms as well as eating behaviour, reward sensitivity and mood have an influence on brain reward systems. The ability to study the neural networks that influence ingestive behaviour in humans has improved immensely with significant advances in imaging technology in the past 15 years. The physical principle of PET, MRI and fMRI are described in this paragraph.

1.3.1. Positron-emission-tomography (PET)

PET is a powerful functional imaging technique that has been used to study the relationship between energy consumption and neuronal activity (Bacharach et al., 2000; Huettel, 2004; Catana et al., 2008). PET uses positron-emitting radioactive tracers that are attached to molecules that enter biological pathways of interest. For example, ^{18}F, a radioactive isotope of fluorine, can be attached to glucose, creating the molecule fluoro-2-desoxy-D-glucose, or FDG. The fluorine tracer does not prevent FDG from entering into the normal pathways for glucose metabolism. Thus, researchers can inject a bolus of FDG into an artery and then use imaging to determine where it is taken up in the cells. As the radioactive isotope decays, it emits a positron (the antimatter counterpart of an electron). When the emitted positron collides with a nearby electron, they are mutually annihilated

and produce two gamma rays that take path 180° apart and are subsequently detected by their near simultaneous impact on opposite sides of a ring of scintillation crystals that surround the subject's head. A computer algorithm then evaluates the number and timing of impact at all of the crystals surrounding head and traces the path taken by the gamma rays back to their origins. Through this method, the distribution of glucose uptake in the brain can be measured, and changes in glucose uptake in different brain regions caused by sensory, motor, or cognitive activity can be determined. PET imaging can also study cerebral blood flow using ^{15}O, a radioactive isotope of oxygen. Certain neurotransmitters can be similarly labeled. For example ^{18}F can be attached to L-DOPA to study its distribution of uptake in the human brain.

1.3.1.1. Limitations

PET scanning is very expensive. The spatial resolution of PET is limited by physical factors associated with positron physics and by the difficulty of acquiring sufficient counting statistics. Furthermore, PET images often lack definitive anatomical information, making interpretation of the precise location of radiotracer accumulation difficult (Catana et al., 2008). Also radiation exposure in human research is carefully regulated, and subjects can participate in only a few PET scans (Huettel, 2004).

1.3.2. Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI or MR scanning) was developed in the 1970s by researchers including Peter Mansfield and Paul Lauterbur. It is a non-invasive and non-ionising imaging technique for creating 3D-images of the soft tissues of the human body. MRI is based on the physical phenomenon of nuclear magnetic resonance (NMR) (Westbrook, 2005).

1.3.2.1. Prinicple

The human body is made of atoms (electrons, protons and neutrons) (Figure 1.6.). Nuclei that have an odd number of protons and neutrons both possess a magnetic moment. These are MR active nuclei (hydrogen, oxygen etc.). Thus, hydrogen nuclei are utilised in MRI as they consist of single protons that move. Hydrogen nuclei have a magnetic field induced around them, and act as small magnets. The north/south axis of each nucleus is represented by a magnetic moment (M_0) and is used as the classical principle of MRI. The magnetic moments of the hydrogen nuclei are randomly orientated. Hydrogen nuclei possess energy in two discrete quantities or populations termed low (+1/2) and high (-1/2) states. Low-energy nuclei align their magnetic moment parallel to the external magnetic field and are termed spin-up nuclei. High-energy nuclei align their magnetic moments in the anti-parallel direction and are termed spin-down nuclei. As soon as a magnetic field is applied, the hydrogen nucleus will tend to align with the external magnetic field (B_0) causing the magnetic moment to follow a circular path around B_0 (precession). This path is called the precessional path and the speed at which they wobble around B_0 is called precessional frequency. The unit of precessional frequency is megahertz (MHz) whereby one Hz is one cycle per second and one MHz is 1 million cycles per second. The value of the precessional frequency is governed by the Lamor equation:

$$\omega_0 = -\gamma B_o$$

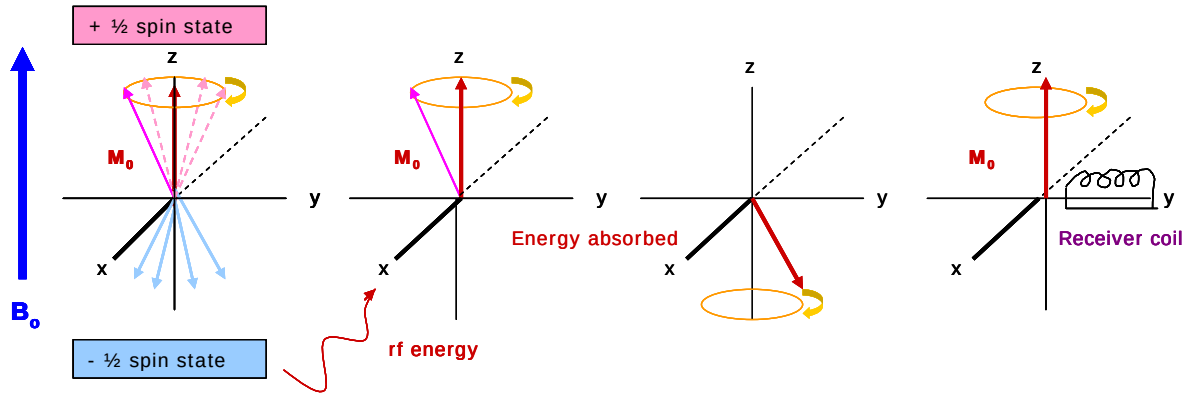
ω_0 = *precessional frequency*
B_o = *magnetic field strength of the magnet*
γ = *gyro-magnetic ratio in MHz/T (relationship between angular moment and magnetic moment of each active nucleus*

Thus, Larmor precession (named after Joseph Larmor) is the precession of the magnetic moments of electrons, atomic nuclei, and atoms about an external magnetic field.

Applying a radio frequency (RF) pulse (with same energy as Lamor frequency of hydrogen), causes an increase in the number of the spin-down hydrogen nuclei as some spin-up nuclei gain energy via resonance and become high-energy nuclei. The result of the resonance is that the magnetic moment moves out of alignment away from B_0, from

the longitudinal to the transverse plane. The angle through which a magnetic moment moves out of alignment is the flip angle. The magnetic moment rotates then in the transverse plane at Lamor frequency. After the RF pulse is switched off the magnetic moment is again influenced by B_0 and tries to realign with it. Spins return to the low energy state by releasing absorbed energy gained from the RF pulse. The process by which hydrogen loses energy is called T1 relaxation. The recovery of longitudinal magnetization is caused by the process termed T1 recovery (= nuclei are giving up their energy to the surrounding environment). The decay of transverse magnetization is caused by a process termed T2 decay (= nuclei exchange energy with neighbouring nuclei). As the magnitude of the transverse magnetization decreases, so does the magnitude of the voltage induced in the receiver coil. Thus, the released energy or MR signal can be measured by the receiver coil. The induction of reduced signal is called the free induction decay (FID) signal. The signal is Fourier transformed and converted into images (from signals to transverse slices) (Westbrook, 2005) (Figure 1.6.).

Figure 1.6. Physical principle of MRI.



Physical principle of MRI: applying a radio frequency (RF) pulse causes the magnetic moment (M_0) to move out of alignment (away from the magnetic field B_0) from the longitudinal to the transverse plane. After the RF pulse is switched off, M_0 is again influenced by B_0 and tries to realign with it. The released energy from the RF pulse or MR signal can be measured by the receiver coil. The signal is Fourier transformed and converted into images.

In brief, the MR images are generated by a complex coordinated series (pulse sequences) of radio frequencies (RF) pulses and magnetic field gradient oscillations in a strong background static magnetic field. A pulse sequence is a combination of RF pulse, gradients

and intervening periods of recovery. The main components of a pulse sequence are repetition time (TR) and echo time (TE). The TR is the time from the application of one RF pulse to the application of the next RF pulse for each slice and is measured in milliseconds (ms). The TR reflects the amount of T1 relaxation that occurrs when the signal is read. The TE is the time from the application of the RF pulse to the peak of the signal induced in the coil and is also measured in ms. The TE determines how much decay of transverse magnetization is allowed to occur.

MRI images are obtained from volumes, so pixels in an MR image are more accurately referred to as voxels. The signal intensity of a voxel within a given pulse sequence can reliably be predicted by knowing three parameters of the region of the object from which the voxel was acquired - the T1, T2 and T2* relaxation times. Most sequences do not isolate an individual parameter, but are instead weighted to being affected by one more than others (McRobbie, 2003). One method, using *T2-weighted echo-planar images* (uses rapid gradient switching following a single exication to fill the entire *k*-space) is functional MRI.

1.3.3. Functional MRI (fMRI)

In the early 1980s, MRI was introduced clinically, and during the 1980s a veritable explosion of technical refinements and diagnostic MR applications to study the brain took place. Scientists soon learned that the large blood flow changes measured by PET could also be imaged by the correct type of MRI. Functional magnetic resonance imaging (fMRI) was born, and since the 1990s, fMRI has come to dominate the brain mapping field due to its low invasiveness, lack of radiation exposure, reduced costs and relatively wide availability. Functional MRI refers to a rapidly developing technology that enables the creation of images which reveal localized neural activity in human brains during sensory, motor, and cognitive activity (Savoy, 1999; Huettel, 2004).

1.3.3.1 Blood Oxygenation Level Dependant (BOLD) signal

The BOLD effect is based on the fact that there are local blood flow and blood oxygenation changes in response to neuronal activity. Applying a sensory, motor, or cognitive stimulus increases neuronal activity. This neuronal activity requires energy in form of adenosine triphosphate, or ATP. Because the brain is able to store limited amounts of energy, it must create the ATP energy through the oxidation of glucose. Both oxygen and glucose are supplied by increased blood flow. Through the increase in blood flow, volume and oxygen, deoxyhaemoglobin flushes from the capillaries, and small veins. Since deoxyhaemoglobin molecules are paramagnetic this alters the spins of nearby diffusing hydrogen nuclei, the presence of deoxyhaemoglobin reduces the MRI signal intensity. By displacing the deoxyhaemoglobin with oxygenated haemoglobin, the increase in blood flow results in a local increase in MRI signal. The magnetic properties and the change of the ratio of oxygenated and deoxygenated haemoglobin can be therefore used to construct images based upon blood-oxygenation-level dependant (BOLD) contrast. In brief, BOLD-fMRI detects a change in the concentration of a paramagnetic molecule (deoxyhaemoglobin) whose levels are correlated with neuronal activity through neurovascular-coupling (Huettel, 2004).

Principle of fMRI preprocessing

After the MRI signal has been recorded, the images need to be reconstructed and quality control assured.

Each imaged slice is acquired at slightly different time points within the TR (e.g. in my studies 44 slices within 3 seconds). Thus **correction for slice timing** is necessary and consistency can be achieved by shifting and then interpolating the data, or including temporal derivatives in the analysis.

Motion correction is important to adjust the time series of images so that the brain is in the same position in every image. For motion correction, image volumes in the same time series are coregistered to a single reference volume by a combination of three

translation (moving the entire image volume along the x-, y-, z-axes) and three rotations (rotating the entire image volume through the roll, yaw and tilt planes).

Geometric distortions caused by inhomogenities in the magnetic field can lead to loss or compression of signal in important brain reward areas such as the OFC as well as temporal cortex due to vicinity to air-filled sinuses. It is possible to correct to some extent for the effects of these inhomogenities by using **field maps** acquiring two images of signal phase with slightly different echo times. The differences between the two images results in an image of the intensity of the magnetic field across space (at any given location) which can be registered with the EPI (echo planar imaging) image and therefore correct geometric distortions, though not recover EPI signal drop-out (Huettel, 2004).

Spatial filtering or smoothing is used to increase the signal to noise-ratio. Each voxel intensity is replaced by a weighted average of neighbouring intensities. As a result the image looks as if it is viewed through a blurred glass and the rate of false-positives is reduced. The image can be smoothed by using a Gaussian kernel (Spatial filter width for fMRI) expressed in millimeters at Full Width Half Maximum (FWHM). Thus, by using this filter, is is possible to average the data over adjacent voxels, reducing the likelihood that a cluster of voxels will pass the same threshold (Huettel, 2004).

Time series from each voxel contain low frequency drifts and high-frequency noise. Drifts are scanner-related and physiological (cardiac cycle, breating, etc.). Both high and low frequency noise hide activation. Using **temporal filtering**, high frequencies, low frequencies or both can be removed without removing signals of interest. For example low-pass filters are used to remove high frequencies.

The differences between functional and structural images of the same brain region are striking. High-resolutional T1 structural brain images appear remarkely detailed with clear boundaries between grey and white matter whereas in functional EPI images boundaries are difficult to visualise. Human brains also have remarkably variable morphology and shape. **Normalisation** or registration to standard space is used to compensate for differences in brain shape. Normalisation of data within a study allows combination of data (brain areas) across individuals. A commonly used standard space

was created by the Monreal Neurological Insitute and consists of an average of 152 T1-weighted brain images. Therefore, the EPI image must first be registered with the individual's T1 image using 6 degrees of freedom which in turn must be registered within standard space using 12 degrees of freedom registration which may be linear or non-linear, and these warping parameters applied to the T1-registered EPI image.

fMRI experimental analysis

Each acquired voxel in an EPI scan contains a time-varying signal (BOLD signal). It is important to model the stimulus-induced changes in BOLD signal (predicted response) in order to find which voxels have signals that match the model. A good match implies activation related to a particular stimulus. The haemodynamic response to a stimulus is blurred and delayed. In order to compare the BOLD signal, the process can be modeled by convolving the activity curve with a haemodynamic response function (HRF), giving the predicted response. A standard General Linear Model (GLM) allows multiple comparisons and correlates the model (predicted response) with each voxel separately. For example, time-series statistical analysis can be carried out using autocorrelation correction including picture onsets (e.g. in my study high-calorie and low-calorie food, object and blurred pictures), with temporal derivatives (to correct for slice timing variation and differences in time of onset of brain activation relative to stimulus in different brain regions) and motion parameters (to deweight scans which may be subject to motion artifact) as co-variates. This gives parameter estimates (beta) and under the null hyothesis beta is 0. If the 'null hypothesis testing' results in a small p-value, the null hypothesis is unlikely and then the voxel would be active. In this way it is possible to create group mean statistical parametric maps for the whole brain or for each region of interest (ROI) to see which voxel in which brain region is significantly activated by one particular stimulus compared to another.

1.4. Signalling in brain reward systems

Although there has been a tremendous progress describing the central homeostatic control of food intake, this system does not work very well in an environment where little effort is necessary to obtain palatable foods with high-calorie density. During the past 40 years, there has been 42% *per capita* increase in the consumption of added fats in foods and a 162% increase in cheese relatively to only a 20% increase in fruits and vegetabales (Frazao and Allshouse, 2003). Reduced costs of sugar and vegetable oils worldwide have also contributed to the production of highly palatable processed foods (Drewnowski, 2000). There is also a "portion-distorted embarassment of food" in today's supermarkets and restaurants (Wansink and van Ittersum, 2007). Evidence in rodents and humans now supports the theory that both drugs of abuse and the consumption of highly palatable foods converge on a shared addiction pathway within brain reward systems to mediate motivated behaviour (Nestler, 2001, Wang et al., 2004, Nestler, 2005). Much of the previous work has focused on the mesocorticolimbic dopamine pathway.

1.4.1. Dopamine

Synthesis

Dopamine is produced in several areas of the brain, including the substantia nigra and the ventral tegmental area of the midbrain. Dopamine signaling on feeding is thought to be mediated by D_1 and D_2 receptors (G protein-coupled receptors) (Schneider, 1989, Kuo, 2002). Tyrosine hydroxylase or tyrosine 3-monooxygenase is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to dihydroxyphenylalanine (DOPA) (Kaufman, 1995, Nagatsu, 1995). DOPA is a precursor for dopamine, which, in turn, is a precursor for noradrenaline and adrenaline. In humans, tyrosine hydroxylase is encoded by the *TH* gene (Nagatsu, 1995).

Function

Mice which lack dopamine, due to the absence of the *TH* gene, have fatal hypophagia. Dopamine replacement into the caudate putamen (dorsal striatum) restores feeding,

whereas replacement into the nucleus accumbens restores preference for a palatable diet (Szczypka et al., 2001). The exact role of dopamine in the process of reward has been vigorously debated in recent years. Berridge has argued that dopamine fosters the motivation to engage in rewarding behaviours - the so called “wanting” of reward, rather than “liking” of reward (Berridge, 2007). In other words, dopamine may not be central to the normal pleasure or hedonic reactions to food or drugs. Dopamine has been hypothesised to encode reward prediction error and would act to shape future behaviour to maximize reward (Montague et al., 2004). Because a reward prediction error forms the basis of Pavlovian learning theories, it has been postulated that dopamine neurons provide an error signal for the learning of stimulus-reward association (Schultz, 2002).

Hypo-dopaminergic functioning

A key focus in research of addiction disorders and obesity is that *hypo*-dopaminergic functioning (Reward Deficiency Syndrome - RDS) in brain reward pathways is a key risk factor for their development (Blum et al., 2000). The premise is that addictive substances, which increase brain dopamine levels, are used as a form of “self-medication” to boost a sluggish dopamine system and increase hedonic capacity and promote excessive behaviour. The same arguments have been recently extended to the risk for obesity; focusing on dopamine D2 receptors. There is reasonable evidence that individuals with the Taq1A+ allele (polymorphism of D2 receptor) have reduced brain dopamine function in the dorsal stritum due to a 30-40% reduction in D2 receptor density in the striatal region (Davis et al., 2008, Stice et al., 2008). Stice and colleagues performed an fMRI study after subjects received a chocolate milkshake or a tasteless solution. Obese individuals, relative to lean individuals, showed a blunted dorsal striatal response to milkshake receipt, and this effect was amplified in those with the A1 allele. Thus, the dorsal striatum was less responsive to food reward in obese, relative to lean, individuals, potentially because obese are suggested to have reduced D2 receptor density and compromised dopamine signaling, which may prompt them to overeat in an effort to compensate for this reward deficit (Stice et al., 2008).

Hyper-sensitivity to reward

The counter argument is that *hyper*-sensitivity to reward contributes to increased risk of obesity because of enhanced motivation to approach pleasurable activities such as eating (Beaver et al., 2006, Davis et al., 2007). Addictive drugs and food could also share a tolerance of their rewarding properties by the downregulation of dopamine receptors in the striatum (Volkow et al., 2002), and a decrease in the function of the amygdala, which produces negative affect and anxiety associated with abstinence (Koob, 2006). There is now evidence that highly palatable foods cause the same neuroadaptions which increase strong cravings, and contribute to the symptoms of withdrawl (Grigson, 2002, Pelchat, 2002).

Davis and Carter suggest a dual vulnerability to addictions whereby both *hypo*-dopaminergic functioning and *hyper*-sensitivity to reward can confer risk, albeit in different individuals and perhaps with different levels of severity (Davis and Carter, 2009).

Dopamine signalling

All common drugs of abuse increase dopamine signaling from the VTA to the NAc, the prefrontal cortex, the hippocampus, and the amygdala. Increasd dopaminergic transmissions occur either by direct action on dopaminergic neurons (cocaine, nicotine) or indirectly through inhibition of GABAergic interneurons in the VTA (alcohol, opiates) (Nestler, 2001, 2005). Natural rewards such as presentation and ingestion of palatable food induces potent release of dopamine into the nucleus accumbens (Nestler, 2001). The mechanism by which food stimulates dopamine signaling is unclear. It does not appear that taste receptors are required, as mice lacking sweet receptors are still able to develop a strong preference for sucrose solutions (de Araujo et al., 2008).

A new concept is that hormones such as leptin, insulin and ghrelin directly affect the dopamine reward pathways. These hormones activate receptors on VTA dopamine neurons, thereby either stimulating (ghrelin) or inhibiting (leptin and insulin) dopamine signaling in the NAc, which is described later in more detail. Other neurotransmitters such as GABA, opioids and serotonin are also integral to reward process and will be described

later (Wang et al., 2004). NPY has been shown to enhance the extracellular levels of accumbal dopamine (Salin et al., 1990). There is also evidence that orexin receptors are located on dopamine neurons, where they couple to the same receptor as ghrelin and microinjection of orexin into the VTA increases the levels of dopamine in the NAc (Narita et al., 2006). Endogenous cannabinoids are an important player in the reward circuits, since they interact with opioid and dopaminergic systems to enhance satisfaction coming from the ingestion of palatable food (Pagotto et al., 2006).

1.4.2. Endocannabinoids

Endocannabinoid receptor CB1 is localised in high concentrations in the hippocamous, cerebellum, prefrontal cortex and striatum, but in low concentrations in the VTA (Herkenham et al., 1990, Tsou et al., 1998). Previous research has shown that CB1 receptor antagonists reduces intake of food and sweet solutions (Arnone et al., 1997; Simiand et al., 1998). CB1-null mice, however, do not show dramatic changes in body weight and food consumption, suggesting that CB1 receptors rather modulate than mediate basic reward functions or other systems compensate for their absence (Ledent et al., 1999, Zimmer et al., 1999, Marsicano et al., 2002). The anti-obesity drug, rimonabant, a CB1-receptor blocker has been shown to inhibit the effect of ghrelin (Kola et al., 2008). In this study, ghrelin was unable to induce an orexigenic effect in CB1-knockout mice suggesting that the endocannabinoid system may mediate ghrelin's actions (Kola et al., 2008).

Exposure to food increases dopamine levels in the NAc and activation of dopamine D2 receptors in this region increases extracellular levels of the endocannabinoid anandamide (Giuffrida et al., 1999, Di Chiara et al., 2004). The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) appear to increase the palatability of food, indicating that they are more important for hedonic aspects than consumatory aspects of food reward (Kirkham et al., 2002, Mahler et al., 2007).

1.4.3. Opiates

Opioids, especially the mu-opioids, also regulate the "hedonics of feeding" – what Berridge calls the "liking" – by their modulation of the palatability of food (Berridge, 1996, 2007). In humans, opiate antagonists are found to reduce food palatability without reducing subjective hunger (Yeomans et al., 1990, Drewnowski et al., 1992). Recent studies have demonstrated a major role for mu-opioid receptors (OPRM1), especially in the ventral striatum and amygdala where their activation enhances positive hedonic reactions to sweet and fatty foods (Olszewski and Levine, 2007). Particularly in the midbrain, mu-opioid receptors are localized on gamma-amino butyric acid neurons (GABAergic signaling) that normally inhibit the dopamine system; mu-opioids inhibit this input, thus disinhibiting the dopamine release in the nucleus NAc and related regions (MacDonald et al., 2004).

1.4.4. Leptin

Early studies showed that leptin receptors are expressed in the VTA (Figlewicz et al., 2003). Co-expression of the leptin receptor with tyrosine hydroxylase, a marker of dopamine neurons has also been demonstrated (Figlewicz et al., 2003, Fulton et al., 2006, Hommel et al., 2006).

The observation that the transcription factor STAT3, is phosphorylated (pSTAT3) in dopamine neurons in response to leptin treatment provides evidence that leptin receptors on dopamine neurons are functional (Fulton et al., 2006, Hommel et al., 2006). In anaesthetized rats, the firing rate of dopamine neurons decreased by ~40% following intravenous infusion of leptin (Hommel et al., 2006), and leptin inhibited dopamine neuron firing when applied to slices containing VTA neurons (Korotkova et al., 2006).

Leptin is also suggested to affect hedonic aspects of feeding behaviour as infusion into the 3rd ventricle and subcutaneous injection reduced sucrose self administration, suppressed the conditional place preference (CPP) for sucrose pellets and reversed CPP for high-fat foods (Figlewicz et al., 2001, Figlewicz et al., 2004). CPP measures the ability of an animal to associate the rewarding aspects of food (or a drug) with a particular

environment. Thus, a reduction in CPP by suggests an interference with subjective reward value and associated learning. Another animal study has shown that inactivation of leptin receptors in the VTA increased feeding, but the mice did not gain weight (Hommel et al., 2006).

A functional MRI study measured brain responses in two human patients with congenital leptin deficiency (Farooqi et al., 2007). They showed images of food before and after 7 days of leptin replacement therapy. Leptin was found to reduce neural activation in key striatal regions (accumbens-caudate and putamen–globus pallidus) in both subjects, suggesting that the hormone acts on neural circuits governing food intake to diminish the perception of food reward. After leptin treatment, hunger ratings in the fasted state decreased, and satiety following a meal also increased (Farooqi et al., 2007). Baicy et al. further reported an fMRI study where leptin-deficient patients (missense mutation in the *OB* gene) with leptin replacement had reduced hunger ratings and reduced activation in brain reward areas (insula, parietal lobe and temporal cortex) when viewing food pictures, but increased activation in self-control areas (prefrontal cortex) (Baicy et al., 2007). A third leptin study measured brain responses to visual food cues in (non-leptin deficient) obese patients who had achieved a 10% reduction in body weight through food restriction (Rosenbaum et al., 2008). Five weeks of twice-daily subcutanous injection of leptin (0.08mg/kg fat mass), following weight-loss (during which leptin levels would naturally decline), increased activation in brain areas known to be involved in the emotional and cognitive control of food intake (brainstem, culmen, parahippocampal gyrus, inferior and middle frontal gyri, middle temporal gyrus, and lingual gyrus) and decreased activation in the hypothalamus, cingulate gyrus, and middle frontal gyrus. Thus, circulating leptin seems to inhibit dopamine signaling in brain reward systems and influence the subjective reward value of food and hence motivation to eat (Palmiter, 2007).

1.4.5. Insulin

Early studies also showed that insulin receptors are co-expressed with tyrosine hydroxylase, a marker of dopamine neurons, in the VTA (Figlewicz, 2003). Downstream substrates of insulin receptors, such as insulin receptor substrate 2 and phosphatidylinositol (3,4,5)-triphosphate, are expressed in dopamine neurons (Pardini et al., 2006). Figlewicz and colleagues showed that intravenous administration of insulin increased the mRNA levels and the functional activity of dopamine transporters; this increase would be expected to facilitate clearing dopamine from synapses and hence reduce dopamine signaling (Figlewicz et al., 1994, Figlewicz et al., 2003).

Insulin is also suggested to affect feeding behaviour as infusion into the 3rd ventricle reversed CPP for high-fat foods (Figlewicz et al., 2004).

Functional MRI research demonstrated the important role of insulin in the modulation of food hedonic responses and related information processing. Guthoff and colleagues showed that activation of brain reward areas (fusiforum gyrus, hippocampus, temporal superior cortex, and frontal middle cortex) by food pictures is down-regulated by intranasal insulin (Guthoff et al., 2009). Thus, circulating insulin also seems to inhibit dopamine signaling in 'reward circuits' and influence the subjective reward value of food and hence motivation to eat (Palmiter, 2007).

1.4.6. PYY_{3-36}

Recent research has also suggested an influence of the gut hormone PYY_{3-36} on 'reward circuits' (Batterham et al., 2007). Unlike the previous described fMRI studies, this study did not use visual food cues. Infusion of PYY_{3-36} to fasted subjects that mimicked post-prandial concentrations of PYY increased the resting BOLD signal in the caudolateral OFC and significantly reduced the number of calories consumed at a subsequent meal. The magnitude of activation in the OFC was inversely correlated with the number of calories eaten; suggesting that PYY-induced activation of the OFC was in part responsible for the reduced appetite. This suggests an inhibiting role of PYY on brain 'reward circuits' but not

traditional homeostatic hypothalamic centres. On the fasted day, saline-induced hypothalamic activation correlated with subsequent caloric intake (Batterham et al., 2007).

1.4.7. Ghrelin

An important novel player in the mesocorticolimbic dopamine reward system is the stomach-derived orexigenic hormone ghrelin. The hypothalamus has the highest concentration of GHS-R in the brain but other regions such as the VTA express this receptor in relatively high concentrations (60-70%) (Abizaid et al., 2006, Zigman et al., 2006). Pharmacological blockade of GHS-R in the VTA decreases feeding in fasted mice (Abizaid et al., 2006). Behavioural data in animals showed that ghrelin injection enhances the willingness to work harder to obtain food (Keen-Rhinehart and Bartness, 2005).

New research suggests that peripheral ghrelin enhances the intake of rewarding food in rodents (Egecioglu et al., 2010), especially sweet taste consumption and preference regardless of its caloric content (Disse et al., 2010). Rats can develop CPP to high fat food after being injected subcutaneously with ghrelin (Jerlhag, 2008, Quarta et al., 2009). Intracerebroventricular administration of ghrelin increases locomotor activity and extracellular levels of accumbal dopamine in mice (Jerlhag et al., 2006). These effects of ghrelin are mediated via central nicotine receptors, suggesting that ghrelin can activate the acetylcholine-dopamine reward link. This reward link consists of cholinergic input from the laterodorsal tegmental area (LDTA) to the mesolimbic dopamine system that originates in the VTA and projects to the nucleus accumbens. Other studies have confirmed that local ghrelin administration into the VTA activates dopamine neurons, subsequently increases dopamine in the nucleus accumbens and locomotor activity, and directly stimulates food intake (Naleid et al., 2005, Jerlhag et al., 2007, Quarta et al., 2009). GHSR-1A is suggested to be expressed in the VTA and LDTA (Jerlhag et al., 2007). Local administration of ghrelin into the LDTA caused a locomotor stimulation and an increase in the extracellular levels of accumbal dopamine (Jerlhag et al., 2007). Taken

together, this indicates that ghrelin might, via activation of GHSR-1A in the VTA and LDTA, stimulate the acetylcholine-dopamine reward link.

Ghrelin does indeed bind to the VTA when directly delivered to these regions (Abizaid et al., 2006). It is important to note that approximately 30% of GABA neurons in the VTA have ghrelin receptors, yet it is unclear how they are affected by ghrelin (Abizaid, 2009). Similarly, ghrelin infusions into brain regions targeted by dopamine cells in the VTA, such as the NAc, amygdala, and hippocampus, produce alterations in feeding responses suggesting that ghrelin acts mostly like a neuromodulator and enhances dopamine release in the synapse (Cowley et al., 2003, Carlini et al., 2004, Naleid et al., 2005, Toth et al., 2008). The possibility must be considered that the effects of ghrelin on the activity and neurotransmitter of the VTA dopamine neurons may be produced indirectly through the actions of ghrelin on afferent systems connected to the VTA (Geisler and Zahm, 2005). Also implicated in activation of VTA dopamine neurons is the peptide neurotransmitter orexin, which is expressed by a population of lateral hypothalamic neurons (Perello et al., 2010).

The presence of GHS-R had already been identified in the hippocampal formation even before ghrelin and its functions were discovered (Guan et al., 1997). Peripheral ghrelin has a robust impact on hippocampal synaptology (Diano et al., 2006). Ghrelin enters the hippocampus and binds to neurons of the hippocampal formation, where it promotes dendritic spine synapse formation and generation of long-term potentiation. Ghrelin enhances learning and memory, whereas the absence of ghrelin due to targeted gene disruption causes impairment of these functions (Diano et al., 2006).

The effect of ghrelin on the amygdala could be also direct, as the amygdala contains ghrelin-positive axon terminals (Cowley et al., 2003), or indirect via the hypothalamus or the VTA, which send dopaminergic projections to the amygdala (Carlini et al., 2004). However, injection of ghrelin into the amygdala failed to increase food intake in one study (Carlini et al., 2004).

A recent study showed that substantia negra pars compacta (SNpc), a brain region where dopamine cell degeneration leads to Parkinson disease, expresses GHS-R. Thus

ghrelin binds to SNpc, activates dopamine neurons, increases tyrosine hydroxylase mRNA and dopamine concentration in the dorsal striatum (Andrews et al., 2009).

One study in humans showed that an i.v. single injection of ghrelin increased the imagination of individual preferred foods (Schmid et al., 2005). Malik and colleagues recently showed that administration of ghrelin, in comparison to saline, produced greater activation in brain reward centres such as the OFC, PFC, amygdala, and anterior insula when subjects viewed food pictures (Malik et al., 2008). Furthermore the magnitude of activation in the OFC and amygdala correlated with increased self-rated hunger levels elicited by ghrelin, suggesting that behavioural effects and BOLD effects of ghrelin were related.

The next paragraph will discuss various factors other then internal hormonal signals such as food preference, nutritional state, gender, obesity and psychological cues and how these factors influence brain reward systems (Figure 1.1., Figure 1.5.).

1.5. Other cues effecting brain reward systems (Table 1.1.-1.8.)

1.5.1. Food preference

The sensory properties of food play a very important role in the way people select their food and how much they eat (Sorensen et al., 2003). The palatability of food is often measured by using visual analogue scales where subjects are asked questions like 'how pleasant was the food' (van Strien, 1986).

Generally, people prefer high-fat over low-fat foods (Mizushige et al., 2007). One study compared the taste of high-fat foods, high-protein foods, and high-carbohydrate foods using 13 strains of mice (Smith et al., 2000). Ten strains of mice preferred the high-fat food over the other selections.

Sweetness is also a major contributor to palatability, although there is an individual difference in optimal level of sweetness. In two studies, the palatability of a test food was

manipulated by using yoghurt with different concentrations of the intense sweetener, aspartame, and sucrose (Monneuse et al., 1991, Perez et al., 1994). In both studies, yoghurt intake was greater at the individual subject's most preferred concentration than at all the other concentrations. Sugars are reinforcing, indicating a high incentive salience (McCaughey, 2008). Rats will run towards a known source of sugar rapidly and will work hard to obtain them (Guttman, 1954, Young and Shuford, 1955). Access to sugar can condition a place preference (Agmo and Marroquin, 1997). There is widespread assumption that sugar consumption can lead to addiction (Benton, 2009). A study by Rolls et al. revealed that pleasantness ratings of chocolate and chocolate-related stimuli had higher positive correlations with fMRI activation in the pregenual cingulate cortex and medial OFC in chocolate cravers than in non-cravers (Rolls and McCabe, 2007). Furthermore a picture of chocolate with chocolate in the mouth produced greater activation in the medial OFC and pregenual cingulate cortex in cravers than non-cravers.

Access to fat can also condition a place preference. One previous study showed that CPP to *high-fat* diet was blocked in ad-lib fed rats given intraventricular insulin or leptin (Figlewicz et al., 2004).

Thus, does the evolutionary effect of eating high-calorie foods in times of plenty in order to store more energy for times without food, lead to a preference for high-calorie, fatty and sugary foods? Obese people might also prefer high-calorie, highly palatable foods in order to compensate for a reduced dopamine signaling in the brain reward circuitry. More research is needed to understand the association between taste preference and development of obesity.

1.5.2. The effect of food stimuli on the human brain (Table 1.2.-1.8.)

Brain activation varies depending on which food stimuli are presented (e.g. high-calorie or low-calorie) and how they are presented (taste, smell, pictures). With the help of new functional imaging research it is now possible to determine the effect of different food types on brain reward systems. Indeed, exposure to highly appetizing food cues can

override satiety signals and promote overeating (Cornell et al., 1989; Bender et al., 2009; Frank et al., 2010).

1.5.2.1. Food versus non-food

PET and BOLD imaging research has shown, in non-obese healthy volunteers, that compared to non-food pictures, visual food stimuli induce greater activity in the amygdala, ACC, hippocampus, insula and OFC, DLPFC and striatum (Gordon et al., 2000, LaBar et al., 2001, Morris and Dolan, 2001, Wang et al., 2004, Holsen et al., 2005, Simmons et al., 2005, St-Onge et al., 2005, Porubska et al., 2006, Fuhrer et al., 2008, Schur et al., 2009). Similar areas are activated when subjects have to smell and/or taste food (O'Doherty et al., 2000, Small et al., 2001, Gottfried et al., 2003).

1.5.2.2. Appetizing versus bland or disgusting foods

Imaging studies also demonstrated that activity is greater in the ventral striatum, OFC, amygdala and insula when viewing appetizing compared with bland or disgusting foods (Beaver et al., 2006, Calder et al., 2007). Activity is also greater in the amygdala and OFC when chosing highly preferred foods from a menu (Hinton et al., 2004; Piech et al., 2009).

1.5.2.3. High-calorie versus low-calorie foods

Compared to low-calorie food pictures, viewing high-calorie food stimuli induced a greater activity in the insula, medial OFC, medial and dorsolateral PFC, when using functional MRI (Goldstone et al., 2009; Gordon et al., 2000; Killgore et al., 2003; Siep et al., 2009; Frank et al., 2010).

1.5.3. Nutritional states (Table 1.2.-1.8.)

Although there are significant variations in the regions reported to be activated by food cues in the individual studies, it is likely that experimental variables such as stimulus

paradigms, gender, BMI, and individual differences in eating behaviour, reward sensitivity and mood, but also *hunger* and *satiety levels* may contribute to these differences. Thus, the sensation of reward is influenced by energy status, as the subject's palatability of food is altered in the fed, compared with the fasting states (Berridge, 1991).

1.5.3.1. Hunger

Food-deprived rats show even larger intake of sugars than do those that are non-deprived (Smith and Duffy, 1957). A human study by Stoeckel and colloeagues found that women (though not men) have enhanced valence of food images when fasted. No evidence was found of hunger-induced enhancement of hedonic ratings of non-foods (Stoeckel et al., 2007; Haase et al., 2009b). In normal weight subjects in a fasted hungry state, visual food versus non-food stimuli is reported to produce greater activation in regions including the amygdala, insula, OFC, striatum, and DLPFC using functional neuroimaging techniques (Gordon et al., 2000, LaBar et al., 2001, Hinton et al., 2004, Wang et al., 2004, Holsen et al., 2005, Simmons et al., 2005, St-Onge et al., 2005, Porubska et al., 2006, Fuhrer et al., 2008, Goldstone et al., 2009, Schur et al., 2009). These areas are also implicated in the neural response to smell and taste (Small et al., 2001; Gottfried et al., 2003; DelParigi et al., 2005; Small et al., 2007; Haase et al., 2009b; Grabenhorst et al., 2010; Smeets et al., 2010).

Brain activity at rest in the insula and OFC also increased under conditions of prolonged fasting (Del Parigi, 2002). The subjective rating of hunger when fasted for at least 5 hours has been shown to correlate with activation in the insula to food pictures (Porubska et al., 2006). Activity in the amgydala and OFC has also been associated with enhanced memory of food stimuli when fasted (Morris and Dolan, 2001).

Another neuroimaging stuy demonstrated that 10% weight loss during a period of maximal 62 days resulted in increased activation in the brainstem and parahippocampal gyrus and pallidum as well as middle temporal gyrus, inferior frontal gyrus, middle frontal gyrus, and lingual gyrus when when viewing food vs. non-food. These areas are involved in reward and decision-making functions (Rosenbaum et al., 2008).

1.5.3.2. Sensory-specific satiety

Interestingly, when a food is repeatedly viewed, smelled or consumed, it drops in ratings of pleasantness relative to foods that have not been eaten and this phenomenon is referred to as sensory-specific satiety (Rolls et al., 1981). Kringelbach et al. showed that a sensory-specific reduction in activation of the OFC correlates with subjective pleasantness when repeatedly eating a liquid food to satiety (Kringelbach et al., 2003). Similar imaging investigations have shown that activation of the OFC is decreased to the odour of a food eaten to satiety (O'Doherty et al., 2000, Small et al., 2001).

Cornier et al. has shown that in the state of positive energy balance (30% overfeeding for two days) activation in brain areas such as the insula and the hypothalamus when viewing foods of neutral hedonic value were reduced in lean individuals. This pattern was reversed in obese individuals and thus no reduced activation in the hypothalamus after two days of overfeeding was observed (Cornier et al., 2009).

1.5.3.3. Hunger versus satiety

A large numer of functional imaging studies using BOLD fMRI or PET have shown differences in neuronal activation in brain reward system when comparing hungry (fasted) and satiated (fed) states in humans. Acute fasting increased activity in the ventral striatum, amygdala, insula and medial OFC in response to food versus non-food stimuli compared with when fed (LaBar et al., 2001, Hinton et al., 2004, Holsen et al., 2005, Uher et al., 2006, Farooqi et al., 2007, Fuhrer et al., 2008, Goldstone et al., 2009).

1.5.4. Gender (Table 1.2.-1.8.)

The prevalence in obesity is higher in women than in men in most countries around the world (Lovejoy and Sainsbury, 2009). The factors driving the greater propensity for excess body weight in women are not well understood. Research has focused on evolution, gonadal hormones, pregnancy, behavioural differences and environment, and their influences on peripheral and central mechanisms that control appetite and body

weight. Women have lower energy expenditure than men (Tooze et al., 2007). Rodent studies have shown that oestrogen treatment in mice increases lipolysis in abdominal fat cells (D'Eon et al., 2005) but differences in eating behaviour might also be important.

Oestrogen deficiency in female animals is associated with hyperphagia and increased body weight and adiposity (Asarian and Geary, 2006, Clegg et al., 2006). Similarly, in women some studies report that menopause is associated with weight gain independent of age (Aloia et al., 1995).

It is also important to note that menstrual cycle is associated with changes in food intake (Davidsen et al., 2007). Functional imaging research has shown that, in women, activation of brain reward system (midbrain, striatum, amygdala, OFC) is increased in the follicular phase compared to the luteal phase (Van Vugt, 2009; Frank et al., 2010).

Using [^{15}O]H_2O PET, Del Parigi and colleagues demonstrated that, in response to hunger, men tended to have greater resting activity in frontotemporal lobe than women (Del Parigi, 2002). In response to satiation (liquid meal), women tended to have greater activation in occipital and parietal sensory association areas and DLPFC than did the men; in contrast, the men tended to have greater activation in the ventromedial PFC than did the women.

An fMRI study showed that gender predicts activation in brain reward centres after chocolate satiation (Smeets et al., 2006). In men, chocolate satiation was associated with increased activation in the ventral striatum, insula, and medial OFC and with decreased activation in somatosensory areas. Women showed increased activation in the precentral gyrus, superior temporal gyrus, and putamen and decreased activation in the hypothalamus and amygdala. Sex differences in the effect of chocolate satiation were found in the hypothalamus, ventral striatum, and medial PFC. A recent study showed that voluntary inhibition of hunger during food stimulation (smelling, viewing and tasting food) in men decreased activation in the amygdala, hippocampus, insula, OFC, and striatum, but not in women, using PET and ^{18}FDG (Wang et al., 2009).

Several fMRI studies have also found that women are more likely to be emotional eaters and have greater activation in brain reward centres such as the amygdala, OFC,

insula, ACC and hippocampus (Born et al. 2010, Koch et al., 2007, Bohon et al., 2009, Schienle et al., 2009). Interestingly, food cravings appear to be a rewarding experience in response to negative feelings in women, and men's food craving appears to be more related to a positive mood (Lafay et al., 2001). Nevertheless, women are more interested in health and taste aspects of foods than are men (Roininen et al., 1999).

1.5.5. Obesity (Table 1.6., 1.7.)

In obese individuals, stimuli associated with high-incentive foods, such as those high in fat and energy density, may possess greater than normal potency for activating reward systems and, as a result, trigger excessive motivation for non-homeostatic eating (Berthoud, 2004). In obese subjects, but not in control subjects, dopamine D2 receptors abundance, is inversely correlated to BMI, suggesting the dopamine system is involved in compulsive food intake (Volkow and Wise, 2005). In humans, imaging studies show reduction in dopamine D2 receptors in the striatum of obese vs. non-obese individuals (Wang et al., 2001). When tasting or viewing food stimuli (food vs. non-food or high-calorie vs. low-calorie food), activation of brain reward centres (DLPFC, medial and ventral PFC, hippocampus, insula, amygdala, medial and lateral OFC, NAc, dorsal striatum) is *increased* in obese subjects compared to lean subjects (Gautier et al., 2000; Gautier et al., 2001; DelParigi et al., 2004; DelParigi et al., 2005; Holsen et al., 2005; Stoeckel et al., 2008; Cornier, 2009; Martin et al., 2009; Stoeckel et al., 2009; Davids et al., 2010). BMI predicts activation in the OFC and insula when viewing high-calorie vs. neutral stimuli in obese compared to control individuals (Killgore and Yurgelun-Todd, 2005, Rothemund et al., 2007).

Meanwhile other studies have shown *decreased* activation in the DLPFC in obese compared to lean men (Le et al., 2006, Le et al., 2007). Lean subjects also show greater activation in the hippocampus, ACC and insula compared to obese subjects (Cornier et al., 2009; Martin et al., 2009; Davids et al., 2010). DLPFC, hippocampus and insula were found to be activated in both lean and obese subjects, and the ACC was only activated in

lean individuals which might be explained by the executive function of the ACC. The discrepancy of these results could arise from using different methods (i.e. PET or fMRI) in each study, but also from different protocols and stimuli paradigms.

Previous fMRI research also revealed increased activation in the premotor area (involved in planning and motor behaviour) on visual and auditory stimuli of binge type foods in obese binge eaters (Geliebter et al., 2006). Another study showed that obese binge eaters have stronger medial OFC activation on viewing food vs. neutral pictures compared to normal weight and overweight controls, and normal weight patients with bulimia nervosa (Schienle et al., 2009).

1.5.6. Psychological cues (Table 1.8.)

Eating behaviour can be triggered by internal signals of energy homeostatis such as hunger and satiety. However, individual differences in reward sensitivity, dietary restraint, emotional and external eating sensitivity, impulsivity and mood can override internal signals of positive or negative energy balance.

1.5.6.1. Reward sensitivity

Obesity, food craving, overeating, binge eating and drug abuse are associated with increased levels of reward drive and sensitivity (Davis et al., 2004, Dawe and Loxton, 2004, Schienle et al., 2009). Interestingly, individual differences in trait reward sensitivity measures have been linked to alcohol and drug use in healthy and clinical populations (Dawe and Loxton, 2004, Franken et al., 2006). The first connection between reward sensitivity and overeating was supplied by questionnaire studies (Franken and Muris, 2005, Davis et al., 2007).

Reward sensitivity can be measured by using different psychological questionnaires, such as the Behavioural Activation Scale (BAS) (Carver and White, 1994; Beaver et al., 2006) (Appendix 4). The BAS scale measures individual's tendency to actively pursue reward. The BAS consists of three BAS questionnaires that measure

personality traits related to reward sensitivity. First, goal drive describes persistence in obtaining desired goals (e.g. “I go out of my way to get things I want”). Secondly, fun seeking describes willingness to seek out and spontaneously approach potentially rewarding experiences (e.g. “I'm always willing to try something new if I think it will be fun”). Thirdly, reward responsiveness measures anticipation and positive response towards reward (e.g. “When I get something I want, I feel excited and energized”). A number of functional imaging studies have analysed the correlation between individual differences in reward drive and neural responses to images of food. Healthy volunteers and binge eating disorder patients with hightened reward sensitivity and reward responsiveness showed increased activation in the ventral striatum, VTA, OFC, and ACC when viewing appetizing vs. bland food or food vs. non-food stimuli (Beaver et al., 2006; Geliebter et al., 2006; Schienle et al., 2009).

1.5.6.2. Eating behaviour

Identifying the neural pathways that regulate cognitive, emotional and appetitive responses to different foods is particularly relevant to understand individual differences in eating behaviour. Adult eating behaviour is strongly correlated with weight gain and BMI (Hays et al., 2002, Drapeau et al., 2003). Three recognized eating behaviour constructs are “dietary restraint”, “disinhibition or emotional eating”, and “external food sensitivity” which are commonly assessed by using psychometric questionnaires such as the Dutch Eating Behaviour Questionnaire (DEBQ) (Appendix 5) or the Three-Factor-Eating Questionnaire (TEFQ) (Stunkard and Messick, 1985, van Strien, 1986).

Dietary restraint is a psychological trait that assesses cognitive self-control of food intake and conscious avoidance of consumption of fattening foods and weight gain (DEBQ restraint: e.g. “I deliberately eat foods that are slimming”, “I take into account my weight with what I eat”) (Wing and Hill, 2001). Dietary restraint is associated with healthier eating habits and long-term weight loss (Wing and Hill, 2001, Moreira et al., 2005, Savage et al., 2009). Psychological programs that emphasize dietary restraint appear to be more

effective interventions for preventing weight gain (Shaw et al., 2005). Altered neural activity in executive control and/or affective reward systems (dorsal PFC and OFC) to food stimuli has been reported in previously obese subjects who are successful dieters, and individuals concerned about the healthiness of food and exerting self-control (DelParigi et al., 2007; Hare et al., 2009; McCaffery et al., 2009). Activation in executive systems (ACC) has also been reported in patients recovered from compared to ongoing anorexia nervosa, where conflict between increased desire to eat and fear of weight gain would be hypothesized to be greater (Uher et al., 2003).

Disinhibition or emotional eating is the tendency to overeat in response to different stimuli, and can occur in a variety of circumstances such as when an individual is presented with an array of palatable foods or is under emotional distress (DEBQ-emotional: e.g. “Do you have a desire to eat when you are depressed or discouraged?”). Disinhibition or emotional eating has been associated with BMI or weight change (Hill et al., 1991, Lindroos et al., 1997, Hays et al., 2002, Drapeau et al., 2003, Provencher et al., 2003). Recent fMRI studies showed that self-reported measures of disinhibition (as measured by the TFEQ) negatively correlated with executive self-control sytem activation (ACC) on viewing food vs. non-food pictures in obese, compared to healthy weight individuals (Martin et al., 2009). Bohon and colleagues demonstrated that female emotional eaters (as measured by the DEBQ) who underwent fMRI showed greater activation in the parahippocampal gyrus and ACC in response to anticipated receipt of milkshake compared to non-emotional eaters (Bohon et al., 2009). Thus, emotional eaters appear to have increased neural response to anticipatory and consumatory food reward, but this effect was only found in the negative mood state.

External cues such as the sight of appetizing foods can also evoke a desire to eat, even in the absence of hunger. This **external food sensitivity** (as measured by the DEBQ-external: e.g. “If food tastes good, I eat more than usual” or “If you walk past the baker I have the desire to buy something delicious”) varies across individuals, and high sensitivity

to external eating has been associated with increased risk of overeating, food craving and obesity when exposed to a food-rich environment (Braet and Van Strien, 1997, Horchner et al., 2002, Burton et al., 2007, Elfhag et al., 2007). An fMRI study by Passamonti and colleagues revealed that individuals with high external food sensitivity show altered brain connectivity to the sight of appetizing food (Passamonti et al., 2009). External food sensitivity (as measured by the DEBQ-external) correlated with changes in connectivity between brain regions implicated in motivational aspects of food processing and in emotional regulation (ventral striatum, amygdala, ACC) on viewing appetizing compared with bland foods. These results are particularly pertinent to recent concerns regarding the power of food advertisements to promote food intake and prefernces.

1.5.6.2. Mood

Fluctuations in emotions or mood can influence the human appetite (Macht, 1999) and food cravings correlate with emotional-related eating (Hill et al., 1991). Substantial individual differences exist in the level of activation of the affective valence system, which are related to an individual's propensity to experience either positive or negative affect in response to emotionally relevant stimuli (Tomarken et al., 1992, Davidson, 2003). For example, since food and drugs can distract from emotional distress, negative emotions are major triggers for both eating and substance use (Macht, 1999). For example, craving intensity is positively correlated with a variety of negative mood states in self-described carbohydrate cravers (Christensen and Pettijohn, 2001). The Positive and Negative Affect Schedule (PANAS) measures the tendency to experience positive and negative affect (Watson et al., 1988, Killgore and Yurgelun-Todd, 2006) (Appendix 6). Research proposes that affect in the human brain is primarily controlled by the PFC (Davidson and Irwin, 1999, Tataranni et al., 1999). Recent evidence suggests that these systems within the PFC may regulate both excitatory and inhibitory reactions to appetitive stimuli (Berthoud and Morrison, 2008). Using fMRI, one study demonstrated that when viewing high-calorie foods, positive affect (as measured by the PANAS) was associated with increased activity in the lateral OFC (Killgore and Yurgelun-Todd, 2006). However, when viewing low-calorie foods, positive affect was associated with increased activity in the medial OFC and the

insula. The opposite pattern was observed for negative affect. This suggests that mood-dependent changes in appetite are directly associated with changes in brain reward activation.

1.5.6.4. Impulsivity

Impulsivity typically refers to behvaiour that incoperates a component of rashness, lack of foresight or planning or as a behaviour that occurs without reflection or carful deliberation. Impulsivity can be measured by using psychological questionnaires like the Barret Impulsivness Scale which describes a tendency to act rashly and without consideration of consequences and assesses frequency of impulsive-related behaviour or cognitions (Patton et al., 1995). The trait of impulsiveness may be another important individual difference variable in identifying those most vulnerable to overating and weight gain (Guerrieri et al., 2007, Lowe et al., 2009). A relationship between obesity and impulsivity has also been frequently described (Nederkoorn et al., 2006b, Lowe et al., 2009). In a study by of obese children with and without binge eating, it was confirmed that the binge eaters were more impulsive on a reward based measure of this personality trait (Nederkoorn et al., 2006b). Several brain areas (ACC, PFC, OFC) have been described as associated with different types of impulsivity (Winstanley et al., 2004, Torregrossa et al., 2008). There is growthing evidence that different measures of impulsivity correlate with food intake and weight gain (Guerrieri et al., 2009, Nederkoorn et al., 2009).

Table 1.2. Human neuroimaging studies in *non-obese* FASTED POPULATIONS (classified by food stimulus).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity (increased if not other indicated)
Fuhrer et al. 2008	fMRI	12; male	Non-obese	Viewing food vs. non-food	Fasted (14h)	Left and right anterior OFC
Holsen et al. 2005	fMRI	9; 4 male	non-obese, Children	Viewing food vs. non-food	Fasted (4h)	Medial and lateral OFC
La Bar et al. 2001	fMRI	17; 9 male	Non-obese, healthy	Viewing food vs. non-food	Fasted (8h)	Amygdala
Morris et al. 2001	$H_2{}^{15}O$ PET	10; 9 male	Non-obese	Viewing food vs. non-food	Fasted (16h)	OFC, amygdale, insula
Porubska et al. 2006	fMRI	12; 7 male	Non-obese	Viewing food vs. non-food	Fasted (5h)	Insula, OFC
St-Onge et al. 2005	fMRI	12; 6 male	Non-obese	Food vs. non-food	Fasted (12h)	Cingulate gyrus, superior temporal gyrus, hippocampus, parahippocampal gyrus, insula
Gordon et al. 2000	^{15}O-CO_2 PET	8; female	Non-obese, healthy	Viewing High-calorie food vs. non-food	Fasted overnight	Decreased insula activation
Killgore et al. 2003	fMRI	13, female	None-obese	Viwing high-and low-calorie and non-foods	Fasted for 1.6-6.8h)	High-calorie foods: Frontal, temporal and occipital gyrus, amygdala, parahippocampus, thalamus, vmPFC, DLPFC, hypothalamus Low-calorie foods: Temporal and lingual gyrus, parahipocampus, OFC, amygdala, vmPFC Non-foods: Frontal and occipital gyrus High > Low:Frontal, temporal and occipital gyrus, thalamus Low > high: temporal, lingual and frontal gyrus
Siep et al. 2009	fMRI	12; female	Non-obese	Viewing food (high-and low-calorie) vs. non-food (objects)	Fasted (18h)	H>O: PCC, lateral and medial OFC, insula, caudate putamen, fusiforum gyrus L>O: medial and lateral OFC H>L: medial OFC, fusiforum gyrus
Schur et al. 2009	fMRI	10; female	Non-obese	Viewing of food (fattening and non-fattening) vs. non-food	Fasted (2-4h)	Food > Objects: Hypothalamus, DLPFC, insula, putamen, ventral striatum, thalamus, occipital lobe Fatteneing > Objects: Hypothalamus, ventral striatum, thalamus, occipital lobe Objects > Fattening: Occipital lobe
Frank et al. 2010	fMRI	12; 6 male	Non-obese, men vs. women	Viewing high- vs low-calorie foods	Fasted (3h)	High-calorie > Low-calorie: OFC, insula Women > men: fusiforum gyrus when hungry
Goldstone et al. 2009	fMRI	20; male	Non-obese	Viewing high-calorie vs. low-calorie food	Overnight fast (1.6h)	Ventral striatum, amygdala, anterior insula, medial and lateral OFC
Simmons et al. 2005	fMRI	9; 3 male	Non-obese , healthy	Viewing of appetizing food vs. non-food	Fasted ?	Appetizing > Objects: Insula, OFC, ACC, inferior temporal, fusiforum gyrus Objects > Appetizing: Fusiforum
Passamonti et al. 2009	fMRI	21; 11 male	Non-obese	Viewing of appetizing or bland food	Fasted (2h)	Viewing appetizing > bland foods: vACC, DLPFC, frontal and temporal gyrus, pCC, ventral striatum, amygdala
Beaver et al. 2006	fMRI	12; 5 males	Non-obese	Viewing appetizing, disgusting, bland foods and objects	Fasted (2h)	Appetizing > Objects: insula, DLPFC, OFC, Parahippocampus Appetizing > Disgusting: OFC, Parahippocampus, DLPFC Appetizing > Bland: ventral striatum, insula, parahippocampus, DLPFC Disgusting > Bland: OFC, ventral striatum, insula Disgusting > Appetizing: insula, OFC Disgusting > Objects: insula, OFC

[a] Adult unless other indicated

Table 1.2. (*CONTINUED*). Human neuroimaging studies in *non-obese* FASTED POPULATIONS (classified by food stimulus).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity (increased if not other indicated)
Uher et al. 2006	fMRI	18; 8 male	Non-obese men and women	Viewing food vs. non-food, and tasting chocolate and chicken	Overnight fast (23h)	Food > Non food: Fusiforum gyrus, insula Tasting chocolate: Hippocampus, precentral sulcus and caudate Taste chicken: Hypothalamus, thalamus, insula, frontal gyrus, precentral sulcus Fasting > Fed: Visula foods: None; taste chocolate: DLPFC; taste chicken: mPFC Gender: Insula (stronger in women) for visual food stimul
Hinton et a. 2004	$H_2{}^{15}O$ PET	12; male	Non-obese	Imagining of restaurant menu (high and low-incentive foods)	Fasted (18h) and Fed comined	All food categories combined: Amygdala and OFC
Piech et al. 2009	fMRI	8; 5 male	Non-obese	Imagining + choosing written food items from restaurant menu (high and low attractive)	Fasted (6h)	Fasted (Food > Non-food): Thalamus, Insula, lateral PFC, Fasted and Fed combined H>L: Amygdala
O'Doherty et al. 2000	fMRI	No info given	Non-obese	Food-related odours (banana)	Fasted (?)	OFC (Olfactory sensory-specific satiety)
Gottfried et al. 2003	fMRI	14; gender?	Non-obese	Visual stimuli before + after odor stimui (Vanilla)	Fasted (6h)	After odor: amygdala, OFC
Wang et al. 2004	PET+ ^{18}FDG	12; 5 male	Non-obese	Viewing, smelling and tasting food vs. non-food	Fasted (17-19h)	Food > Non-food: Postcentral gyrus, superior temporal cortex, insula, OFC
Koch et al 2007	fMRI	40; 21 male	Non-obese, women vs. men	Positive or negative olfactory stimuli	?	Negative olfactory stimuli in women > men: Insula, frontal and temporal gyrus.
Wang et al. 2009	PET+ ^{18}FDG	23; 10 men	Non-obese , men vs. women	Viewing, smelling and tasting food with cognitive inhibition task	Overnight fast (17-19h)	When cognitive inhibition in men (not women): decreased amygdala, hippocampus, insula, OFC, striatum → inhibition correlated with decreased self-reports of hunger
Grabenhorst et al. 2010	fMRI	12; 9 male	Non-obese	Tasting pleasant vanilla drink + unpleasant strawberry drink	Fasted (3h)	Ventral PFC correlates with subjective pleasantness ratings
Haase et al. 2009		18; 9 male	Non-obese	Tasting caffeine, citric acid, GMP, saccharin, sucrose, NaCl	Fasted (12h)	For all tastes: Hunger: increased insula, thalamus, and substantia nigra Satiety: decreased parahippocampus, hippocampus, amygdala, ACC, Activation varies when tasting sucrose only
Rolls et al. 2007	fMRI	16; female	Non-obese cravers vs. non cravers	Taste and sight of chocolate	Fasted	Sight chocolate in cravers > non-cravers: medial OFC, ventral striatum; sight+taste in cravers > non-cravers: medial OFC, pregenual cingulated cortex Cravers > non-cravers: Positive correlation of pleasantness ratings of chocolate with activation in the pregenual cingulated cortex and medial orbitofrontal cortex
Small et al. 2001	$H_2{}^{15}O$ PET	9; 4 male	Non-obese, chocolate lovers vs. non-lovers	Tasting chocolate	Fasted (3-4h)	Insula, medial OFC, striatum
Bender et al. 2009	fMRI	15; 6 male	Non-obese	Tasting sweet, sour, salty or tasteless solution	Fasted ?	Taste vs tasteless: Insula and operculum
Del Parigi et al. 2002	$H_2{}^{15}O$ PET		Non-obese, men vs women	Tasting liquid meal	Fasted (36h) before tasting	Hunger: men > women: DLPFC, middle tempiral gyrus, posterior cingulated and parahippocampal gryus

[a] Adult unless other indicated

Table 1.3. Human neuroimaging studies in *non-obese* FED POPULATIONS (classified by food stimulus).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity
Fuhrer et al. 2008	fMRI	12; male	Non-obese	Viewing food vs. non-food	Fed (1h)	Parietal lobe, posterior middle temporal gyrus
Holsen et al. 2005	fMRI	9; 4 male	non-obese, Children	Viewing food vs. non-food	Fed	None
La Bar et al. 2001	fMRI	17; 9 male	Non-obese, healthy	Viewing food vs. non-food	Fed	None
Morris et al. 2001	$H_2{}^{15}O$ PET	10; 9 male	Non-obese	Viewing food and non-food	Fed during scanning	Reduced insula activation
Siep et al. 2009	fMRI	12; female	Non-obese	Viewing food (high-and low-calorie) vs. non-food (objects)	Fed (lunch)	L>O: PCC, lateral and medial OFC, insula, caudate putamen, fusiforum gyrus L>H: PCC, medial and lateral OFC
Frank et al. 2010	fMRI	12; 6 male	Non-obese, men vs. women	Viewing high- vs low-calorie foods	Fed	None
Goldstone et al. 2009	fMRI	20; male	Non-obese	Viewing high-calorie vs. low-calorie food	Fed	None
Piech et al. 2009	fMRI	8; 5 male	Non-obese	Imagining + choosing written food items from restaurant menu (high and low attractive)	Fed	Food > Non-food: Caudate, lateral PFC
Kringelbach et al. 2003	fMRI	10; male	Non-obese	Tasting food vs neutral solution	Fed with solution	Pleasantness ratings correlated negatively with activation in the OFC (sensory specific satiety)
Haase et al. 2009		18; 9 male	Non-obese	Tasting caffeine, citric acid, GMP, saccharin, sucrose, NaCl	Fed	For all tastes: (Satiety) decreased parahippocampus, hippocampus, amygdala, ACC, Activation varies when tasting sucrose only
Small et al. 2001	$H_2{}^{15}O$ PET	9; 4 male	Non-obese, chocolate lovers vs. non-lovers	Tasting chocolate	Fed (3-4h)	Parahippocampus gyrus, lateral OFC, PFC (sensory-specific satiety)
Smeets et al. 2006	fMRI	24; 12 male	None-obese men vs. women	Tasting chocolate milk	Fed (Senosry-specific satiety)	Men: increased in ventral striatum, insula, and medial OFC and decreased somatosensory areas Women: increased in precentral gyrus, superior temporal gyrus, and putamen and decreased in hypothalamus and amygdala Men vs women: hypothalamus, ventral striatum, and medial PFC
Smeets et al. 2010	fMRI	10; male	None-obese, healthy	Tasting of orangeade	Fed (caloric or non-caloric orangeade)	Amygdala more activated by non-caloric vs. caloric orangeade; Striatum more activated by caloric vs. non-caloric orangeade
Del Parigi et al. 2002	$H_2{}^{15}O$ PET		Non-obese, men vs women	Tasting liquid meal	Fed after liquid meal	Satiety women > men: DLPFC, precuneus Satiety men > women: ventromedial PFC

[a] Adult unless other indicated

Table 1.4. Human neuroimaging studies in *non-obese* FASTED > FED POPULATIONS (classified by food stimulus).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity
Fuhrer et al. 2008	fMRI	12; male	Non-obese	Viewing food vs. non-food	Fasted > Fed	Amygdala, ACC
Holsen et al. 2005	fMRI	9; 4 male	non-obese, Children	Viewing food vs. non-food	Fasted > Fed	Amygdala, medial and lateral OFC, medial frontal cortex, basal operculum/insula, parahippocampal gyrus, cingulate and fusiform gyrus
La Bar et al. 2001	fMRI	17; 9 male	Non-obese, healthy	Viewing food vs. non-food	Fasted > Fed	Parahippocampal gyrus, anterior fusiform gyrus
Hinton et a. 2004	$H_2{}^{15}O$ PET	12; male	Non-obese	Imagining of restaurant menu (high and low-incentive foods)	Fasted (18h) and Fed	All fod categories combined: hypothalamus, amygdala, insula High > Low incentive: OFC

[a] Adult unless other indicated

Table 1.5. Human neuroimaging studies in *non-obese* FED > FASTED POPULATIONS (classified by food stimulus).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity (increased if not other indicated)
La Bar et al. 2001	fMRI	17; 9 male	Non-obese, healthy	Viewing food vs. non-food	Fed v> Fasted	None
Hinton et a. 2004	$H_2{}^{15}O$ PET	12; male	Non-obese	Imagining of restaurant menu (high and low-incentive foods)	Fasted (18h) and Fed	All fod categories combined: OFC

[a] Adult unless other indicated

Table 1.6. Human functional neuroimaging studies in *non-obese vs. obese* (FASTED AND FED) populations (classified by food stimulus).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity (increased if not other indicated)
Davids et al. 2009	fMRI	22 obese; 7 male 22 NW*; 10 male	Obese vs. non-obese children	Viewing food pictures	Fasted (2h)	Obese > NW: DLPFC Nw > Obese: Fusiforum gyrus, ACC, caudate, hippocampus, parahippocampus gyrus, thalamus
Martin et al. 2009	fMRI	10 in each group, 5 male in each group	Obese vs. lean	Viewing of food vs. non-food	Fasted (4h) Fed (500kcal)	Fasted: Obese > lean: mPFC, frontal gryus, ACC, fusiforum gyrus, Lean > Obese: Temporal gryus Fed: Obese > Lean: mPFC, frontal + temporal gyrus, hippocampus, precuneus Lean > Obese: None
Geliebter et al. 2006	fMRI	10 in each group; female	Obese and lean, binge eaters vs. non-binge reaters	Viewing binge eating and none binge eating foods and non-foods	Fasted (3h)	**Viewing binge eating foods:** Obese binge eaters: ventral premotor cortex, precentral gyrus, frontal and fusiforum gyrus, lingual gyrus Obese non-bingers: None Lean bingers: None Lean non-bingers: occipital gyrus and lingual gyrus **Viewing none-binge eating foods:** Obese binge eaters: fusiforum and frontal gyrus Obese non-bingers: None Lean bingers: None Lean non-bingers: occipital, lingual and temporal gyrus **Non-food stimuli:** None
Rothemund et al. 2007	fMRI	13 obese, 13 lean; female	Obese vs. Non-obese	Viewing food pictures (high-calorie and low-calorie)	Fasted (3-4h)	BMI correlated with activation in putamen, caudate, insula, cingulate cortex, pallidum, lateral OFC Obese > control HC: putamen, caudate, insula, hippocampus LC: Frontal and temporal gyrus Objects: Frontal gyrus and parietal lobe H>L: Putamen Control > Obese: HC and LC: None Objects: Caudate
Stice et al. 2009	fMRI	33; female*	Obese vs. Non-obese adolescents	Anticipation and tasting of milkshake and tastless solution	Fasted (4-6h)	Obese > lean: Anticipation and tasting of milkshake > tasteless: insula increased, caudate decreased
Stoeckel et al. 2008	fMRI	12 obese, 12 lean; female	Obese vs. lean	Viewing high-calorie +low-calorie food and objects	Fasted (8-9h)	Obese > controls: H>O: Medial/ lateral OFC, amygdala, ventral striatum, mPFC insula, ACC, hippocampus L>O: Putamen, caudate Controls > obese: H>O: mPFC L>O: lateral OFC, mPFC, ACC
Stoeckel et al. 2009	fMRI	12 obese + 12 NW*; female	Obese vs. lean	Viewing of food (high-vs low-calorie) vs. non-food	Fasted (8-9h)	Obese > non-obese for all food categories: Reduced connectivity from amygdala to OFC and NAc; Increased connectivity from OFC to NAc Controls but not obese: Greater connectivity between amygdala and OFC when viewing high-calorie foods

[a] Adult unless other indicated, *normal weight

Table 1.6. (*CONTINUED*). Human functional neuroimaging studies in *non-obese vs. obese* (FASTED AND FED) populations (classified by food stimulus).

Author/ year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Brain reward activity (increased if not other indicated)
Schienle et al. 2008	fMRI	67; female	Overweight bingers (BED), bulemics and overweight and lean controls	Viewing high-caloric, distgust-inducing and non-foods	?	**Food > Non-food** All groups: Occipital, ACC, OFC, insula BED > Bulemics: lateral and medial OFC BED > overweight/ lean: medial OFC Bulemics > BED/ overweight/lean: insula, ACC **Disgusting > Non-food:** All groups: amygdala, insula, lateral OFC Bulemics > overweight controls: insula Lean > BED: insula and lateral OFC
Killgore et al. 2005	fMRI	13; female	Non-obese	Viewing low-calorie, high-calorie and non-food	?	High-calorie: BMI negatively related with ACC, OFC; Low-calorie: BMI negatively related with OFC; Non-food: BMI positively related with OFC
McCaffery et al. 2009	fMRI	~17 in each group, ~ 2 male in each group	Obese, weightloss maintainers (SWLs) vs. normal weight	Viewing food pictures (high and low-calorie) and non-foods	Fasted for 4h	**High-and low calorie foods > non-foods:** SWLs > NW/ obese: frontal and temporal regions Obese > SWLs, NW: precentral gyrus **Viewing high-calorie foods only:** NW > Obese: Frontal gyrus, precuneus NW > SWLs: cingulate gyrus SWLs > Obese: occipital, lingual, frontal gyrus, pre- and postcentral, insula, cuneus Obese > SWLs: ACC
Del Parigi et al. 2004	$H_2{}^{15}O$ PET	23 obese; 11 male 11 postobese; 3 male; 21NW*; 11 male	Post-obese, obese, lean	Tasting liquid meal after 36h fast	Fed	Obese and postobese: greater insula + lower hippocampus; Obese > lean: insula, amygdala, posterior CC, hippocampus
Del Parigi et al. 2005	$H_2{}^{15}O$ PET	21 obese; 10 male 20 lean; 10 male	Obese vs. lean	Tasting liquid meal	Fasted (36h)	Obese > Lean: Increase: insula, midbrain Decrease: OFC, posterior cingulate gyrus and temporal gyrus Lean > Obese: None
Gautier et al. 2000	$H_2{}^{15}O$ PET	11 obese, 11lean; male	Obese vs lean, men	Taste of liquid after fasting	Fed	Satiation in both obese and lean: Increase in vmPFC and DLPFC and decrease in striatum, hippocamous, temporal lobe, precuneus; Satiation in obese > lean: In crease in PFC, hypothamaus and thalamus
Gautier et al. 2001	$H_2{}^{15}O$ PET	12 obese, 10 lean; female	Obese vs lean, women	Taste of liquid after fasting	Fed	Lean and obese: Increase PFC and decreased thalamus, insula, parahippocampus and temporal cortex Obese only: Decreased hypopthalamus, cingulate, ventral striatum and amygdala Obese > lean: Increased ventral PFC and decreased frontal and temporal cortex
Le et al. 2006	PET	9 obese, 9 lean; male	Obese vs. Non-obese	Tasting liquid meal after fasting	Fed	Obese > Lean: Less DLPFC activation
Le et al. 2007	PET	9 obese, 10 lean, 8 postobese; female	Lean, obese and postobese	Tasting liquid meal after fasting	Fed	Obese > lean and post-obese: less DLPFC

[a] Adult unless other indicated, *normal weight

Table 1.7. Human functional neuroimaging studies with experimental hormonal or dietary manipulation (classified by hormone).

Author / year	Type	Subjects[a] (n, sex)	Manipulation and BMI	Food stimulus	Nutritional state	Brain reward activity (increased if not other indicated)
Baicy et al. 2007	fMRI	3 ; gender ?	Leptin-deficient subjects; Leptin supplementation	Viewing of food (high and low-calorie) vs. non-food	Fed (3h)	Leptin supplementation: Decreased: insula, parietal and temporal cortex Increased: PFC
Farroqui et al. 2007	fMRI	1 male, 1 female	Leptin-deficient subjects, before + 7 days after leptin treatment	Viewing food vs. non-food	Fasted Fed	Leptin-deficient state > leptin-repleaced state: ventral striatum Leptin-deficient state: Positive correalation of Food Liking Ratings and activation in the ventral striatum *when fasted and fed* Leptin-replaced state: Positive correalation of Food Liking Ratings and activation in the ventral striatum *only when fasted*
Rosenbaum et al. 2008	fMRI	6; 2 male	Obese > 10% weight loss; Leptin > saline injection after weight loss	Viewing of food vs. non-food	?	Weight loss > Weight Baseline: Frontal and temporal gryus, parahippocampus gyrus Weight Baseline > Weight loss: Amygdala, ACC, CC, fusiforum gyrus, hypothalamus, frontal grysu, parahippocampal gryus Weight loss leptin > Weight Baseline placebo: CC, hypothalamus, frontal gyrus, temporal gyrus, putamen, precuneus, thalamus Weight Baseline placebo >Weight loss leptin: Frontal gyrus, insula, parahippocampal gyrus, precuneus
Batterham et al. 2007	fMRI	8; male	Non-obese, PYY and Saline infusion	Viewing of food vs. non-food	Overnight fast	PYY infusion: increased activation in VTA , insula, ACC and OFC Correlation analysis: After PYY infusion OFC predicts food intake at lunch; After Saline infusion hypothalamus predicts food intake at lunch
Malik et al. 2008	fMRI	20 ; male	Non-obese, Ghrelin iv. infusion	Viewing food vs. scenery	Fasted (3h)	Amygdala, OFC, anterior insula Effect of ghrelin on the amygdala and OFC response correlated with self-rated hunger ratings
Cornier et al. 2007	fMRI	25; 1 men	Eucaloric diet (2 days) and 30% overfeeding (2 days)	Viewing foods (high (H) and neutral (N) hedonic value) and non-foods (NF)	Fasted overnight	Eucaloric diet: N>NF: Insula and DLPFC H>N: Hypothalamus, hippocampus and parietal lobe Overfeeding: H>N: Decreased hypothalamus Eucaloric diet > Overfeeding: H>N: Hypothalamus; N>NF: None
Cornier et al. 2009	fMRI	22 thin; 12 male 19 reduced weight; 9 male	Thin vs reduced obese, eucaloric or 30% overfeeding diet	Viewing high-calorie food, neutral food and non-food	Overnight fast	**Lean:** Eucaloric: HC > non-food → insula, OFC, ventral striatum, hippocampus Eucaloric >Overfeeding: HC >non-food →decrease insula and hypothalamus Overfeeding > Eucaloric: None **Reduced Obese:** Eucaloric >Overfeeding: high-calorie > non-food: None **Reduced Obese > Thin:** Eucaloric: High-calorie > non-food: Only visual cortex **Thin > Reduced Obese:** Eucaloric: High-calorie > non-food: None Eucaloric >Overfeeding: High-calorie food >non-food →decrease in visual cortex, insula and hypothalamus

[a] Adult unless other indicated

Table 1.8. Human functional neuroimaging studies with individual differences in psychological traits (classified by co-variable).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Co-variable	Brain reward activity (increased if not other indicated)
Born et al. 2009	fMRI	9; females	Non-obese,	Visual food stimuli	Fasted and Fed	Stressed vs. rest	When Fasted and stressed: Redcued amygdala, hippocamous and ACC activation When Fed and stressed: Reduced Putamen activation
Calder et al. 2007	fMRI	12; 5 male	Non-obese	Viewing disgusting, appetizing + bland foods	Fasted ?	Disgust sensitivity questionnaire	Correlation with disgust sensitivity questionnaire: Disgusting > Non-food: insula, ventral pallidum Disgusting > Bland: ventral pallidum Disgusting > Appetizing: insula, pre-and postcentral gyrus
Martin et al. 2009	fMRI	10 in each group, 5 male in each group	Obese vs. lean	Viewing of food vs. non-food	Fasted (4h) Fed (500kcal)	TEFQ - disinhibition	Fasted: Obese > lean: mPFC, frontal gryus, ACC, fusiforum gyrus, Lean > Obese: Temporal gryus Fed: Obese > Lean: mPFC, frontal + temporal gyrus, hippocampus, precuneus Lean > Obese: None Correlations with questionnaire scoers: disinhibition correlated negatively with ACC
Del Parigi et al. 2005	$H_2{}^{15}O$ PET	21 obese; 10 male 20 lean; 10 male	Obese vs. lean	Tasting liquid meal	Fasted (36h)	TEFQ - disinhibition	Obese > Lean: Increase: insula, midbrain Decrease: OFC, posterior cingulate gyrus and temporal gyrus Correlations: TEFQ correlates with insula Lean > Obese: None
Del Parigi et al. 2007	$H_2{}^{15}O$ PET	9 dieters, 20 non-dieters; female	Sucessful dieters (non-obese) vs. non-dieters (obese)	Resting scan or after tasting meal or solution	Overnight fast, Fed	TEFQ - restraint	Solution > Fasted: Dieters > non-dieters → none Non-dieters > Dieters → Hippocampus Meal > Fasted: Dieters > non-dieters → DLPC, dorsal striatum, Non dieters > Dieters →OFC Correlations: Restraint positively coorelated with DPFC and negatively correlated with OFC
Bohon et al. 2009	fMRI	21; female	Non-obese, emotional vs. non-emotional eaters	taste of chocolate milk vs. tasteless solution, and anticipated receipt of milkshake	Fasted 4-6h	DEBQ – emotional, PANAS:	**Negative > neutral mood:** Anticipated receipt of milkshake: Emotional eaters → Parahippocampal gyrus, ACC Non-emotional eaters → all ROIs decreased Receipt of milkshake: Emotional eaters → ACC, pallidum, thalamus
Passamonti et al. 2009	fMRI	21; 11 male	Non-obese	Viewing of appetizing or bland food	Fasted (2h)	DEBQ - external	Viewing appetizing > bland foods: vACC, DLPFC, frontal pole, temporal gyrus, pCC, ventral striatum, amygdala Correleations when vieing appetizing > bland foods: External food sensitivity correlates with ventral striatum, amygdala, and ACC

[a] Adult unless other indicated

Table 1.8. (*CONTINUED*). Human functional neuroimaging studies with individual differences in psychological traits (classified by co-variable).

Author / year	Type	Subjects[a] (n, sex)	BMI	Food stimulus	Nutritional state	Co-variable	Brain reward activity (Increased if not other indicated)
Beaver et al. 2006	fMRI	12; 5 males	Non-obese	Viewing appetizing, disgusting + bland food	Fasted (2h)	BAS – reward sensitivity	Appetizing > Objects: insula, DLPFC, OFC, Parahippocampus Appetizing > Disgusting: OFC, Parahippocampus, DLPFC Appetizing > Bland: ventral striatum, insula, parahippocampus, DLPFC Disgusting > Bland: OFC, ventral striatum, insula Disgusting > Appetizing: insula, OFC Disgusting > Objects: insula, OFC **Correleations:** Correlates for appetizing food > bland foods/ non-foods s in ventral striatum, OFC, amygdala + midbrain (VTA), ventral pallidum
Schienle et al. 2008	fMRI	67; female	Overweight bingers (BED), bulemics and overweight and lean controls	Viewing high-caloric, distgust-inducing and non-foods	?	BAS/ BIS without subscales, BED scales	**Food > Non-food** All groups: Occipital, ACC, OFC, insula BED > Bulemics: lateral and medial OFC BED > overweight/ lean: medial OFC Bulemics > BED/ overweight/lean: insula, ACC Correleations with reward sensitivity: BED: ACC and medial OFC Bulemics: ACC, medial OFC, insula Correlations with BED: Insula **Disgusting > Non-food:** All groups: amygdala, insula, lateral OFC Bulemics > overweight controls: insula Lean > BED: insula and lateral OFC
Killgore et.al. 2006	fMRI	13; female	Non-obese	Viewing of high-calorie and low-calorie	?	PANAS - mood	**Viewing high-calorie foods:** Positive affect: lateral OFC Negative affect: medial OFC, ACC and insula **Viewing low-calorie foods:** Positive affect: medial OFC, insula Negative affect: lateral and medial OFC, and insula
Uher et al. 2004	fMRI	19; female	Healthy, anorexic and bulimic pateints (ED)	Viewing food and non-food	Fasted min. 3h	?	**Food > Non-food:** ED > Healthy: vmPFC Healthy > ED:DLPFC Annorexia > Healthy: vmPFC Healthy > Annorexia: Parietal lobe Bulemic > Healthy: vmPFC Healthy > Bulemic: DLPFC Annorexia > Bulemic: PFC Bulemic > Annorexia: None
Hare et al. 2009	fMRI	18 self-controllers, 19 non-self-controlers	Self-controllers vs. non self-controllers	Choosing tasty vs. healthy foods	?	?	Self-controllers when choosing tasty > healthy foods: vmPFC, DLPFC

[a] Adult unless other indicated

1.6. Summary introduction

Evidence is accumulating that brain reward systems can interact with peripheral and central appetite signals that encode energy balance. Functional neuroimaging studies have established that brain activity, as measured by BOLD fMRI or PET, is increased in reward systems in response to imagining, smelling, tasting and viewing food cues. Differences are seen under fed or fasted (nutritional state) conditions in normal weight and obese subjects that provide new insights into the control of ingestive behaviour. Altered activity in reward systems is also associated with gender and individual differences in eating behaviours. Primarily pre-clinical studies have demonstrated that circulating insulin, leptin and PYY are able to inhibit and ghrelin to stimulate dopamine signaling in reward systems and thus influence the subjective reward value of food and hence motivation to eat. However, it is still unclear how nutritional state, circulating hormones and different food stimuli interact in humans (Van Vugt, 2009).

1.7. Hypotheses of thesis

1.7.1. Fed-fasted fMRI study

- Fasting biases brain reward systems towards high-calorie foods in humans.
- Individual differences in eating behaviour and reward sensitivity alter the response of brain reward systems to high-calorie foods.

1.7.2. Ghrelin fMRI study

- The bias in brain reward systems to high-calorie foods when fasted is mediated by increases in circulating ghrelin.

1.8. Aims of thesis

1.8.1. Fed-fasted fMRI study

- To investigate the interaction between nutritional state (fasted or fed) and caloric value of food stimuli on the appeal of food and on activity within brain reward systems using fMRI in healthy non-obese adults.
- To investigate whether individual differences in activation in brain reward systems to different food stimuli when fasted are influenced by individual differences in eating behavior and reward sensitivity.

1.8.2. Ghrelin fMRI study

- To investigate whether ghrelin administration to fed healthy non-obese adults mimics the effect of fasting to bias brain reward system to high-calorie foods.

Chapter 2

MATERIALS AND METHODS

2.1. Fed – fasted fMRI study

2.1.1. Participants

Twenty-five subjects (mainly PhD student working in the department) were recruited by word-of mouth at the Robert Steiner MRI Unit, Hammersmith Hospital, London. To avoid confounding factors and contraindications among participants in their response to experimental stimuli, individuals were excluded based on the following criteria: a) a positive eating disorder history by SCOFF questionnaire (Appendix 3) (Morgan et al., 1999; Van Vugt, 2009; Born et al., 2010)), b) food preferences that were inconsistent with our food images e.g. vegetarian, vegan, gluten or lactose intolerant, d) recent change in weight (>5% change in the preceeding 3 months), e) smoking, f) left-handedness, g) claustrophobia, h) pregnancy or breastfeeding, i) neurological or psychiatric history, j) use of street drugs, k) any metallic objects in subject's body excluding MRI, e.g. pacemaker. Twenty healthy, non-obese volunteers with normal or corrected to normal vision completed the study (10 male, 10 female, mean ± SEM age 26 ± 1 years, range 19-35; body mass index (BMI) 22.1 ± 0.5 kg/m2, range 18.2-27.1, with only one subject having BMI>24.9).

The protocol was approved by the Local Research Ethics Committee (ethics number: 06/Q0406/18), and was performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

2.1.2. Protocol

Subjects were scanned after an overnight fast (15.9 ± 0.3h since supper) and avoided alcohol and vigorous exercise the day before and day of the study. They were told to eat their normal meals the day before the study but eat or drink nothing after supper other than water. On the day of the study, subjects were asked to continue fasting or eat a filling breakfast of their choice and keep a detailed food diary including saving of food packaging. Breakfast calorie content and macronutrient composition was determined using

standard dietary calculators in DietPlan6 (Forestfield Software Ltd, West Sussex, UK). Breakfast intake (mean ± SEM) was 724 ± 59 kCal (47 ± 4% of estimated resting energy expenditure using Schofield equation (Schofield, 1985) (age, weight-adjusted): RMR (kcal/24hrs) = 239 x ((0.062 x weight (kg) + 2.036), if age 18-29, RMR (kcal/24hrs) = 239 x ((0.034 x weight (kg) + 3.538), if age 30-59, with macronutrient content: 51 ± 4% carbohydrate, 33 ± 3% fat, 15 ± 1% protein.

Before and after scanning, volunteers completed 10cm visual analogue scales of appetite (VAS) containing the questions: “How hungry do you feel right now?” (hunger), “How full do you feel right now?” (full), “How pleasant would it be to eat right now?” (pleasantness), “How sick do you feel right now?” (nausea) and “How much do you think you could eat right now?” (volume able to eat) (Flint et al., 2000) (Appendix 10). VAS rating scores for before and after fMRI scanning were averaged for each subject to allow for changes in ratings over the period of the scanning session. Subject’s height and weight were also recorded.

2.1.2.1. Questionnaires

Subjects completed the Dutch Eating Behaviour Questionnaire (DEBQ) to measure eating restraint or extent of dieting regime (DEBQ-restraint; e.g. “I do deliberately eat less in order not to become heavier”), and external influences on eating behaviour (DEBQ-external; e.g. “If I walk pass the bakery I have the desire to buy something delicious”) (van Strien, 1986) (Appendix 5). In addition, participants completed the Behavioural Activation / Behavioural Inhibition Scales (BAS/BIS). This questionnaire consists of three BAS scales that measure personality traits related to rewards sensitivity (goal drive, fun seeking and reward responsiveness) and one BIS scale related to responsiveness to punishment cues (Carver and White, 1994) (Appendix 4).

2.1.3. MRI scanning protocol

In the MRI scanner subjects wore ear plugs and head phones to block of the hammering noise of the MRI scanner, had a keypad in right hand to rate fMRI pictures (see in detail below), and an alarm buzzer in left hand, while their head was padded with foam to avoid excessive head movement.

At the beginning of each scanning session, the patient tested that each button on the keypad worked and practiced rating the appeal of test animal and blurred pictures for about 2 minutes.

Following this, four types of colour photographs were presented in a block design: (1) 54 high-calorie foods (e.g. burgers, cakes and chocolate), (2) 54 low-calorie foods (e.g. salads, fruits and vegetables, fish), (3) 54 non-food related household objects (e.g. furniture, clothing, electrical equipment) and (4) 192 Gaussian blurred images of the high-calorie foods, low-calorie foods and object pictures (Figure 2.1.). Pictures were obtained from freely available websites and the International Affective Picture System (IAPS, NIMH Center for the Study of Emotion and Attention, University of Florida, Gainesville, FL, USA). Food and object pictures were of similar luminosity and resolution.

Food images were selected to represent familiar foods which are typical to the modern Western diet. The total caloric load, caloric density and macronutrient composition of the foods were determined using standard dietary calculators in DietPlan6 (Forestfield Software Ltd, West Sussex, UK) and are as follows - high calorie foods: 855 ± 108 kCal, 317 ± 14 kCal/100g, 42 ± 1 % fat, 48 ± 2 % carbohydrate, 10 ± 1 % protein; low-calorie foods: 274 ± 33 kCal, 87 ± 9 kCal/100g, 35 ± 3 % fat, 43 ± 4 % carbohydrate, 23 ± 3 % protein; high-calorie vs. low-calorie foods: $P<0.001$ for energy content, density and % protein; $P=0.03$ for % fat; but not for % carbohydrate ($P=0.14$).

Pictures of non-food household objects and blurred pictures were included as non-specific control stimuli in order to examine the effect of nutritional state (fed or fasted) on brain activation during other non-food related tasks.

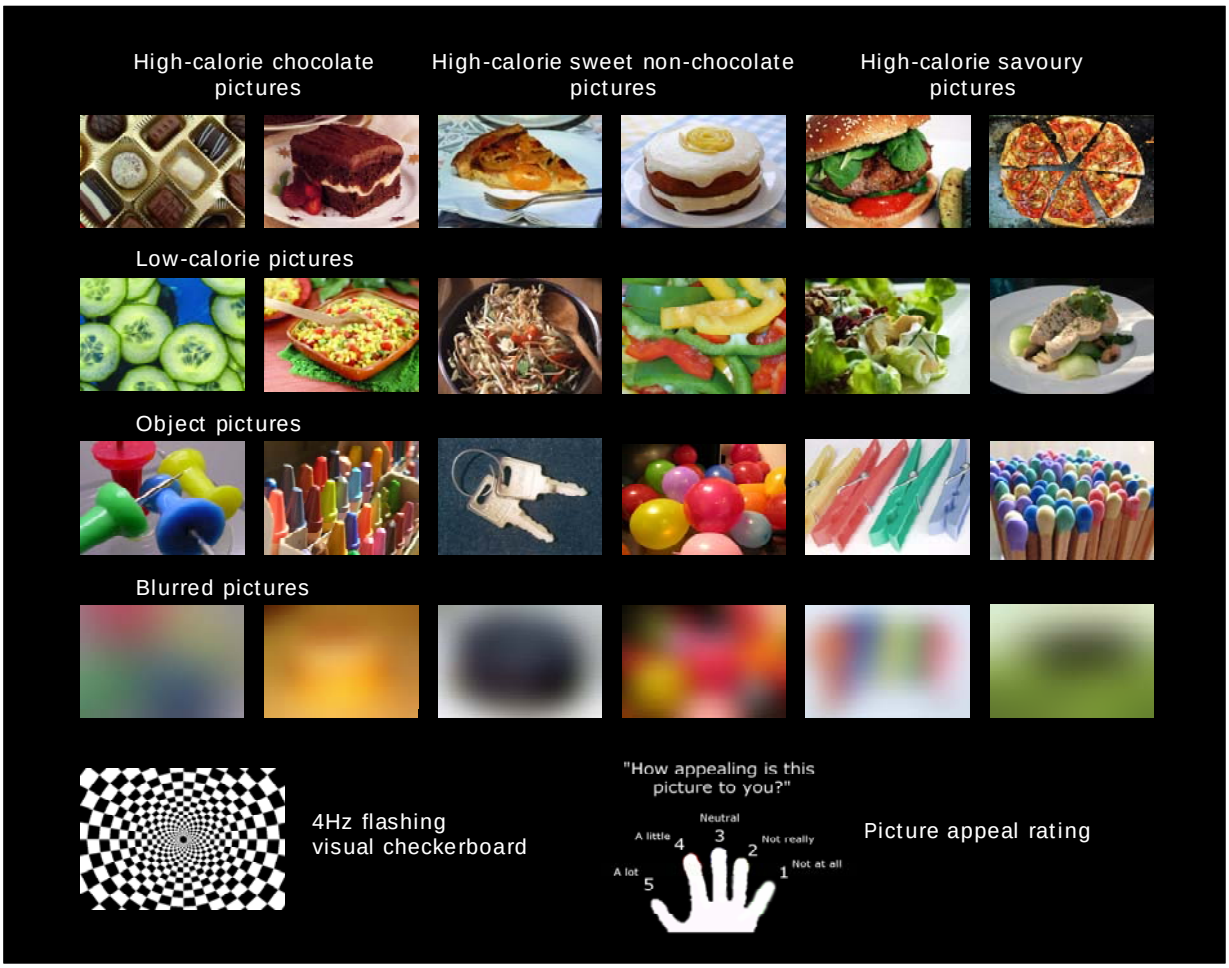
Photographs were presented in 18 second blocks in a single run lasting approximately 17 minutes. Each block contained 6 different images from the same

category (high-calorie foods, low-calorie foods, household objects), with a total of 9 blocks of each type shown in one of two pseudorandom block orders with a randomised picture order within each block. Each high-calorie food block consisted of equal numbers of foods containing chocolate, non-chocolate sweet and savoury non-sweet foods (n=2 of each). Each image was displayed for 2500 ms, followed by a 500ms inter-stimulus interval of a fixation cross. Each food and object block was followed by a similar duration block of 6 blurred pictures. The block of blurred pictures therefore allowed return of BOLD signal to baseline after blocks of categorical stimuli.

Images were viewed via a mirror mounted above an 8 channel RF head coil which displayed images from a projector using the IFIS image presentation system (In Vivo, Wurzburg, Germany) and ePrime 1.1 software (Psychology Software Tools Inc, Pittsburgh, PA, USA). Whilst each image was on display to subjects in the scanner, they were asked to immediately rate how 'appealing' each picture was to them at that moment on a scale of 1 to 5 using a hand-held keypad (1 = not at all, 2 = not really, 3 = neutral, 4 = a little, 5 = a lot). The appeal rating was thus made and recorded simultaneously with the stimulus presentation used for fMRI activation (Figure 2.1.).

A 4Hz flashing visual checkerboard (alternating with a fixation cross in 5 blocks of 24 seconds each) was viewed at the end of each scanning session as a control visual stimulus to look for non-specific changes in fMRI activation between fasted and fed states (Figure 2.1.). This scan lasted around 5 minutes.

Figure 2.1. functional MRI stimulation paradigm.



Functional MRI stimulation paradigm showing blocks of high-calorie pictures (chocolate, sweet non-chocolate and savoury pictures), low-calorie pictures, object- and blurred pictures in a pseudorandomised design. Subjects are instructed to simultanously rate the appeal of each picure on a scale from 1-5. The 4Hz flashing checkerboard was used as a control stimulus.

2.1.4. Imaging acquisition

Imaging was conducted on a 3T Philips Intera whole body scanner. Whole-brain data were acquired with T2* weighted gradient-echo echoplanar imaging with an automated higher-order shim procedure (44 ascending contiguous 3.25mm thick slices, 2 x 2 mm voxels; SENSE factor 2 repetition time (TR) 3000 ms; echo time (TE) 30 ms; 90º flip angle; FOV 190x219, matrix 112x112), slice acquisition angle was -30º from AC-PC line to reduce frontal lobe signal drop out due to the air sinuses, with a z-shim gradient correction to compensate for through-plane susceptibility gradients (Deichmann et al., 2003). The first 5 volumes of each fMRI run were discarded to allow for equilibrium effects. No

neuroanatomical abnormalities were seen on a high-resolution T1-weighted turbo field echo structural scan collected at each visit (TE 4.6 ms; TR 9.7 ms; flip angle 8°; FOV 240 mm; voxel dimensions, 0.94 x 0.94 x 1.2 mm).

2.1.5. Image pre-processing and statistical analysis

SPM5 (update 826, Wellcome Dept. of Imaging Neuroscience, UCL, UK) was used for individual pre-processing. EPI images were corrected for slice timing as each slice is acquired at different time points. ArtRepair (Mazaika, 2007) was used to quantify relative head motion. A mean EPI image was created for each subject and registered to a standard EPI template in stereotatctic space using MNI coordinates (Monreal Neurological Institute, Quebec, Canada). The normalized images were smoothed with a three-dimensional isotropic Gaussian kernel of 8 mm full-width half-maximum (FWHM). A general linear model (GLM) was applied by convolution of the individual block onsets with the haemodynamic response function including the motion parameters as a regressor, with subsequent random effects contrast analysis (high-calorie vs. object, low-calorie vs. object, high-calorie vs. low-calorie, object vs. blurred, and visual checkerboard vs. fixation cross) (Beaver et al., 2006).

Since excessive head motion in general can produce artefactual brain activation, a cut off for head motion in this study was less than 10% of scans having greater than 0.3 mm motion (average of all 6 directions) per scan. Two additional subjects were excluded from the analysis because of excessive head movement or image artefacts during fMRI scanning.

2.1.5.1. Second-level group analysis for the fed – fasted visit

Second level group random effects analysis was performed separately on the fasted and fed scans. A statistical threshold of $P<0.001$ uncorrected and cluster extent > 5 voxels (2 x 2 x 2 mm) was used for activation using whole brain analysis with correction for multiple

comparisons made using false discovery rate (FDR) and family-wise error rate (FWER) at $P<0.05$. For a *priori* regions of interest (ROI) threshold was $P<0.005$ uncorrected with cluster extent > 5 voxels, with small volume correction (SVC) for multiple comparisons using FWR at $P<0.05$.

Anatomical labelling of activations was checked with reference to neuroanatomical atlases (Duvernoy, 1995, 1999). For visualising activations, group maps were overlaid on the average of the mean EPI scans for each subject, averaged separately for the 1st and 2nd visits, and then combined.

In a separate group analysis, BMI was also included as a co-variate in the second level group random effects analysis to identify whether the activation in any of the ROIs for the high-calorie vs. low-calorie food contrast was influenced by BMI when fasting.

Co-ordinates of peak voxel activation within each ROI were determined at the group level for fasted and fed visits. The co-ordinates of peak voxel activation within each ROI for the high-calorie vs. low-calorie foods contrast (or flashing checkerboard vs. fixation cross) at the group level for the relevant visit (or at just the fasted visit if no significant activation see at the fed visit) were then used to separately extract data on the magnitude of activation (beta value BOLD contrast) at the fasted and fed visits for individual subjects for the high-calorie vs. object, low-calorie vs. object, high-calorie vs. low-calorie and object vs. blurred picture (or flashing checkerboard vs. fixation cross) contrasts. Beta values were extracted separately for each hemisphere, and also combined to give average bilateral activation.

Comparison between groups was performed using 2-way paired Student's t-test (fasted vs. fed). 2-way repeated measure ANOVA with post-hoc Student-Newman-Keuls test was performed to look at the interaction of breakfast consumption (fasted vs. fed) and food picture category (high-calorie vs. low-calorie) on BOLD activation in each ROI and appeal ratings. Equal variance between groups was confirmed using the Levene Median Test with a threshold $P=0.01$.

Linear regression analysis was used to examine the relationship (Pearson correlation coefficient r) between the effect of breakfast consumption on brain activation

in each ROI and the effect of breakfast consumption on appeal bias for high-calorie foods. The dependent factor was the individual average bilateral BOLD contrast values for high-calorie vs. low-calorie food contrasts in the fed state subtracted from values obtained in the fasted state. The independent factor was the individual appeal rating difference between high-calorie and low-calorie foods in the fed state subtracted from the rating difference obtained in the fasted state.

Unpaired t-test, or 2-way repeated measures ANOVA was used to compare between genders (male vs. female) and food category (high-calorie vs. low-calorie), the appeal of food pictures and brain activation in each ROI. The relationship between appetite VAS ratings and food picture appeal was examined using linear regression analysis.

Statistics was performed using SigmaStat 2.03 (SPSS, San Rafael, CA, USA). All data is presented as mean ± standard error of mean (SEM). Significance was taken as $P<0.05$.

2.1.5.2. Brain activation correlation with questionnaire scores

Statistical parametric maps (for high-calorie greater than low-calorie food picture contrast) were constructed to determine those voxels within each *a priori* ROI whose activity was significantly correlated with individual questionnaire scores (BAS-Drive, BAS-Fun Seeking, BAS-Reward Responsiveness, BIS, DEBQ-Restraint, DEBQ-External) by their inclusion as covariates of interest in separate 2nd level group general linear models by random effects analysis.

The *a priori* ROI threshold for this analysis was $P<0.005$ uncorrected with cluster extent > 3 voxels, with small volume correction (SVC) for multiple comparisons using FWR at $P<0.05$. For visualising activations, group maps were overlaid on the average mean EPI scans for each subject. The co-ordinates of significant peak voxels for each correlation in each ROI were used to extract the magnitude of activation (BOLD contrast) for individual subjects for the high-calorie vs. low-calorie food contrast.

Simple linear regression was used to correlate questionnaire scores with brain activation in each ROI. The dependent factor was the individual unilateral BOLD contrast value for high-calorie vs. low-calorie food contrast and the independent factor was the individual questionnaire score. These co-ordinates were also used to separately extract data on the individual magnitude of activation for the object vs. blurred picture contrast followed by simple linear regression to correlate questionnaire scores with brain activation in each ROI for this contrast. Gender and visit number were also included as co-variates in multiple linear regression analysis. Statistics were performed using SigmaStat 2.03 (SPSS, San Rafael, CA, USA). All data is presented as mean ± SEM. Significance was taken as $P<0.05$.

2.1.5.3. Regions of interest

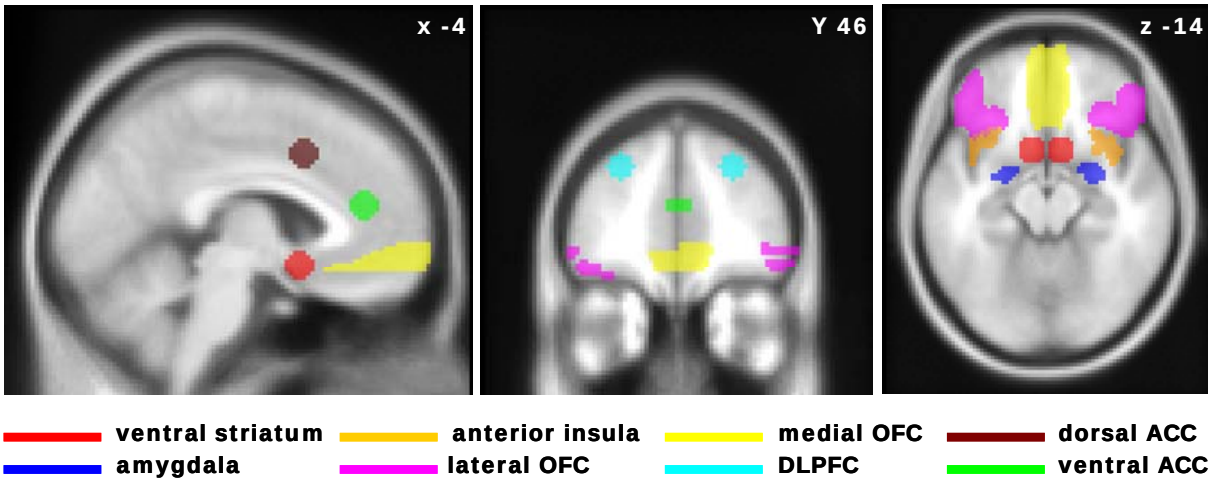
The following *a priori* ROIs were choosen for the first analysis (Figure 2.2., 2.3.): ventral striatum, anterior insula, amygdala, medial and lateral orbitofrontal cortex (OFC) for the picture viewing; lingual gyrus, calcarine sulcus and lateral geniculate nucleus for the flashing checkerboard.

The following *a priori* ROIs were choosen for the second analysis (2.1.5.2.) (Figure 2.2.): ventral striatum, anterior insula, amygdala, medial and lateral OFC, dorsolateral prefrontal cortex (DLPFC), dorsal and ventral anterior cingulate cortex (ACC) for the picture viewing.

All ROIs were selected according to previous publications and standardized atlases. ROIs for the amygdala, anterior insula, and medial and lateral OFC used masks generated from the WFU Pickatlas (version 2.4), using the automated anatomical labeling atlas, using only voxels with $y > 0$ to define the anterior insula, the medial section of the middle orbital frontal cortex for the medial OFC, the inferior orbital frontal cortex for the lateral OFC (Tzourio-Mazoyer et al., 2002, Maldjian et al., 2003). The OFC ROIs were also masked with the overall EPI mask for the whole group at second level analysis for all visits to ensure that each voxel in the ROI was present for each subject at each visit to

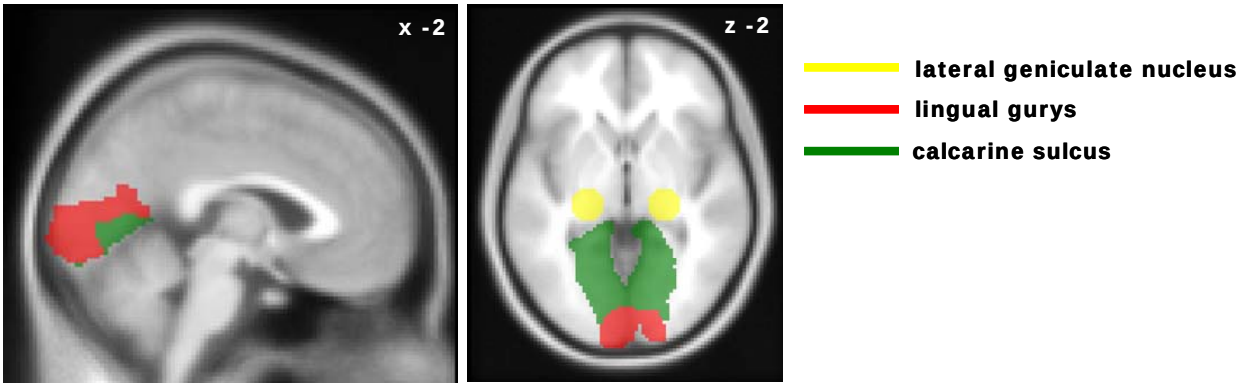
compensate for varying degrees of signal drop out in the OFC between subjects and visits. The ventral striatum ROI used two 8mm diameter spheres centred at MNI co-ordinates x ± 8 y 10 z -12 taken from a previous fMRI study of viewing appetizing vs. bland food pictures (Beaver et al., 2006). The dorsolateral prefrontal cortex (DLPFC) ROI used two 10mm spheres centred at MNI co-ordinates x ± 26 y 52 z 34 taken from a previous fMRI study of viewing high-calorie vs. low-calorie food pictures (Killgore et al., 2003). The dorsal ACC ROI used a 14mm sphere centred at MNI co-ordinates x -2 y 12 z 40 taken from a previous fMRI study of viewing fearful vs. neutral face distractors (Bishop et al., 2007). The ventral ACC ROI used two 14mm spheres centred at MNI co-ordinates x ± 15 y 36 z -12, taken from a previous fMRI study of viewing negative and neutral words (Whalen et al., 1998). The lateral geniculate nucleus ROI used two 10mm spheres centred at MNI co-ordinates x ± 22 y -24 z -5 taken from a previous fMRI study viewing a flashing checkerboard (Schneider et al., 2004). To define the lingual gyrus and calcarine sulcus masks were generated from the WFU Pickatlas (version 2.4).

Figure 2.2. ROIs for brain food reward and executive systems.



All ROIs (amygdala, anterior insula, medial and lateral OFC, ACC and DLPFC) were selected according to previous publications and standardized atlases (WFU Pickatlas within SPM5).

Figure 2.3. ROIs for brain visual processing.



All ROIs (lateral geniculate nucleus, lingual gyrus and calcarine sulcus) were selected according to previous standardized atlases (WFU Pickatlas within SPM5).

The VTA is very difficult to study in humans using functional neuroimaging because of its small size, proximity to major blood vessels, susceptibility to motion artefacts related to cardiac and respiratory cycles, and difficulties in image registration to standard space. Therefore this ROI was not included in the analysis of this study (D'Ardenne et al., 2008).

2.1.6. Summary fed-fasted fMRI study

Twenty-five subjects were screened for this study but only 20 non-obese healthy adults were scanned on two separate mornings between 11am and noon, once after eating a filling breakfast (fed), and once after an overnight fast (fasted) in a randomised cross-over design.

Using functional magnetic resonance imaging (fMRI), the interaction between nutritional state (fed and fasted) and different food stimuli on brain food reward systems was analysed. This study also examined how blood oxygen level dependent (BOLD) activity within *a priori* ROI varied while viewing pictures of high-calorie and low-calorie foods. Pictures of non-food household objects and flashing checkerboard were included as control visual stimuli. During scanning, subjects rated the appeal of each picture.

Questionnaires (BIS/BAS and DEBQ) were used to measure individual differences in reward sensitivity and eating behaviour to examine their influence on individual differences in activation in brain reward and executive systems.

2.2. Ghrelin fMRI study

2.2.1. Participants

Fourty-seven adults were screened for this study. Participants were recruited via poster, email and web-based advertisements and word of mouth. To reduce variability or confounds among participants in their response to experimental stimuli, individuals were excluded based on various criteria: abnormal eating habits (assessed by the Eating Attitude Test (Garner et al., 1982), SCOFF questionnaire (Morgan et al., 1999) (Appendix 3), Dutch Eating Behaviour Questionnaire (DEBQ) (van Strien, 1986) (Appendix 5), 3-day dietary record (Appendix 7)), missing breakfast (more than 3 days per week), history of addiction within the last 5 years and severe depression (Beck Depression Inventory (BDI-II>28)). Further exclusion criteria were as described previously for the fed-fasted fMRI study (see 2.1.1.).

Twenty healthy, non-obese adults with normal or corrected to normal vision were eligible and agreed to take part in the study. Two participants withdrew from the study without any specified reason after completing 2 visits. Eighteen subjects completed the study (mainly students and health professionals working in the hospital; mixed gender (13 male, 5 female as only women with regular menstrual cycle for the fMRI scanning were included); mixed ethnicity (white British n=9, other White n=1, Indian n=2, Chinese n=3, other Asian n=2, White and Asian n=1)). All subjects were either born in the UK or used to a Western diet.

The study was performed on Ward C2 (Sir John McMichael Centre Clinical Investigation Unit) and the Robert Steiner MRI Unit at Hammersmith Hospital London. All subject's characteristics are to be found in Table 2.1.

The study was approved by the Local Research Ethics Committee Hammersmith Hospital (ethics number: 07/Q0406/19), and was performed in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent.

Table 2.1. Subjects characteristics (n = 18).

	Mean ± SEM	**Range**
Age (years)	24.3 ± 1.5	19-41
Height (m)	1.80 ± 0.0	1.59-1.91
Weight (kg)	76.1 ± 3.7	50.7-104.5
BMI (kg/m^2)	24.3 ± 0.7	19.2-30.2
Body fat (%)	16.9 ± 1.6	6.1-31.6
LBM (kg)	63.3 ± 3.4	45.0-86.1

2.2.2. Protocol

2.2.2.1. Screening visit

After a short telephone screening which included major inclusion and exclusion criteria (see 2.1.1.), subjects were invited for a 2 hour screening visit. Participants underwent a physical examination (including ECG). Height, weight and general screening blood tests (haematology, renal, liver and thyroid function) were measured. Body fat was measured by bio-electrical impedance analysis which measures electrical resistance to determine fat free mass (Bodystat 1500, Isle of Man, UK). Pregnancy was excluded using a urine HCG dipstick testing. Subjects had to fill in a metal check form to ensure they had no metal inside their body (Appendix 8). A three-day food diary to be completed at home and different questionnaires (see 2.1.1) were used to screen for mood and abnormal eating behaviour (Appendix 3 to 7). Subjects also had to choose a study lunch (macaroni cheese or chiken tikka masala curry) that they would be given at each study visit, with subjects initially given the pasta. The final meal was chosen from that with a pleasantness ratings of at least “Like moderately” at the screening visit (Appendix 2).

2.2.2.2. Randomisation

On the first study visit subjects were not given breakfast (remained fasted) and had saline administered (*Fasted Dummy visit*). This Fasted-Dummy visit was performed to habituate individuals to the study environment (chapter 4).

During the subsequent three visits, participants were fed or fasted in a block randomised cross-over design and administration of ghrelin or saline during each fed visit was double-blinded and each fasted visit single-blinded, as follows: *Fasted Saline, Fed Ghrelin, Fed Saline*. There was an average of 20 (5-151) days (median, range) between each study visit.

2.2.2.3. Day before each study visit

Subjects completed a 3-day dietary record starting from the morning prior to the study day in order to confirm similar nutritional states and food intake between visits. Volunteers were told to avoid rigorous exercise and alcohol intake and to eat supper at 8.00pm on the day before each study visit. They were advised to eat a similar supper before each study visit.

2.2.2.4. Study day

After an overnight fast (nothing to eat or drink other than water), subjects arrived between 8am and 9.30am to Ward C2 of the Hammersmith Hospital. They had to remove all their metal and changed into blue hospital scrubs. They had to fill in a metal check form and mood/depression assessment questionnaires at each visit in order to ensure depression had not developed at the time of scanning (Appendix 8 and 9). All female volunteers were confirmed not to be pregnant via urine HCG dipstick testing. Females were only scanned in the follicular phase (day 2-10) of their menstrual cycle to avoid hormone-induced changes in food selection as food intake and fMRI responses to rewarding stimuli may vary over the menstrual cycle in response to changes in ovarian steroid hormone levels (Buffenstein et al., 1995, Dye and Blundell, 1997, Frank et al., 2010).

Subjects had their height, weight and body fat (bio-electrical impedance analysis) measured. A peripheral intravenous catheter was inserted into the subject's left or right antecubital fossa to allow serial blood sampling at time points (t) = -15 ,0 ,40, 70, 150,

180 minutes with at least 30 minutes between insertion and first blood sampling to avoid stress responses (Figure 2.4.).

2.2.2.5. Visual Analogue Scales

Visual Analogue scale (VAS) ratings (0-10 cm) were recorded at 8 different time points: t = -15, 0, 40, 55, 70, 150, 180 and 210 minutes to measure hunger, sickness, pleasantness to eat, anxiety, volume of food wanting to eat, fullness, stress and sleepiness (Flint et al., 2000; Blundell et al., 2010) (Figure 2.4.) (Appendix 10). Subjects were asked to rate their hunger at 3 time points (after the Test and Rest scan and before the AMV scan while in the MRI scanner, using a 5 fingers keypad rating from 1) not at all to 5) a lot (Figure 2.5.).

2.2.2.6. Blood pressure

At each time point (t = -15, 0, 40, 55, 70, 150, 180 and 210 minutes) pulse, systolic and diastolic blood pressure was measured to ensure patient safety.

2.2.2.7. Study breakfast

Subjects did not know whether they would be given breakfast until t=0 minutes. On the first study visit, subjects were not given breakfast. During the subsequent three visits, participants were fed or fasted (not given any breakfast) in a randomised cross-over design (see 2.2.2.2.). On the fed visit, subjects consumed a fixed breakfast of 730 Kcal (14% protein, 31% fat and 55% carbohydrate) at t = 0 minutes consisting of 220ml orange juice, 40g Kelloggs bran flakes, 170ml semi-skimmed milk, 2 slices of wholemeal bread, each with 10g of margarine, one with 10g of Robertson strawberry jam and the other with one slice of Leerdamer cheese (8g) (Figure 2.4.). Subjects were told to finish all of the given food. They rated how tasty and pleasant the breakfast was on a 10cm scale. The average time to eat breakfast was 9.0 ± 1.1 minutes (mean ± SEM).

2.2.2.8. Ghrelin injection

At t = 55 minutes, subcutanous saline or octanylated ghrelin was injected into the abdomen at a dose of 3.6 nmol/kg (Figure 2.4.), as with earlier studies as the smallest effective dose from a dose-response pilot study performed in healthy volunteers (Dr. N. Neary and Dr. M. Druce, Department of Metabolic Medicine, Imperial College London, UK, personal communication, November 2003), in a concentration of 548 nmol/ml (Druce et al., 2005, Wynne et al., 2005a). All vials were coded by a member of the department not involved in the study, so that ghrelin and saline injections were blinded to the researchers. Human octanylated ghrelin was purchased from Penninsula Laboratories, St. Helens, Mereyside, UK. Bioactivity of the ghrelin vials had been demonstrated by acute feeding studies in mice as part of a previous human ghrelin feeding study (Ashby et al., 2009).

Preparation and Sterility of ghrelin

The peptide was aliquoted into sterile glass vials under aseptic conditions and freeze-dried. Although the material is packed and stored under vacuum, which destroys any active (aerobic) microorganisms, the freeze-dried peptide was also sent to the HHNT Microbiology Dept. for culture of all common pathogenic microorganisms as a further safety check. Absence of pyrogen contamination of every batch of peptide was confirmed by highly sensitive measurements of endotoxin content using a Limulus Amoebocyte Lyaste (LAL) test, performed by Associates of Cape Cod International, 3a Newton Court, Wavertree Technology Park, Liverpool.

Potential toxicity of ghrelin

For each batch of peptide aliquots, toxicity studies were carried out in mice. Greater than 10 times the maximum dose to be given to man was administered by intraperitoneal injection to a minimum of 20 mice and compared to a saline injected control group. The animals were observed and, if no adverse effects were seen, one group of 10 mice was killed by a schedule 1 method after around 48 hours observation and the remaining

animals were killed after at least 14 days. Full necropsies with histological examination of all important internal organs, including lungs, heart, brain, and kidneys were performed under the supervision of an independent experienced rodent pathologist and a formal report was issued.

2.2.2.9. Blood sampling

Blood samples drawn at each time point were immediately transferred to chilled lithium heparin polyprooylene tubes, containing 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (A8456 Sigma-Aldrich), a protease inhibitor to prevent the breakdown of active ghrelin, and aprotinin (Nordic Phama UK), a protease inhibitor to prevent breakdown of other gut hormones, to give a final AEBSF and aprotinin concentration of 1 mg/ml whole blood and 200 kIU/ml whole blood respectively (Figure 2.4., Appendix 11).

Blood samples were centrifuged at 4ºC, 4000 rpm for 10 minutes. 150µl aliquots of separated plasma was immediately mixed with HCl (to prevent breakdown of active ghrelin) to give a final concentration of 0.05M, which was frozen and stored at -80ºC for future assaying of acylated ghrelin. One milliliter aliquots of seperated plasma were not mixed with HCl, frozen and stored at -80ºC for future assaying of adipocytes and gut hormones. The samples have not yet been analysed for gut hormones as additional subjects have been recruited to the study to improve statistical power and all blood samples need to be assayed together.

At each time point blood samples were taken to measure plasma glucose, and serum insulin, growth hormone, cortisol, prolactin, and triglycerides. All metabolic paramteres were measured in the Department of Clinical Biochemistry, Hammersmith Hospital and Charing Cross Hospital, using either an Abbott Architect ci8200 analyser (Abbott Diagnostics, Maidenhead, UK) or an Axsym analyser (Abbott Diagnostics, Maidenhead, UK) respectively. Intra-assay co-efficients of variation of all metabolic parameters were 1.0–5.0%.

2.2.2.10. Lunch

After MRI scanning (t = 150 mins) each subject received the same and excess amount of lunch (macaroni cheese (per 100g: 205kCal, 6.5g protein, 18.9g carbohydrate and 11.5g fat; or chicken tikka masala (per 100 g: 150kCal, 6.6g protein, 13g carbohydrate, 8g fat). Men were presented with 2000g and women were presented with 1500g of excess lunch. Subjects were instructed to eat as much as they wanted until they felt comfortably full (Figure 2.4.; Appendix 2). Subjects rated how tasty and pleasant the lunch was on a 10 cm scale.

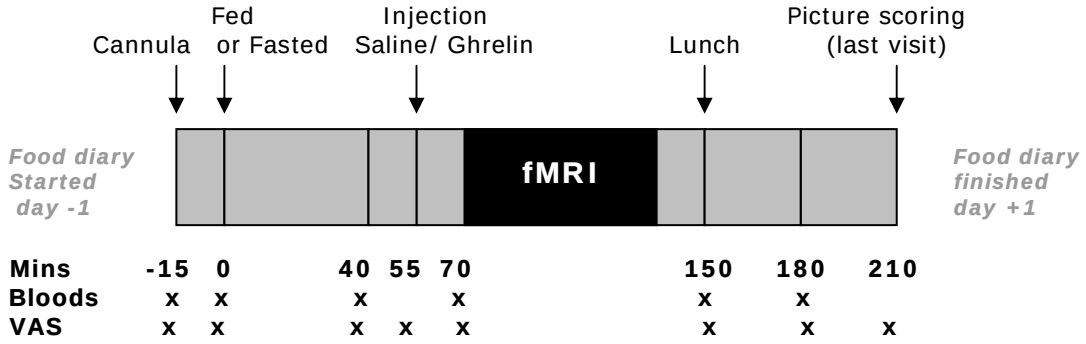
2.2.2.11. Three-day dietary record

Subjects finished the 3-day dietary record at home until the evening after the study day (Figure 2.4.). Food diaries were analysed using dietary analysis software DietPlan6 (Forestfield Software Ltd, West Sussex, UK).

2.2.2.12. Picture scoring

After the 4th study visit volunteers named all of the food pictures viewed during the fMRI scanning to ensure subjects were familiarized with the foods (percentage correctly recognised). Subjects also scored all of the food pictures viewed during the fMRI scanning to rate *how much they usually like the food*: hate a lot (score -3), hate somewhat (score -2), hate a little (score -1), neither like nor hate (score 0), like a little (score +1), like somewhat (score +2) or like a lot (score +3); and *how often they usually eat the food*: never (score 0), 3 times or less per year (score 1), 6 times or less per year (score 2), once per month or less (score 3), 2-3 times per month (score 4), once per week (score 5), 2-3 times per week (score 6), 4-6 times per week (score 7) or once per day or more (score 8). Scores were added separately for high-calorie and low-calorie foods to assess usual preferences and consumption of the high-calorie vs. low-calorie foods (Figure 2.4.).

Figure 2.4. Ghrelin study protocol.

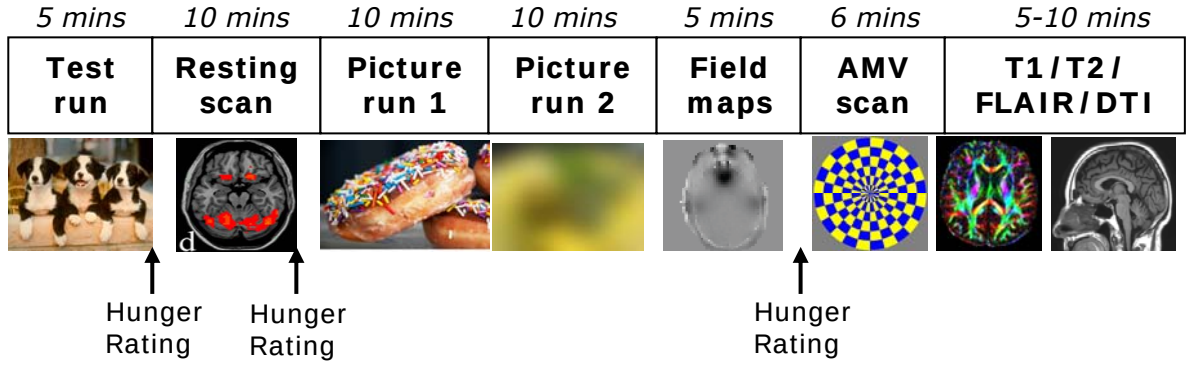


Ghrelin study protocol: Each subject completed 4 study visits. After an early morning cannulation, subjects received breakfast or remained fasted. Before the fMRI scanning session, subjects received an injection of saline or ghrelin. After 1 hour of scanning, an *ad libitum* lunch was given. Blood samples were taken and Visual Analogue Scales filled in throughout the study morning. Food diaries had to be completed for 3 days starting the day prior to the study. On the last study visit subjects were instructed to score the food pictures seen in the MRI scanner.

2.2.3. MRI scanning protocol

After the injection, each subject underwent one hour fMRI session, starting at t = 70 mins (Appendix 12).

Figure 2.5. Scanning protocol



Functional MRI scanning protocol lasting for about 60 mins: 1) Test run to get subjects used to the keypad button pressing, 2) Resting scan to look at brain activity in the absence of a task, 3) Two picture runs showing blocks of food- and object pictures alternating with blurred pictures, 4) Filed maps to reduce signal drop-out, 5) AMV – Audio-Motor-Visual task used as a control task, 6) Anatomical scans to look for abnormalities in the brain.

The scanning protocol was similar to the fed-fasted fMRI study scanning protocol (see 2.1.3.). Additions to that protocol in this study were as follows (Figure 2.5.):

(1) In the MRI scanner, subjects had a respiratory belt to measure breathing and a pulse oximeter to measure heart rate and blood oxygenation to monitor subject's safety.

(2) The software to display images in the MRI scanner was updated from ePrime 1.1 to ePrime 2.0 (Psychology Software Tools Inc, Pittsburgh, PA, USA).

(3) Fruit pictures as well as salad pictures showing excessive oil or dressing were removed from the low-calorie food picture paradigm and replaced by 14 new pictures as their sweetness or calorie content was felt to be too high to be included in the low-calorie pictures. The percentage of carbohydrates in the low-calorie pictures was now reduced and the amount of protein increased. The new total caloric load, caloric density and macronutrient composition of the foods were reassessed using Dietplan6 (Foresfield Software Ltd, West Sussex, UK) - high calorie foods: 834 ± 100 kCal, 321 ± 13 kCal/100g, 42 ± 2 % fat, 48 ± 1 % carbohydrate, 10 ± 1 % protein; low-calorie foods: 157 ± 18 kCal, 64 ± 5 kCal/100g, 35 ± 3 % fat, 35 ± 3 % carbohydrate, 29 ± 3 % protein; high-calorie vs. low-calorie foods: $P<0.001$ for energy content, density, % protein and % carbohydrate; and $P=0.03$ for % fat.

(4) The high-calorie and low-calorie food and object picture number was increased from 54 to 60 pictures per category to allow equal numbers of blocks per run.

(5) The food picture run (17 minutes) was divided into two runs each of 9 minutes to increase subject's concentration and attention. Each run contained different pictures and contained 5 blocks each of high-calorie and low-calorie foods and objects interleaved with 31 blocks of blurred pictures (6 pictures per block). A different pseudorandomised block run order was used between each visit.

(6) Fieldmaps were used to correct for geometric distortions caused by inhomogenities in the magnetic field which can lead to compression of the BOLD image or signal drop out in important brain reward areas such as the OFC (due to its air-filled sinuses). Therefore, two images of signal phase with slightly different echo times were acquired (Figure 2.5). The differences

between the two images results in an image of the intensity of the magnetic field across space (at any given location) which can be registered with the EPI image to correct geometric distortions (Huettel, 2004). The imaging parameters to acquire fieldmaps were as follows: TR 800 ms; TE 20ms, 90º flip angle; FOV 190x219, 44 ascending contiguous 3.25mm thick slices, 2 x 2 mm voxels, ∂TE 0 and 2.5.

(7) The software to analyse fMRI data was changed from SPM5 to FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Advantages for using this software to analyse fMRI data included: ease of use, ability to easily script pre-processing steps for multiple visits, improved visualization of registration steps, non-linear registration, and maintenance of results within subject space until the end of the analysis when registration to the T1 anatomical scan and hence to standard space was performed.

(8) Instead of slice timing correction, this study now used temporal derivatives which measures the relative change of activation over time and is applied to the GLM to correct for differences in both slice timing and in the onset of the HRF after stimulus delivery between different voxels.

(9) After the Test Run, subjects had a resting state fMRI scan for 10 minutes during which they were instructed to keep their eyes shut, not to fall asleep nor think of anything in particular (Figure 2.5.). This scan allows assessment of resting connectivity between brain regions, which shows how strongly coordinated activity is within brain networks in the absence of a task (Damoiseaux et al., 2006).

(10) An auditory-motor-visual (AMV) task was performed for 6 minutes at the end of each scanning session as control stimuli to look for non-specific changes in fMRI activation between fasted and fed states, and fed saline and fed ghrelin injections (Figure 2.5.). The following brain areas will be assessed with this task: visual cortex (lingual gyrus, calcarine sulcus) and lateral geniculate nucleus, primary and secondary auditory cortex, primary motor cortex and

supplementary motor area. Subjects were told to perform two tasks simultanously:

a. Watching a 4Hz colour flashing checkerboard while listening to a story, or
b. Watching a 4Hz flashing checkerboard while pressing the right index finger once every second, or
c. Listening to a story while pressing the right index finger once every second.

The AMV scan fMRI data has not been analysed for this thesis.

(11) After the Test and Resting scan and before the AMV scan subjects rated how hungry they felt using the hand-held keypad (5 = a lot, 4 = a little, 3 = neutral, 2 = not really, 1 = not at all) (Figure 2.5.).

(12) Image acquisition (2.1.4) was similar to the first fed-fasted study except that the first 6 volumes rather than 5 volumes were discarded to allow for equllibrium effects. The number of volumes per scan was as follows: Rest scan 192 volumes, Food picture run each 192 volumes, AMV scan 113 volumes.

(13) Additionally to the first fed-fasted study, a second T1 image was collected as anatomical brain image used for image registration in case there were artefacts with the first T1 scan.

(14) Fluid attenuation inversion recovery (FLAIR) was added into the protocol which is a special inversion recovery sequence with long TI to remove the effects of fluid from the resulting images. The FLAIR sequence was used to look at anatomical brain abnormalities and is described as follows: TR/TI=11000/2800, TE=125, Slices=30, Slice gap=1mm, acquired voxel size=0.65x0.97x4mm, rest slab=1, TSE factor=27.

The "pathology weighted" T2 (turbo spin echo) sequence is important as most pathology contains more water than normal tissue around it which lights usually brighter on a T2 sequence (TR=3000, TE=80, Slices=28, Slice gap=1mm, acquired voxel size 0.57x0.72x4mm). The T2 and FLAIR scans were included to improve detection and assessment of any neurological abnormality that might be found as incidental findings (Figure 2.5.).

2.2.4. Image pre-processing

FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) version 5.98, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). The following preprocessing was applied: motion correction using MCFLIRT (Jenkinson et al., 2002); fieldmap-based EPI unwarping using PRELUDE+FUGUE (Jenkinson, 2003, 2004); non-brain removal using BET (Smith, 2002); spatial smoothing using a Gaussian kernel of FWHM 6.0mm; grand-mean intensity normalization of the entire 4D dataset by a single multiplicative factor and highpass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma=100.0s).

Time-series statistical analysis was carried out using FILM with local autocorrelation correction including picture onsets, temporal derivative and motion parameters as co-variates (Woolrich et al., 2001). Registration to high resolution T1 structural and/or standard space images was carried out using FLIRT (Jenkinson and Smith, 2001, Jenkinson et al., 2002). Registration from high resolution structural to standard space was then further refined using FNIRT nonlinear registration (Anderson et al., 2007a, b). For the food pictures, higher level analysis was carried out using a fixed effect model to combine the 2 runs, by forcing the random effects variance to zero in FLAME (FMRIB's Local Analysis of Mixed Effects) (Beckmann et al., 2003, Woolrich et al., 2004). One subject (20 year old male BMI 21.2 kg/m^2) was removed from further analysis because of excess head movement (over 10% of scans showing motion >0.3mm/TR or average relative motion per scan >0.3mm).

2.2.5. Image statistics

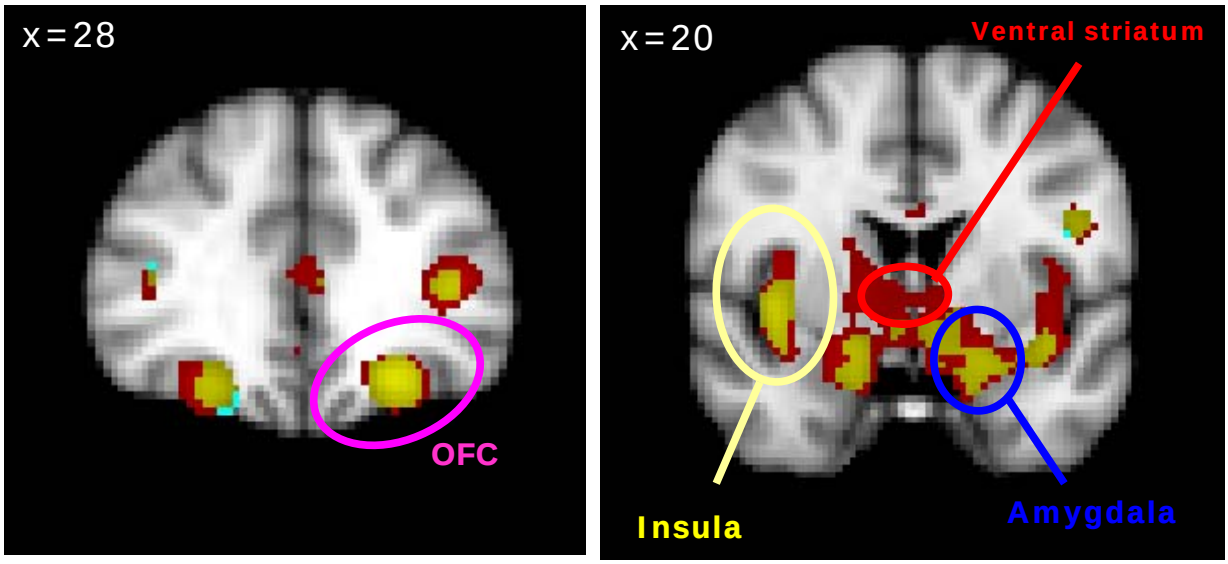
Average group activation for each visit separately

Higher level group whole brain analysis using FEAT version 5.98 with random effects analysis was performed to identify those voxels in the brain which are significantly more activated when viewing high-calorie foods (vs. objects) or low-calorie foods (vs. objects) for each visit separately, with correction for multiple comparisons made using false discovery rate (FDR) at $P<0.05$.

Combined activation averaged across 3 visits

Significant activated voxels in the brain when viewing high-calorie foods > objects averaged across all three study visits (Fasted Saline, Fed Saline and Fed Ghrelin) were then determined using higher level group analysis using FEAT version 5.98 with random effects analysis and a whole brain statistical threshold of FDR P<0.05 (Figure 2.6.). Similar activation maps averaged across all visits were performed for the low-calorie foods > object contrast, and generally were contained within the mask of those voxels activated for the high-calorie > food contrast.

Figure 2.6. Combined whole brain activation averaged across 3 visits.



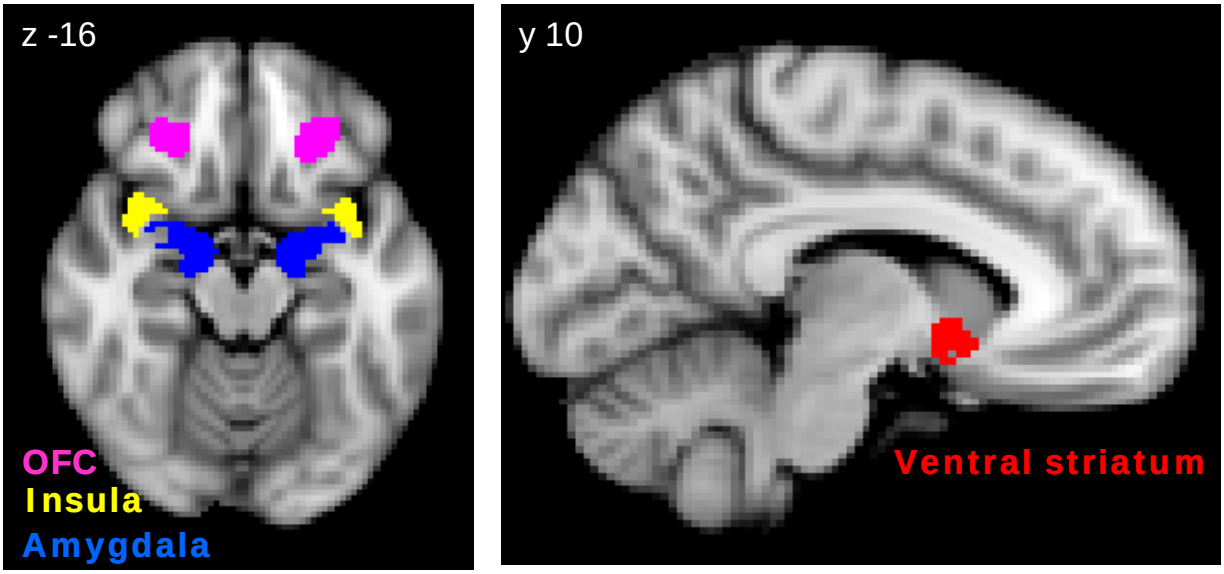
Combined whole brain activation across 3 visits (Fasted Saline, Fed Saline and Fed Ghrelin visit). Various brain areas were activated when viewing different pictures contrasts: RED when viewing high-calorie foods > object pictures, BLUE when viewing low-calorie foods > object pictures, YELLOW showing the overlap of activation of viewing high-calorie and low-calorie foods > object pictures.

2.2.6. Regions of Interest (ROIs)

A priori ROIs for this study were as follows: ventral striatum, amygdala, insula and anterior OFC. For the former the activation map produced for the high-calorie food > object contrast averaged across all three study visits at FDR P<0.05 (see 2.2.5) was masked with the ventral striatum, amygdala and insula ROIs determined by the cortical and subcortical structural Harvard FSL atlas (Shenton et al., 1995; Dreher et al., 2007; Van Vugt, 2009) (Figure 2.7.). For the OFC a cluster of activation in the anterior OFC

(y>74) was used from the high-calorie food > object contrast across all three visits (Figure 2.7.).

Figure 2.7. ROIs averaged across 3 visists.



A priori ROIs (ventral striatum, amygdala, insula and anterior OFC): The activation map produced for the high-calorie food > object contrast averaged across all three study visits at FDR P<0.05 was masked with the ventral striatum, amygdala and insula ROIs determined by the Harvard FSL atlas within FSL. For the OFC a cluster of activation in the anterior OFC was used from the high-calorie food > object contrast across all three visits.

Extraction of magnitude of activation for each visit separately

These ROI activation masks were then used to extract the average magnitude (beta values) of BOLD activation within each *a priori* ROI for each of the four visits separately (Fasted Dummy, Fasted Saline, Fed Saline and Fed Ghrelin) for each individual subject for each of the contrasts: high-calorie food > object, low-calorie > object, and object > blurred (as control) using FEAT query in FSL. Beta values were extracted separately for each hemisphere. Average BOLD activation for each of these contrasts within each ROI was then compared between visits using paired Students t-test or Wilcoxon Signed Rank test outside FSL using SigmaStat 2.03.

2.2.7. Statistical analysis

Results are presented as mean value ± standard error of the mean (SEM). VAS scores and blood parameters were calculated as the total area under curve (AUC) in millimeters for the time course: -15 mins to 210 mins (VAS) and -15 mins to 180 mins (blood parameters). The study population demographics, dietary records, VAS, blood parameter and appeal ratings were compared between (i) Fasted Dummy and Fasted Saline, (ii) Fasted Saline and Fed Saline, or (iii) Fed Ghrelin and Fed Saline using paired Student's t-test or Wilcoxon Signed Rank *post-hoc* test if not normally distributed. Unpaired t-test was used for analysis of picture scores (recognition, liking, frequency) to compare between high-calorie and low-calorie foods or Mann-Whitney-Rank Sum test if not normally distributed.

The relationships between hunger ratings (independent variable) and brain activation (dependant variable) or brain activation / VAS appetite scores (independent variable) and *ad libitum* lunch intake (dependant variable) were examined using linear regression analysis.

Analyses were performed using Prism version 4.03 software (Graphpad Software, San Diego CA, USA), Microsoft Office Excel 2003 and SigmaStat 2.03. $P<0.05$ was considered to be statistically significant.

2.2.8. Summary ghrelin fMRI study

Eighteen non-obese healthy adults were scanned on 4 separate mornings starting between 10.36 am and 12.40 pm, twice after eating a fixed 730kCal breakfast (Fed Saline or Fed Ghrelin), and twice after an overnight fast (Fasted Dummy or Fasted Saline) in a randomised cross-over design. They were either given a subcutaneous injection of saline (Fasted Dummy, Fasted Saline, Fed Saline) or ghrelin (Fed Ghrelin). Using fMRI, the interaction between nutritional state (Fasted Saline vs. Fed Saline visit) and different food stimuli on brain food reward systems was analysed. It was further examined whether the effect of fasting on food reward can be mimicked by the orexigenic hormone ghrelin (Fed

Ghrelin vs. Fed Saline visit). This study further investigated the effect of activation in brain reward systems on subsequent food intake (*ad libitum* lunch). In order to measure appetite and hunger subjects were asked to complete VAS and three-day food diaries. During scanning, subjects rated their appeal of each picture and hunger on a 5-point scale. Blood samples were also taken throughout the study morning to measure glucose, insulin, triglycerides and growth hormone as well as stress responses (cortisol and prolactin). Finally this study investigated the reproducibility of fMRI data by comparing the Fasted Dummy and Fasted Saline visit.

Chapter 3

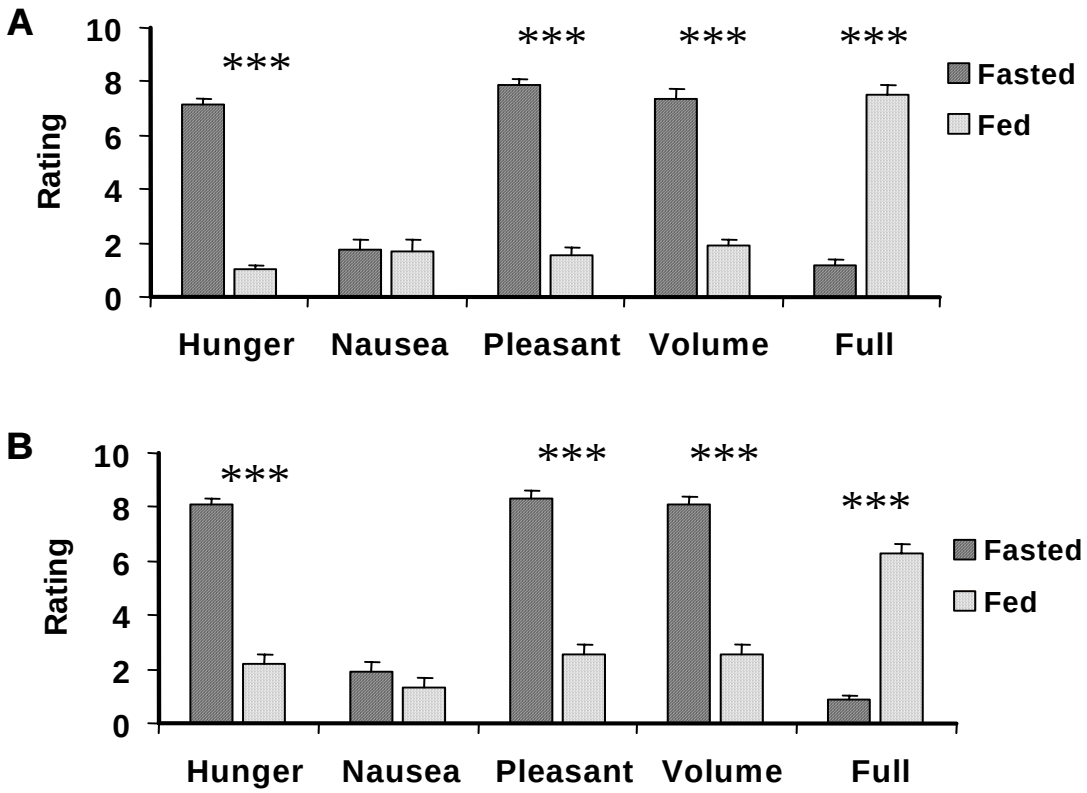
RESULTS

FED – FASTED fMRI STUDY

3.1. Appetite visual analogue scales

Subjects rated higher scores for “hunger”, “pleasantness to eat”, and “volume able to eat” and lower scores for “fullness” when fasted compared to when fed both before and after the fMRI scan (P<0.001) (Figure 3.1., Table 3.1.). There was no significant difference in the change in VAS from before to after the fMRI scan between fasted and fed visits, although there was a tendency for fullness to decline more on the fed than fasted visit (P=0.08) (Table 3.1.).

Figure 3.1. Pre (A) and post (B) – fMRI scan Visual Analogue Scales.



Subjects rating of appetite in fasted (hatched bar) and fed states (dotted bar) (*** P<0.001).

Table 3.1. Appetite visual analogue scale (VAS) ratings.

	Fed	Fasted
Appetite VAS Rating [a]		
Hunger	1.5 ± 0.2	7.7 ± 0.2 ***
Nausea	1.6 ± 0.4	2.0 ± 0.4
Pleasant	2.0 ± 0.3	8.1 ± 0.2 ***
Volume	2.0 ± 0.2	7.8 ± 0.3 ***
Full	6.9 ± 0.4	1.0 ± 0.2 ***
Change in VAS over fMRI scan [b]		
Hunger	1.0 ± 0.4	0.9 ± 0.2
Nausea	- 0.4 ± 0.4	0.2 ± 0.2
Pleasant	0.8 ± 0.4	0.4 ± 0.3
Volume	0.6 ± 0.3	0.8 ± 0.3
Full	- 0.2 ± 0.3	- 1.2 ± 0.4

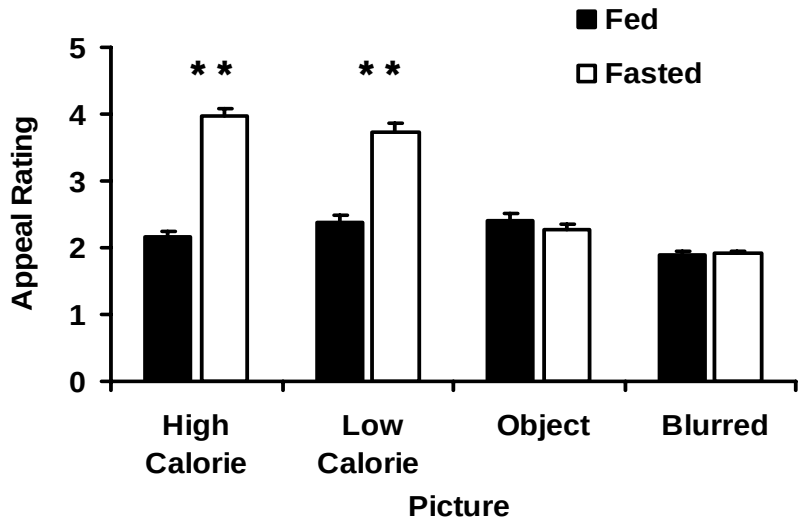
[a] Appetite visual analogue scale (VAS) ratings (1-10) using average of values obtained before and after fMRI scan. Questions asked: "How hungry do you feel right now?" (hunger), "How sick do you feel right now?" (nausea), "How pleasant would it be to eat right now?" (pleasant), "How much do you think you could eat right now?" (volume), and "How full do you feel right now?" (full). [b]After minus before fMRI scan. Data shown as mean ± SEM, n=20 per group: *** P<0.001 fed vs. fasted.

3.2. Food picture appeal rating

Both high-calorie and low-calorie food pictures (but not object and blurred pictures) were more appealing when fasted than when fed (P<0.001) (Figure 3.2., 3.3., Table 3.2., 3.4.). Furthermore, high-calorie and low-calorie foods were more appealing relative to object (and blurred) pictures when fasted compared to when fed (P<0.001) (Figure 3.2., 3.3., Table 3.2., 3.4.). However, there was no significant difference in the appeal of object relative to blurred pictures when fasted compared to when fed (P=0.14) (Table 3.2.).

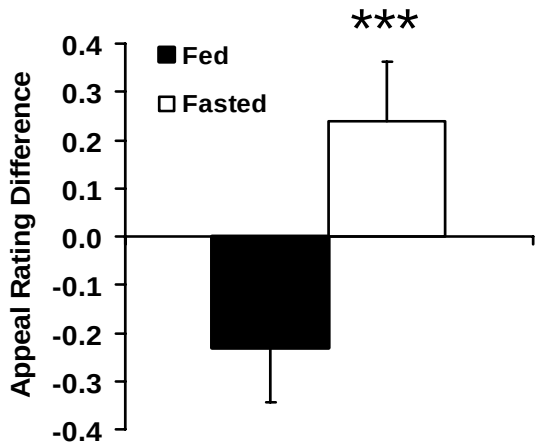
Fasting affected the preference for high-calorie over low-calorie foods (P<0.001). High-calorie foods were less appealing than low-calorie foods when fed, but more appealing than low-calorie foods when fasted (P<0.001 fasted vs. fed) (Figure 3.2., 3.3, Table 3.2., 3.4.). These differences were irrespective of the type of high-calorie food in the picture: chocolate, sweet non-chocolate or savoury (Table 3.2.).

Figure 3.2. Appeal rating of pictures.



Appeal rating of pictures (high-calorie foods, low-calorie foods, non-food related household objects, blurred) when fed (solid bar) and fasted (white bar). ** P<0.001 fasted vs. fed.

Figure 3.3. Appeal rating difference.



Difference between appeal ratings for high-calorie and low-calorie foods when eating (fed, solid bar) and skipping (fasted, white bar) breakfast. *** P<0.001 fed vs. fasted.

Table 3.2. Food picture appeal rating.

Picture Appeal Rating	**Fed**	**Fasted**
Absolute rating [a]		
High-calorie foods	2.15 ± 0.19	3.98 ± 0.13 ***
Low-calorie foods	2.39 ± 0.16	3.74 ± 0.15 ***
Household objects	2.41 ± 0.14	2.26 ± 0.16
Blurred	1.91 ± 0.18	1.91 ± 0.19
Chocolate high-calorie foods	2.29 ± 0.22	3.98 ± 0.18 ***
Sweet non-chocolate high-calorie foods	2.27 ± 0.20	3.94 ± 0.15 ***
Savoury high-calorie foods	2.00 ± 0.18	3.99 ± 0.13 ***
Difference in rating between [b]		
High-calorie foods & objects	- 0.26 ± 0.27	1.72 ± 0.23 ***
Low-calorie foods & objects	- 0.03 ± 0.22	1.48 ± 0.23 ***
Chocolate high-calorie foods & objects	- 0.12 ± 0.28	1.72 ± 0.26 ***
Sweet non-chocolate high-calorie foods & objects	- 0.14 ± 0.28	1.68 ± 0.23 ***
Savoury high-calorie foods & objects	- 0.41 ± 0.26	1.73 ± 0.23 ***
High-calorie & low-calorie foods	- 0.23 ± 0.11	0.24 ± 0.12 ***
Chocolate high-calorie & low-calorie foods	- 0.10 ± 0.15	0.25 ± 0.19 **
Sweet non-chocolate high- & low-calorie foods	- 0.12 ± 0.14	0.20 ± 0.11 *
Savoury high-calorie & low-calorie foods	- 0.39 ± 0.10	0.25 ± 0.12 ***
Objects & blurred	0.51 ± 0.21	0.35 ± 0.19

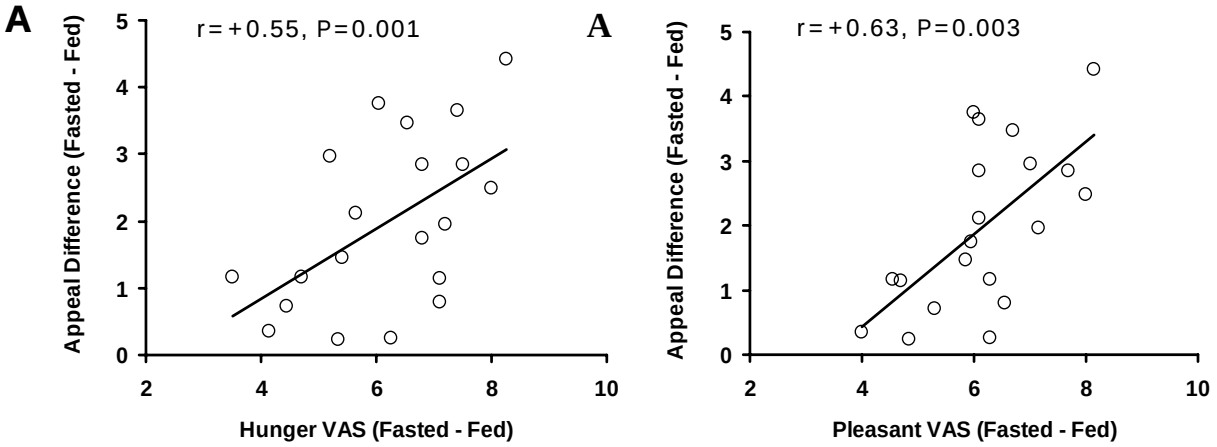
Food picture appeal rating ([a]1=not at all, 2=not really, 3=neutral, 4=a little, 5=a lot; [b]1st category minus 2nd category). Data shown as mean±SEM, n=20 per group: * P<0.01, ** P<0.005, *** P<0.001 fed vs. fasted.

3.2.1. Relationship between Appeal and VAS

The individual differences in the appeal of high-calorie foods (compared to objects) between fasted and fed states also correlated with the individual differences in 'hunger' (r=+0.55, P=0.01) and 'pleasantness to eat' (r=+0.63, P=0.003) (Figure 3.4.).

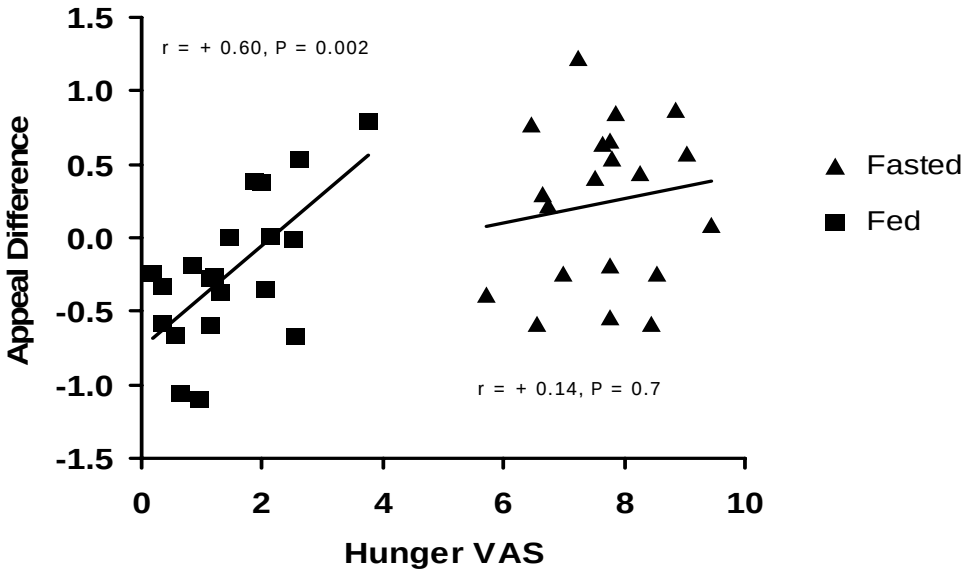
Inividual differences in hunger positively correlated with individual differences in appeal bias towards high-calorie foods in the fed state (r=+0.60, P=0.002) but not in the fasted (r=+0.14, P=0.7) state when viewing high-calorie over low-calorie foods (Figure 3.5.).

Figure 3.4. Correlation between differences in appeal ratings for high-calorie foods and appetite VAS between fasted and fed visits.



Correlations for high-calorie foods (relative to objects) between fasted and fed states and changes in (A) 'hunger' and (B) 'pleasantness to eat' VAS ratings between fasted and fed states.

Figure 3.5. Relationship between absolute hunger ratings and appeal rating bias.



Relationship between VAS hunger ratings and individual differences in appeal ratings when viewing high-calorie over low-calorie foods at the fasted (black triangles) and fed (black squares) visit.

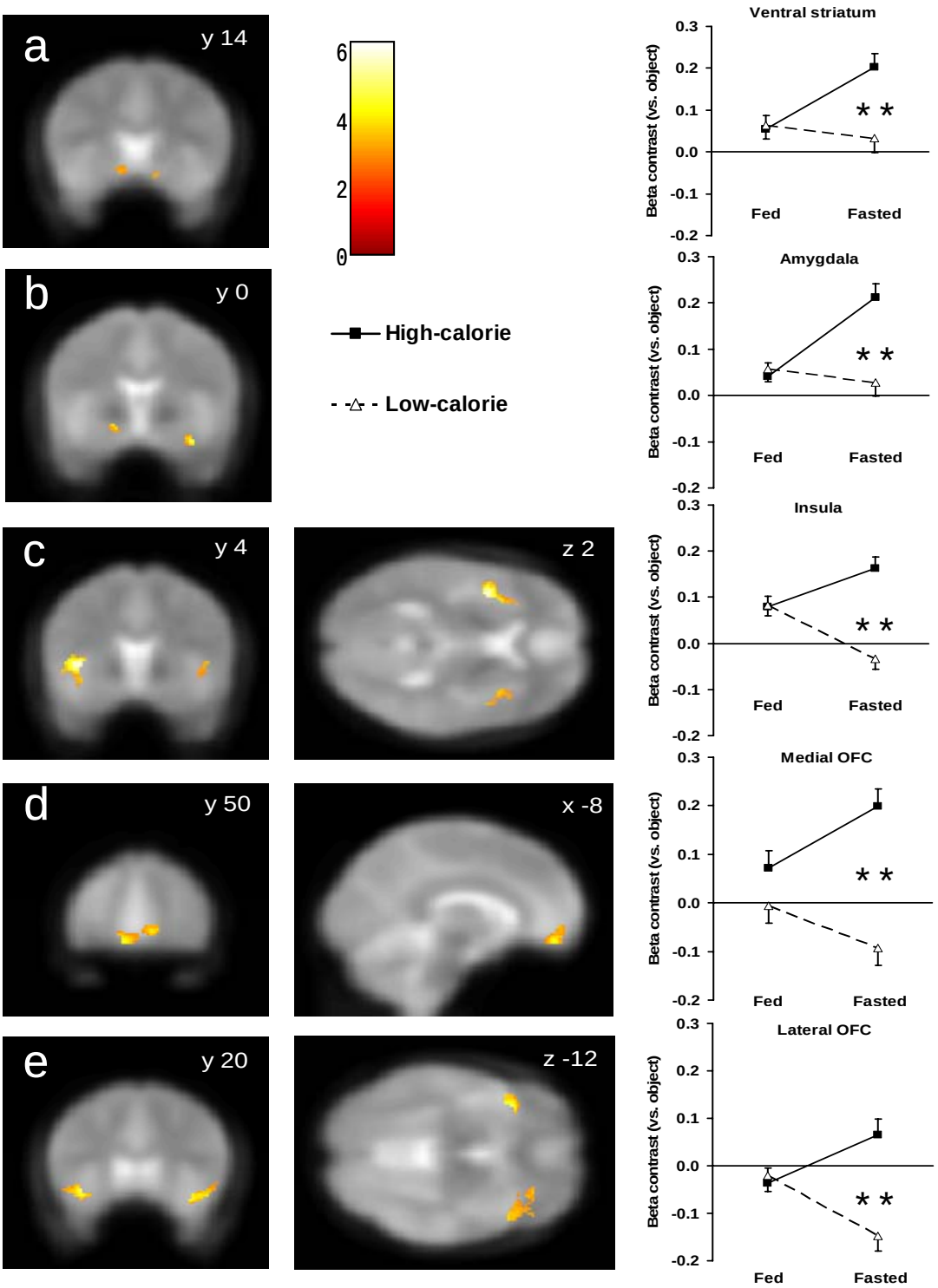
3.3. ROI analysis

The nutritional state and caloric value of the food stimuli had a significant interactive effect on brain activation in each ROI (Figure 3.6., Table 3.4.).

There was no significant activation of the ventral striatum, anterior insula, amygdala, medial OFC and lateral OFC on viewing high-calorie compared to low-calorie foods when fed (P=0.4-0.9). When fasted, there was significantly greater activation bilaterally in each of these ROIs on viewing high-calorie compared to low-calorie foods (P<0.01 to <0.001) (Figure 3.6., Table 3.3.). The magnitude of activation was also significantly greater for high-calorie than low-calorie foods when fasted but not when fed (P=0.03-0.002) (Figure 3.6., Table 3.4.).

There were no voxels within any of the ROIs whose activation showed a significant correlation with BMI for the high-calorie vs. low-calorie food contrast when fasted (surviving SVC when including BMI as a covariate in the GLM).

Figure 3.6. Brain activation in the fed and fasted state to food pictures.



Left hand side: area of (a) ventral striatum, (b) amygdala, (c) anterior insula, (d) medial OFC and (e) lateral OFC showing increased group activation for viewing high-calorie relative to low-calorie foods when fasted. Color bar indicates T values. Activations are thresholded at P<0.005 uncorrected, minimum cluster size 5 voxels, overlaid onto the average EPI scan for all subjects. Co-ordinates are given in standard MNI space. By contrast, no voxels were activated above this threshold when fed (statistical parametric maps not shown). Right hand side: average bilateral activation (contrast beta value relative to objects) in peak activated voxel in each ROI for high-calorie (black square, solid line) and low-calorie (white triangle, dotted line) foods when fed and fasted. ***P<0.001 high-calorie vs. low-calorie.

Table 3.3. Co-ordinates of peak activated voxel with ROIs.

ROI	H	Fed					Fasted				
		x	y	z	v	Z	x	y	z	v	Z
Ventral striatum	L	-	-	-	-	ns	-12	10	-12	55	3.02 * [a]
	R	-	-	-	-	ns	14	14	-14	9	3.03 * [b]
Amygdala	L	-	-	-	-	ns	-18	0	-12	22	3.31** [b]
	R	-	-	-	-	ns	34	2	-24	29	4.47 ** [d]
Insula	L	-	-	-	-	ns	-40	4	2	655	4.45 ** [c]
	R	-	-	-	-	ns	38	16	-14	38	3.80 ** [b]
Medial OFC	L	-	-	-	-	ns	-8	50	-12	144	3.56 ** [b]
	R	-	-	-	-	ns	12	46	-6	215	3.52 ** [b]
Lateral OFC	L	-	-	-	-	ns	-38	20	-12	133	3.91 ** [b]
	R	-	-	-	-	ns	30	34	-16	403	3.90 ** [b]

Acivation within ROI at 2nd level group analysis for high- vs. low-calorie food picture contrast in fasted and fed state (n=20 per group). Results represent co-ordinates of peak statistical voxel (x, y, z in MNI space) for each hemisphere (H), number of voxels within cluster (v, 2 x 2 x 2 mm), and Z statistic using statistical threshold P<0.005, minimum 5 voxel cluster size. Uncorrected: *P= 0.001, **P<0.001; family wise error (FWE) small volume correction (SVC): [a]P=0.05, [b]P<0.05, [c]P<0.005, [d]P<0.001.

Table 3.4. Appeal ratings and magnitude of activation within ROI.

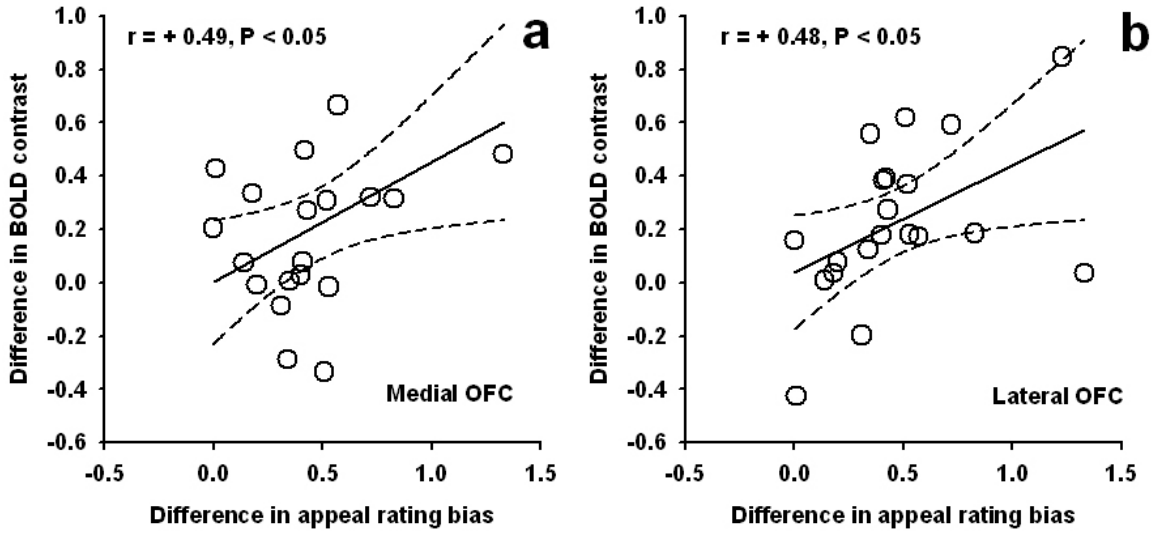
ROI	H	Fed		Fasted	
		High-Calorie	Low-calorie	High-Calorie	Low-calorie
Appeal rating		-0.258 ± 0.270	-0.028 ± 0.222	1.716 ± 0.227 [a] ***	1.475 ± 0.226 ***
Ventral striatum	L	0.044 ± 0.046	0.085 ± 0.057	0.206 ± 0.060 [b]	0.056 ± 0.048
	R	0.064 ± 0.054	0.043 ± 0.055	0.201 ± 0.057 [c]	0.007 ± 0.050
Amygdala	L	0.004 ± 0.047	0.045 ± 0.045	0.323 ± 0.075 [b] **	0.129 ± 0.047
	R	0.078 ± 0.038	0.071 ± 0.041	0.100 ± 0.060 [c]	-0.073 ± 0.069
Insula	L	0.066 ± 0.053	0.044 ± 0.044	0.197 ± 0.050 [c]	-0.032 ± 0.058
	R	0.093 ± 0.054	0.121 ± 0.053	0.128 ± 0.071 [c]	-0.034 ± 0.070 *
Medial OFC	L	0.038 ± 0.111	-0.078 ± 0.114	0.245 ± 0.098 [c]	- 0.131 ± 0.110
	R	0.107 ± 0.060	0.067 ± 0.053	0.153 ± 0.045 [c]	-0.055 ± 0.051
Lateral OFC	L	-0.088 ± 0.104	- 0.045 ± 0.105	0.044 ± 0.079 [b]	-0.239 ± 0.090
	R	0.014 ± 0.060	0.002 ± 0.063	0.087 ± 0.065 [c]	- 0.055 ± 0.067

Appeal rating difference (compared to object pictures) and magnitude of activation at peak voxel within ROI for each hemisphere (H) depending on nutritional state (fasted vs. fed) and food picture (high-calorie vs. low-calorie). Co-ordinates of peak voxel determined from high-calorie vs. low-calorie foods contrast when fasted. 2-way repeated measures ANOVA using nutritional state and food picture as factors, and ß values extracted at peak voxel for individual subject contrast estimates (vs. objects) for high-calorie and low-calorie foods (n=20 per group): [a]P<0.05, [b]P<0.01, [c]P<0.001 for high-calorie vs. low-calorie foods within fed or fasted; *P<0.05, **P<0.01, ***P<0.001 for fed vs. fasted within low-calorie or high-calorie.

3.3.1. Relationship between brain activation and food appeal

ROI activations were correlated with subjective appeal scores in individual subjects to examine the relationship between the effect of nutritional state (fed or fasted) on brain reward systems and food appeal. The increase in appeal rating bias for high-calorie over low-calorie foods with fasting was positively correlated with the change in activation in both the medial OFC (r=+0.49, P=0.029), and lateral OFC (r=+0.48, P = 0.034) for the high-calorie vs. low-calorie food contrast with fasting (Figure 3.7.). However no significant correlations were seen for any of the other ROIs.

Figure 3.7. Relationship between brain activation and food appeal.



Difference in activation (contrast beta value) within lateral (a) and medial (b) OFC (average bilateral) on viewing high-calorie vs. low-calorie foods between fasted and fed states correlates with difference in appeal ratings for high-calorie relative to low-calorie foods between fasted and fed states.

3.4. Whole brain analysis

Additional brain areas were activated when viewing high-calorie than low-calorie foods when fasted but only few areas when fed using whole brain analysis (Table 3.5., 3.6.). More reward areas were activated at the fasted visit but not at the fed Saline visit. Visual areas were activated at both visits.

Table 3.5. Whole brain analysis in fasted state.

Region	H	*Z*	v	x	y	z
Cerebellum	L	5.25 [a b]	2014	-36	-46	-32
Cerebellum	R	3.94 [a]	50	18	-74	-24
Inferior temporal/cerebellum	R	5.07 [a b]	2577	28	-40	-30
Posterior cingulated	L	4.85 [a]	176	-8	-24	50
Ventroanterior insula	L	4.49 [a]	928	-36	10	-16
Ventroanterior insula	R	4.47 [a]	177	34	2	-24
Ventroposterior insula	R	4.45 [a]	92	38	-10	-14
Pre-frontal/orbitofrontal cortex	L	4.30 [a]	84	-26	48	34
Premotor cortex/dorsal cingulate	L/R	4.24 [a]	492	2	2	70
Dorsolateral pre-frontal cortex	L	4.15 [a]	19	-24	36	54
Premotor/motor cortex	L	4.13 [a]	329	-46	4	54
STG/temporal pole	L	4.06 [a]	44	-38	18	-28
Motor cortex	L	3.98 [a]	9	-16	-8	54
Posterior hippocampus	L	3.97 [a]	33	-20	-38	2
Hippocampus (posterior)/lingual gyrus	L	3.81 [a]	129	-14	-46	-4
Somatosensory cortex	L	3.97 [a]	195	-34	-40	64
Visual cortex (cuneus)	L	3.94 [a]	305	-8	-78	0
Orbitofrontal cortex (medial)	R	3.90 [a]	48	30	34	-16
Orbitofrontal cortex (medial)	L	3.89 [a]	92	-20	66	6
Orbitofrontal cortex (lateral)	R	3.86 [a]	42	42	40	-2
Somatosensory	R	3.86 [a]	30	54	-24	44
Orbitofrontal cortex (medial)	L	3.75 [a]	59	-14	56	-8
Dorsolateral pre-frontal cortex	R	3.73 [a]	20	26	34	54
Dorsal medial pre-frontal cortex	R	3.63 [a]	10	10	28	62
Lateral occipital/inferior parietal lobule	L	3.72 [a]	19	-54	-72	18
Motor cortex	R	3.72 [a]	28	56	-10	24
Cerebellum	R	3.68 [a]	40	12	-54	-52
Premotor cortex	R	3.66 [a]	65	58	14	34
Motor cortex	L	3.59 [a]	16	-20	-8	74
Insula/parietal operculum	L	3.57 [a]	11	-50	-24	24
Dorsal anterior cingulate	R	3.66 [a]	147	8	20	30
Dorsal anterior cingulate	L	3.56 [a]	28	-10	14	36

Activation within whole brain at 2nd level group analysis for high-calorie vs. low-calorie food picture contrast when fasted (n=20 per group). Results represent co-ordinates of peak statistical voxel (x, y, z in MNI space) within each cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm), and *Z* statistics using statistical threshold P<0.001, minimum 5 voxel cluster size. Correction for multiple corrections: [a]FDR P<0.05; [b]FWE P<0.05.

Table 3.6. Whole brain analysis in fed state.

Region	H	*Z*	v	x	y	z
Central operculum/insula	R	4.34	13	36	-8	18
Visual cortex	R	4.08	116	50	-62	-12
Anterior fusiform cortex/ temporo-occipital boundary	L	3.63	51	-38	-50	-14
Visual cortex	L	3.50	44	-48	-66	-2

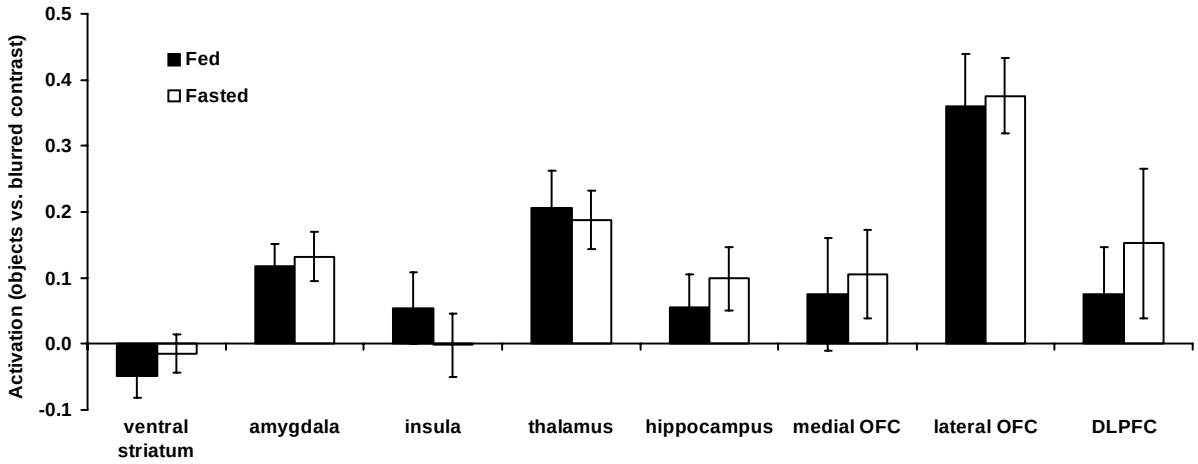
Activation within whole brain at 2nd level group analysis for high-calorie vs. low-calorie food picture contrast when fed (n=20 per group). Results represent co-ordinates of peak statistical voxel (x, y, z in MNI space) within each cluster for each hemisphere (H), number of voxels within cluster (v), and *Z* statistics using statistical threshold P<0.001, minimum 5 voxel cluster size. No peak voxels survived correction for multiple corrections: FDR or FWE P<0.05.

3.5. Control activation

3.5.1. Object vs. blurred picture contrast

The nutritional state had no significant effect on the activation in each ROI when viewing household objects vs. blurred pictures (P=0.3-0.8) (Figure 3.8.).

Figure 3.8. Brain activation when viewing objects vs. blurred pictures.

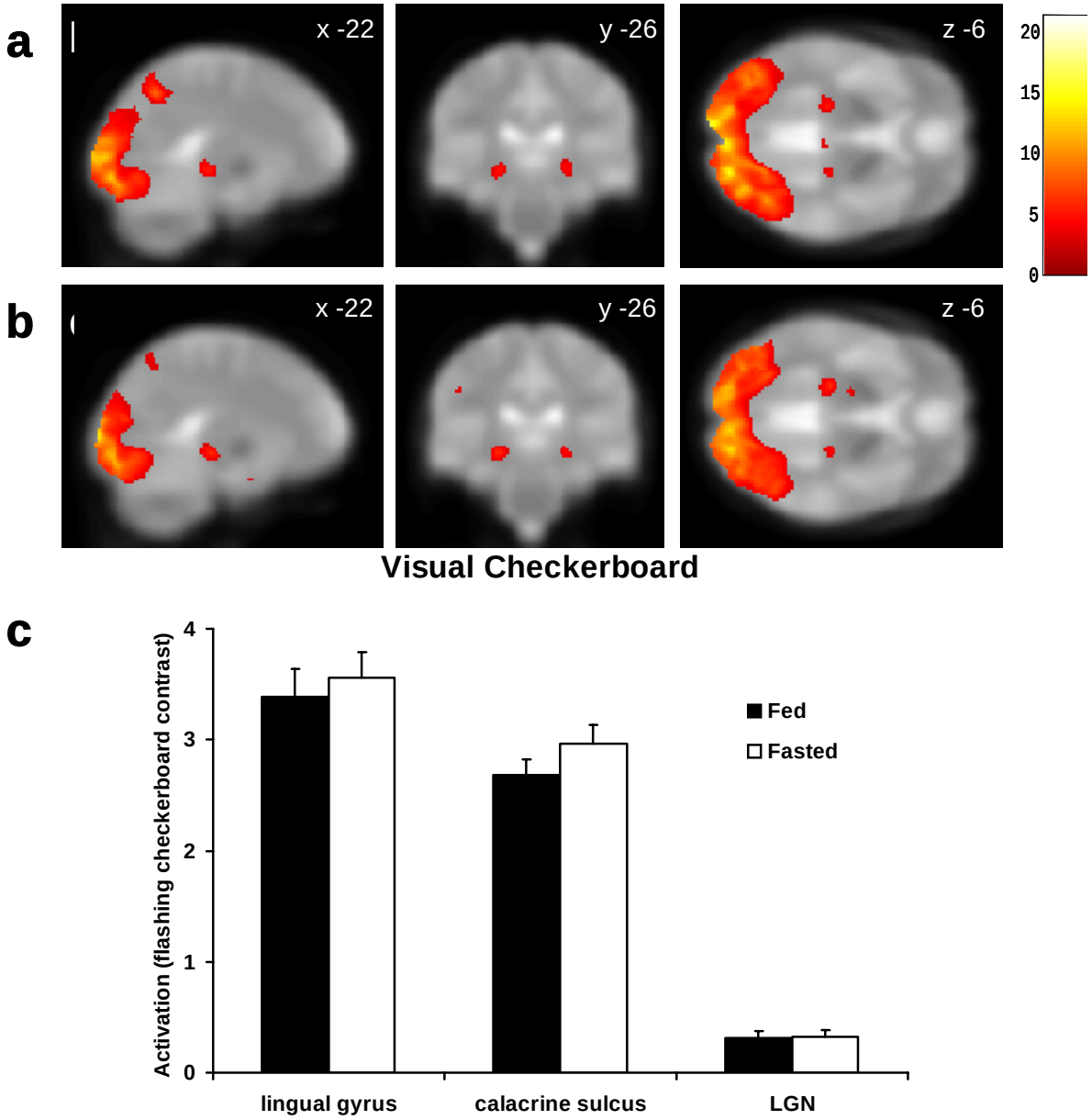


Activation (contrast beta value) in peak activated voxel (from high-calorie vs. low-calorie foods contrast in fasted state) within each ROI (average bilateral) on viewing objects relative to blurred pictures when fed (solid bar) and fasted (white bar). *P<0.05 fasted vs. fed.

3.5.2. Visual checkerboard

The occipital cortex and lateral geniculate nucleus showed no significant difference in activation on viewing a 4Hz flashing checkerboard between fed and fasted states (P=0.1-0.9) (Figure 3.9. a-c, Table 3.7.).

Figure 3.9. Brain activation when viewing 4Hz visual checkerboard.



(a,b) Group brain activation in occipital cortex and lateral geniculate nucleus when viewing flashing checkerboard (relative to fixation cross) when (a) fed and (b) fasted. Colour bar indicates T values. Activations are thresholded at *P<0.05 uncorrected, minimum cluster size 5 voxels. Co-ordinates are given in standard MNI space. (c) Activation (contrast beta value) in peak activated voxel (from fasted state) within each ROI (lingual gyrus, calacrine sulcus, lateral geniculate nucleus (LGN), average bilateral) on viewing flashing checkerboard (relative to fixation cross) when fed (solid bar) and fasted (white bar). *P<0.05 fasted vs. fed.

Table 3.7. Co-ordinates of activated voxels when viewing 4Hz flashing checkerboard.

ROI	H	Fed					Fasted				
		x	y	z	v	*Z*	x	Y	z	v	*Z*
Calacrine	L	-10	-102	-10	734	7.67 ** [d]	-12	-98	-10	749	6.98 ** [d]
sulcus	R	22	-94	-4	423	6.90 ** [d]	22	-94	-2	444	6.98 ** [d]
Lingual	L	-14	-98	-12	558	6.35 ** [d]	-14	-98	-12	623	6.58 ** [d]
gyrus	R	24	-94	-8	797	6.35 ** [d]	10	-92	-10	814	6.82 ** [d]
Lateral	L	-20	-28	-4	98	4.13 ** [c]	-22	-26	-6	106	4.33 ** [c]
geniculate n.	R	24	-28	-2	67	3.91 ** [b]	24	-26	-6	31	3.55 ** [a]

Activation within regions of interest (ROI) at 2nd level group analysis for flashing checkerboard vs. fixation cross contrast in fasted and fed state (n=20 per group). Results represent co-ordinates of peak statistical voxel (x, y, z in MNI space) for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm), and *Z* statistic using statistical threshold P<0.005, minimum 5 voxel cluster size. Uncorrected: **P<0.001; FWE small volume correction (SVC): [a]P<0.05, [b]P<0.01, [c]P<0.005, [d]P<0.001.

3.5.3. Reaction time for appeal rating

There was no significant difference in the reaction time for appeal rating of any of the picture categories between the fasted and fed state (P=0.4-0.9) (Table 3.8.), suggesting the absence of non-specific effect of nutritional state on the picture viewing and rating task.

Table 3.8. Picture rating reaction time.

Picture Rating Reaction Time (msec)	Fed	Fasted
High-calorie foods	1271 ± 57	1282 ± 48
Low-calorie foods	1318 ± 57	1292 ± 51
Household objects	1280 ± 50	1207 ± 56
Blurred	1058 ± 69	1024 ± 69

Data shown as mean ± SEM, n=20 per group.

3.6. Relationship with questionnaire scores

3.6.1. Behavioural Activation and Inhibition Scale (BAS / BIS)

Mean ± SEM (range) questionnaire scores were as follows: BIS: 21.8 ± 0.86 (15-28); BAS drive: 10.6 ± 0.49 (range 8-15); BAS fun seeking: 12.2 ± 0.61 (7-16); BAS reward responsiveness: 16.9 ± 0.45 (14-20).

BAS-drive scores were significantly positively correlated with activation to high-calorie relative to low-calorie foods in the ventral striatum (x 12, y 10, z -16: 57 voxels, SVC FWE P=0.025; r=+0.68, P<0.001), but no other ROIs (Figure 3.10. a). BAS-drive did not differ between genders (male 10.3 ± 0.5 vs. female 10.9 ± 0.9, P=0.56) and the positive correlation between ventral striatum activation and BAS-drive remained significant (P=0.001), when including gender and visit number as independent variables (though they were not significant variables themselves: P=0.7 and 0.4 respectively).

3.6.2. Dutch Eating Behaviour Questionnaire (DEBQ)

Mean ± SEM (range) questionnaire scores were as follows: DEBQ-restraint 19.8 ± 1.1 (11-32); DEBQ-external: 34.8 ± 0.87 (27-43).

DEBQ-restraint score was positively correlated with activation to high-calorie relative to low-calorie foods in the DLPFC (-32, 54, 28: 9 voxels, P<0.001, SVC FWE P=0.04; r=+0.69, P<0.001) and dorsal ACC (-6, 10, 36: 12 voxels, P=0.001, SVC FWE P=0.04; r=+0.63, P=0.003) (Figure 3.10. b,c)..

By contrast, DEBQ-restraint was negatively correlated with activation for high-calorie relative to low-calorie foods in the amygdala (-30, -4, -22: 4 voxels, P=0.001, SVC FWE P=0.04; r=-0.53, P=0.015), and left lateral OFC (-38, 28, -22: 243 voxels, P<0.001, SVC FWE P=0.003; r=-0.83, P<0.001) (Figure 3.10. d,e).

There was a tendency for a negative correlation of DEBQ-restraint with activation in the right lateral OFC but it did not survive SVC for multiple comparisons (2 clusters: 38, 36, -10: 7 voxels, P<0.001, SVC FWE P=0.16; r=-0.68, P=0.001; and 44, 30, -18: 28 voxels, P=0.001, SVC FWE P=0.26; r=-0.64, P=0.002).

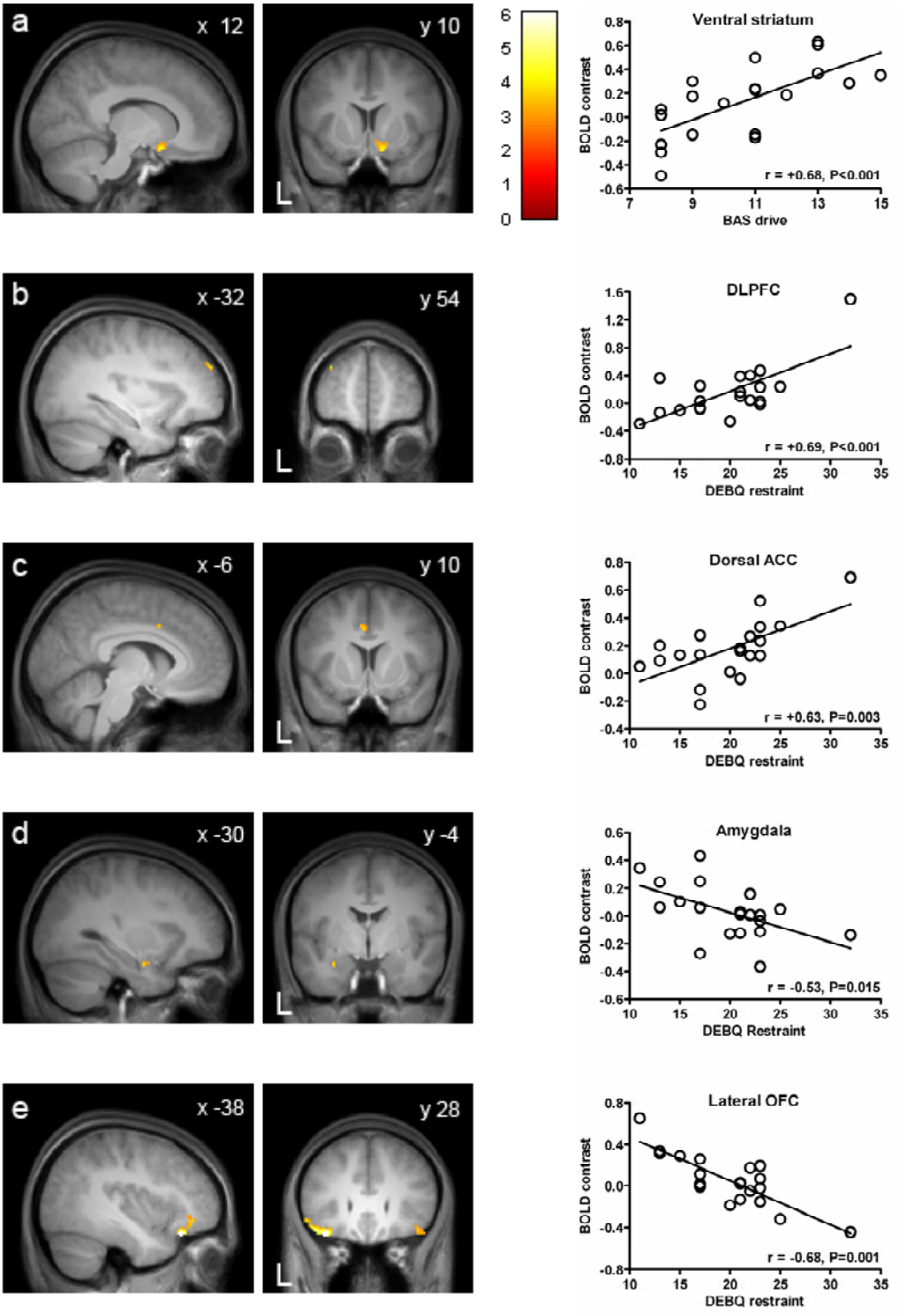
Dietary restraint did not differ significantly between genders (male 18.0 ± 1.4 vs. female 21.6 ± 1.5, P=0.10). Furthermore the negative correlations between dietary restraint and amygdala and left lateral OFC activation (P=0.015 and <0.001 respectively), and the positive correlations with DLPFC and dorsal ACC activation (P=0.004 and 0.005 respectively), remained significant when including gender and visit number as independent variables, though they were not significant variables themselves (gender: P=0.3-1.0, visit number: P=0.1-0.9).

Examination of the spread of dietary restraint between subjects, revealed one outlier with particularly high dietary restraint (score of 32) (Figure 3.10. b-c). Exclusion of this outlier from the linear regression analysis did not qualitatively alter the correlations between DEBQ-restraint and activation to high-calorie relative to low-calorie foods in the ROIs although the results for DLPFC and dorsal ACC did not quite reach statistical significance (amygdala: r=-0.52, P=0.02; left lateral OFC: r=-0.78, P<0.001; DLPFC: r=+0.45, P=0.05; dorsal ACC: r=+0.43, P=0.06).

There was no significant correlation between BAS-drive nor DEBQ-restraint with activation to viewing object compared to blurred pictures in any ROI (r=-0.02 to +0.31, P=0.2-0.9). There was no significant correlation between the difference in appeal rating between high-calorie and low-calorie foods and BAS-drive (r=+0.13, P=0.6) nor DEBQ-restraint (r=+0.10, P=0.7).

There was no significant correlation seen between activation in any ROI and DEBQ-external eating behaviour when including the latter as an independent variable in the GLM.

Figure 3.10. Relationship between brain activation and questionnaire scores.



Left hand side: Statistical parametric maps indicating significant group correlations between brain activation in *a priori* regions of interest when viewing high-calorie compared to low-calorie food pictures when fasted, and (a) reward sensitivity (BAS drive) in ventral striatum, or dietary restraint in (b) DLPFC, (c) dorsal ACC, (d) amygdala and (e) lateral OFC. Colour bar indicates T values. Activations are thresholded at P<0.005 uncorrected, minimum cluster size 3 voxels, overlaid onto the average EPI scan for all subjects. Co-ordinates are given in standard MNI space. n=20 per group. Right hand side: Correlation of individual differences in brain activation for high-calorie vs. low-calorie food contrast at peak voxel in (a) ventral striatum, (b) DLPFC, (c) dorsal ACC, (d) amygdala and (e) lateral OFC, and (a) reward sensitivity (BAS drive) or (b-e) dietary restraint for each individual subject (n=20).

3.7. Summary results fed – fasted fMRI study

In twenty non-obese healthy adults, fasting selectively increased activation to pictures of high-calorie over low-calorie food in the ventral striatum, amygdala, anterior insula, and medial and lateral orbitofrontal cortex (OFC). Furthermore, fasting enhanced the subjective appeal of high-calorie more than low-calorie foods, and the change in appeal bias towards high-calorie foods was positively correlated with medial and lateral OFC activation.

Individual differences in reward drive positively correlated with activation in the ventral striatum when viewing high-calorie compared to low-calorie foods. Dietary restraint positively correlated with activation in the dorsolateral prefrontal cortex (DLPFC) and dorsal anterior cingulate cortex (ACC) when viewing high-calorie compared to low-calorie foods, but negatively correlated with activation in the amygdala and lateral orbitofrontal cortex (OFC).

3.8. Limitations of fed – fasted fMRI study

For the fed visit, the size of the breakfast consumed by the subject was variable. Subjects were told to have a filling breakfast of their choice one hour before they arrived for fMRI scanning. The breakfast intake was 724 ± 59 kCal (mean ± SEM). In order to compare brain activity between subjects it is important to have the same macronutrient composition consumed by each of them. For that reason, questionnaire scores were not correlated to brain activation in this study.

The subject's appetite on the study day could also be influenced by food intake the night prior to the study day and irregular eating habits habits (not eating meals at typical times or not eating breakfast). Indeed, research underlined that the effect of skipping breakfast on subsequent intake depends on the usual breakfast habits (Schlundt et al., 1992). This study did not control for food intake nor assess participant's normal eating habits by using dietary records. However, only one subject reported to eat breakfast only 3 days per week, 2 eat breakfast 4 days a week, and 70% eat breakfast every day.

Females were not scanned in the same phase of their menstrual cycle. There is evidence for hormone-induced changes in food selection in the luteal phase of the menstrual cycle. Thus, food intake as well as fMRI responses to rewarding stimuli can vary over the menstrual cycle in response to changes in ovarian steroid hormone levels (Buffenstein et al., 1995; Dye and Blundell, 1997; Dreher et al., 2007; Van Vugt, 2009, Frank et al., 2010).

In this study, there was no blood collection to measure gut hormones involved in appetite regulation. It is therefore impossible to relate changes in brain activation in the fed and fasted state to changes in anorexigenic and orexigenic gut hormones.

Geometric distortions caused by inhomogenities in the magnetic field can reduce brain activation in certain areas such as the frontal cortex due to vicinity to air-filled sinuses. It is possible to correct the inhomogenities by using field maps (Huettel, 2004). In this study, field maps have not been acquired due to delayed sequence development. Signal such as activation in the orbitofrontal cortex may have been lost in this study which then could have biased the significance of the results.

Studies in animals and humans have shown that anxiety and stress can alter feeding behavior (Adam and Epel, 2007; Chandarana et al., 2009; Born et al., 2010). The participants in this study had no dummy visit to acclimatize to the study and scanning, although many subjects had previously experienced MRI scans. Therefore, stress and anxiety may have influenced the study results and especially the activation in important brain reward systems. Nevertheless, the order of the study visits was counterbalanced and so the differences between the fed and fasted visits should remain the same even when including a sham visit.

In the subsequent Ghrelin fMRI study, these limitations were corrected: every subject had the same breakfast, subjects were instructed to eat the same dinner the night before each study visit and to keep a food diary of intake the day before the study to improve and check for similar prior food intake. Blood was collected throughout the study morning to

measure gut hormones. Field maps were acquired during each fMRI scanning session. A dummy visit was also included in the ghrelin fMRI study. The following chapter shows the importance of dummy visits in feeding studies.

Chapter 4

REPRODUCIBILITY OF EATING BEHAVIOUR STUDIES

4.1. General limitations of eating behaviour studies

Measuring eating behaviour can be very challenging. Self-report measures of appetite such as Visual Analaogue Scale (VAS) have been previously questioned in terms of their reproducibility. However, VAS are easily used and translated, and appear to be valid, sensitive and unbiased measurement tools for regular use in standard practice and have been therefore used in this Ghrelin fMRI study (Blundell et al., 2010).

Furthermore, appetite studies are mostly used within laboraties and therefore not performed under real-life circumstances which could influence eating behaviour (Blundell et al., 2010). For example, venepuncture to withdraw bloods on different time points has been shown to acutely increase circulating cortisol concentrations because of stress-induced cortisol release related to the novelty of the first study visit (Chandarana et al., 2009). The effects of unfamiliar study protocols and venepuncture could alter gut hormone concentrations or appetite. Several research studies including the Ghrelin fMRI study have adopted the standard protocol of waiting for subjects to "recover" after cannula insertion prior to commencing assessments (Chandarana et al., 2009). Additionally, feeding behaviour studies in animals and humans have shown that anxiety and stress can alter *ad libitum* food intake. Functional MRI studies have previously demonstrated that stress can alter responses in brain reward systems (Adam and Epel, 2007; Chandarana et al., 2009; Blundell et al., 2010; Born et al., 2010). In the Ghrelin fMRI study, participants had one dummy visit to acclimatize to the hospital environment, research study protocol and MRI scanning protocol. The following chapter will analyse different measures of appetite and fMRI responses comparing the 2 identical fasted study fMRI visits.

4.2. Fasted Dummy versus Fasted Saline visit (sub-study, ghrelin fMRI study)

The initial Fasted Dummy visit has been compared with the subsequent Fasted Saline visit for the following parameters: VAS hunger, fullness, pleasantness, stress, anxiety, and sickness VAS ratings, serum cortisol and prolactin levels, lunch intake and pleasantness, fMRI appeal rating and fMRI BOLD signal to food pictures. Each measure was also used to

examine the correlation between the two fasted visits. Study characteristics and other comparisons are shown in the following table (Table 4.1.). There was a median interval of 18 days between the 2 visits (range 6-151 days). Percentage intake of fat was significantly less at supper prior to the study visit and intake of carbohydrates significantly increased at the Fasted Dummy visit compared to the Fasted Saline visit ($P<0.05$). The time of injection to the first food picture run and time of injection to lunch was significantly longer at the Fasted Dummy visit ($P<0.05$).

Table 4.1. Subject characteristics.

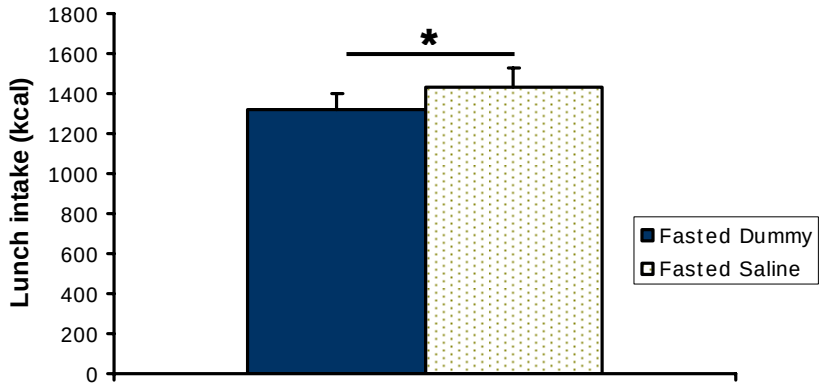
	Fasted Dummy	**Fasted Saline**
N	17	17
Age (*years)*	24.6 ± 1.6	24.6 ± 1.6
Height (*m)*	1.80 ± 0.0	1.80 ± 0.0
Weight (k*g)*	76.1 ± 3.7	76.3 ± 3.6
BMI ($kg/m^{2)}$	24.2 ± 0.7	24.1 ± 0.7
Body fat (*%)*	16.6 ± 1.7	17.1 ± 1.6
LBM (*kg)*	63.7 ± 3.5	64.6 ± 3.3
Day of menstrual cycle *(females)*	5.2 ± 1.2	5.2 ± 1.2
Food intake at supper prior to study		
Energy *(kcal)*	962 ± 135	1012 ± 179
Protein *(%)*	19 ± 3	21 ± 2
Fat *(%)*	30 ± 4	40 ± 5*
Carbohydrate *(%)*	51 ± 5	38 ± 5*
Food intake whole day prior to study		
Energy *(kcal)*	2142 ± 191	2645 ± 397
Protein *(%)*	16 ± 1	17 ± 1
Fat *(%)*	29 ± 3	37 ± 4
Carbohydrate *(%)*	55 ± 3	46 ± 3
Time fasting since supper (hrs)	17.4 ± 0.3	17.1 ± 0.3
Time sleep (hrs)	7.4 ± 0.2	7.0 ± 0.3
Time injection to first food fMRI scan *(hrs)*	0.8 ± 0.1	0.6 ± 0.0*
Time injection to lunch *(hrs)*	1.7 ± 0.1	1.6 ± 0.0*
Absolute Head motion *(mm)*	0.4 ± 0.1	0.2 ± 0.0

n=17 per group, Mean ± SEM, *P<0.05.

4.2.1. VAS appetite scores and food intake

Analysis of VAS ratings revealed that there was no significant difference between the Fasted Dummy and the Fasted Saline visit in hunger ratings (P=0.65) but there was a trend for fullness to be greater (P=0.07), and pleasantness-to-eat to be lower (P=1.0) on the Fasted Dummy visit (Figure 4.1.). There was an 8% increase in energy intake (P=0.04) at the Fasted Saline visit compared to the Fasted Dummy visit (Figure 4.2., Table 4.2.). There was no significant difference in lunch tastiness (P=0.18) but a trend for lunch pleasantness to be greater at the Fasted Saline visit (P=0.07) (table 4.2.).

Figure 4.2. *Ad libitum* lunch intake.



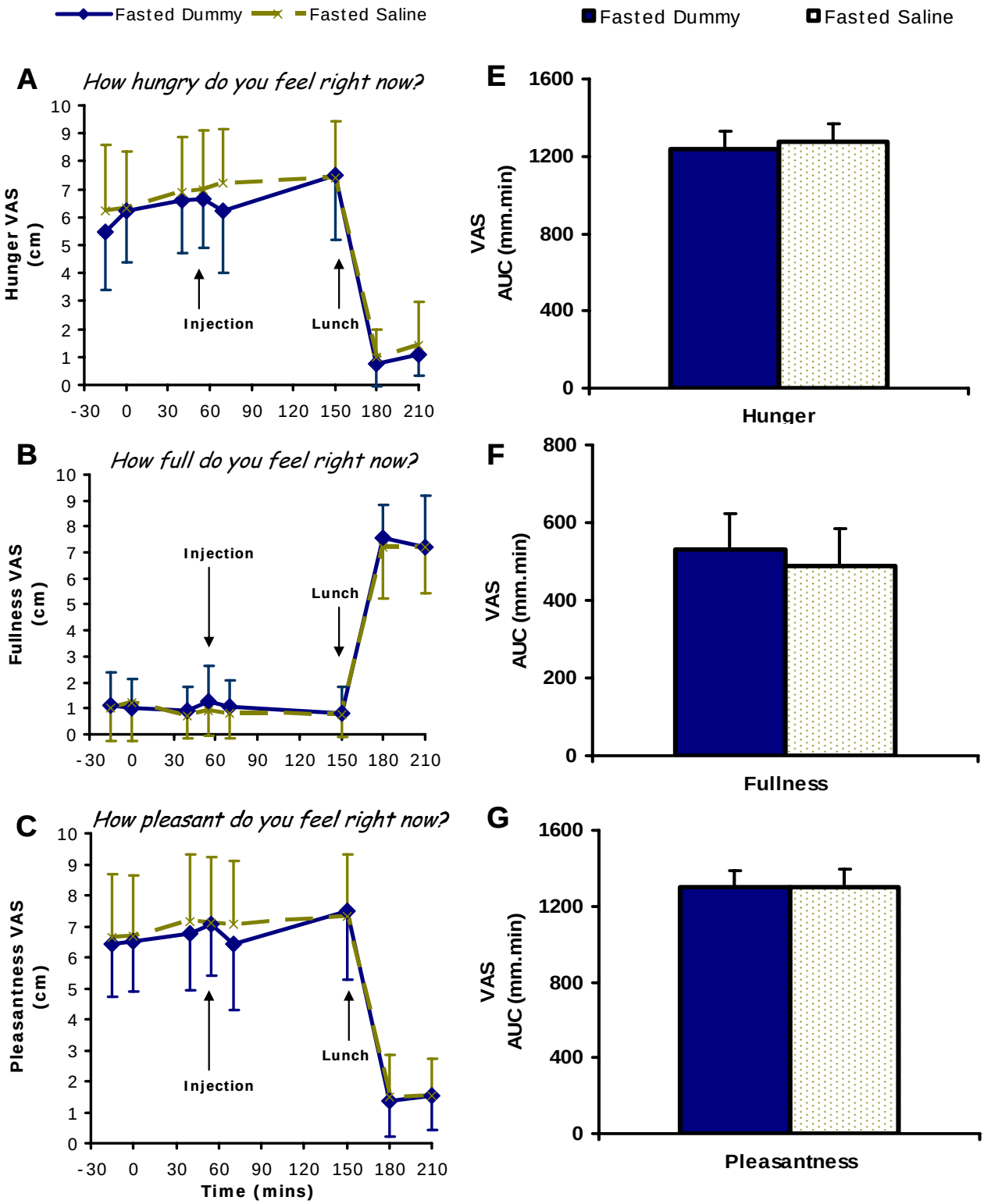
Ad libitum lunch intake for fasted visits (Fasted Dummy, dark blue bar and Fasted Saline, green dotted bar), n=17 per group. Data are expressed as mean ± SEM, *P<0.05.

Table 4.2. *Ad libitum* lunch intake for fasted visits.

	Fasted Dummy	Fasted Saline
Lunch intake		
Total lunch food (*kcal)*	1320 ± 81	1428 ± 101*
Lunch tastiness	5.9 ± 0.3	6.4 ± 0.3
Lunch pleasantness	6.0 ± 0.4	6.8 ± 0.3

n=17 per group, data shown as mean ± SEM, *P<0.05 Fasted Dummy vs. the Fasted Saline.

Figure 4.1. VAS appetite scores.

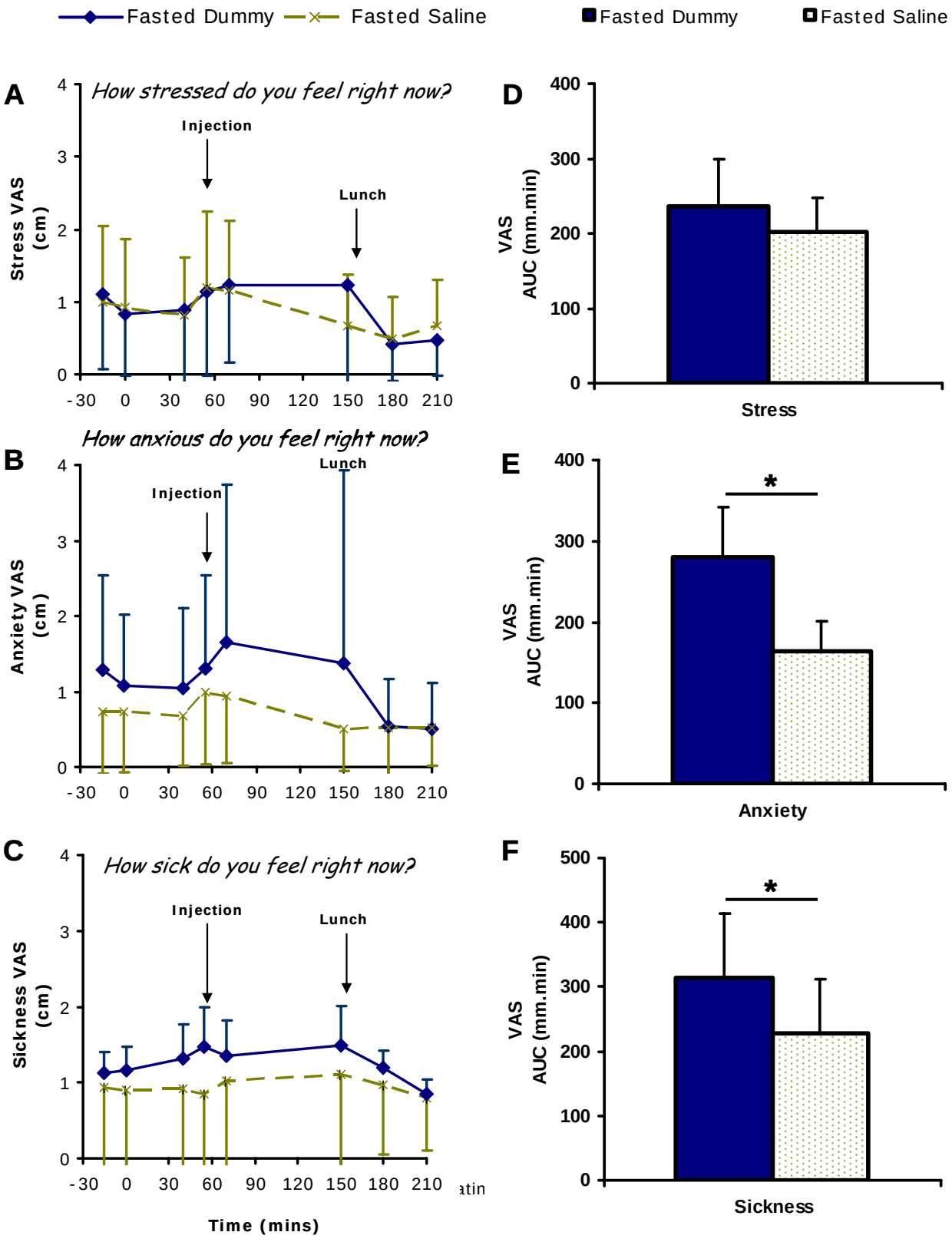


Absolute VAS scores for hunger, fullness and pleasantness to eat ratings for fasted visits (Fasted Dummy, dark blue bar and Fasted Saline, green dotted bar), (A-C) time course of VAS scores, (D-F) mean AUC for each VAS score. n=15 per group (2 subjects have been excluded from the analysis because of incomplete data sets), data are expressed as mean ± SEM, *P<0.05.

4.2.2. Stress ratings

Analysis of VAS stress ratings revealed that there was no significant difference between the Fasted Dummy and the Fasted Saline visit in stress (P=0.26), but anxiety (P=0.01), and sickness (P=0.01) ratings were significantly higher at the Fasted Dummy visit (Figure 4.3.).

Figure 4.3. VAS stress scores.

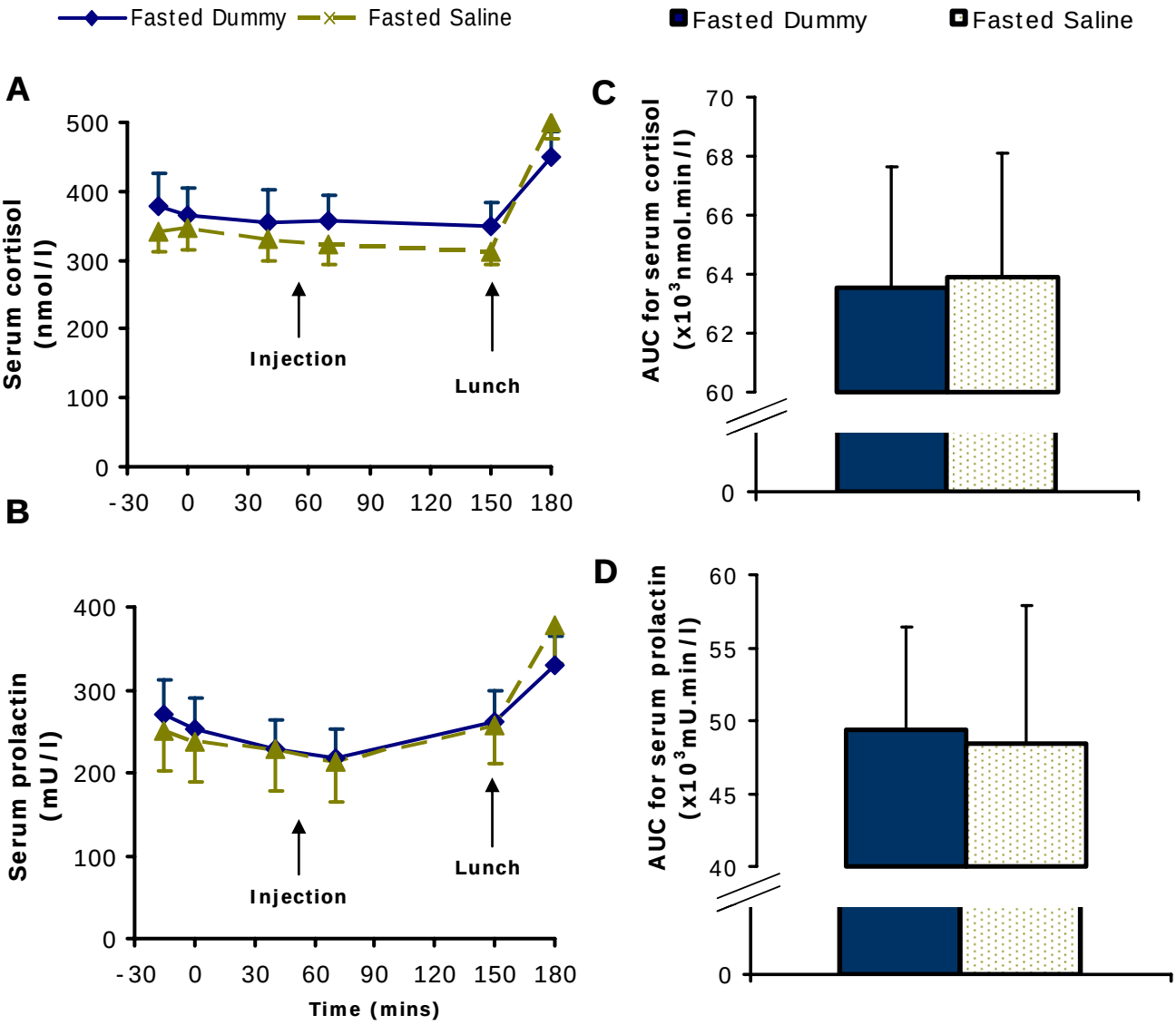


Fasted Saline, green dotted bar), (A-C) time course of VAS stress scores, (D-F) AUC for each VAS stress score, n=15 per group (2 subjects have been excluded from the analysis because of incomplete data set), data are expressed as mean ± SEM, *P<0.05.

4.2.3. Serum stress hormones

There was no significant difference between the Fasted Dummy and the Fasted Saline visit in serum cortisol (P=0.95), or prolactin (P=0.58) levels (Figure 4.4.).

Figure 4.4. Cortisol and prolactin serum levels for fasted visits.



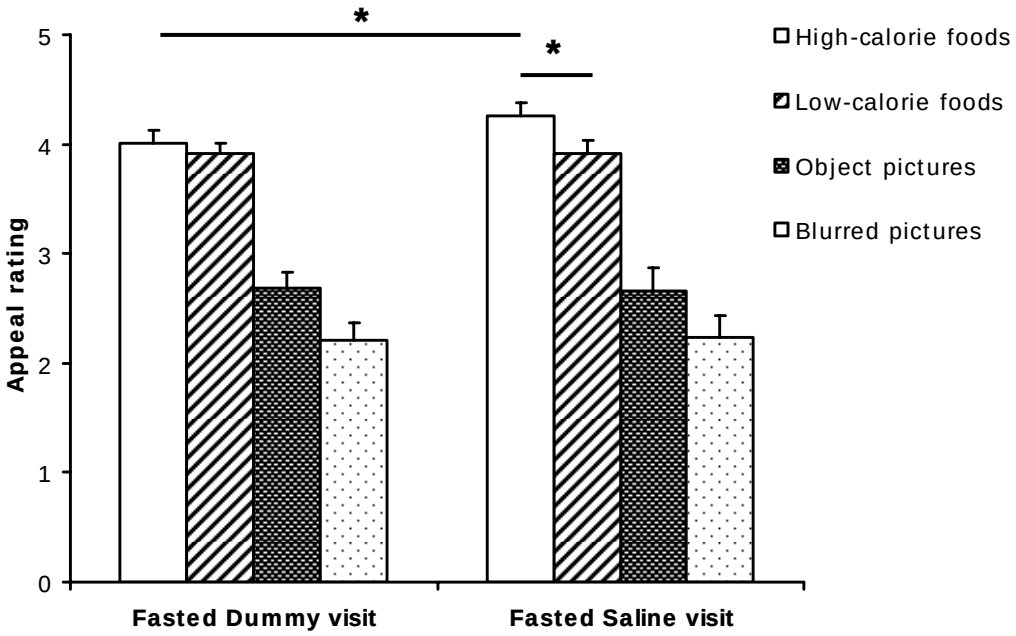
Cortisol and prolactin serum levels for fasted visits (Fasted Dummy, dark blue bar and Fasted Saline, green dotted bar), (A, B) time course of serum cortisol and prolactin levels, (C, D) AUC for each serum stress hormone, n=17 per group, data are expressed as mean ± SEM, *P<0.05.

4.2.4. Food picture appeal rating

High-calorie foods were less appealing at the Fasted Dummy visit compared to the Fasted Saline visit (P<0.05) (but not low-calorie food, object and blurred pictures (P=0.77-0.96) (Figure 4.5.; Table 4.3.). High-calorie foods were more appealing relative to low-calorie foods at the Fasted Saline (P=0.01), but not at the Fasted Dummy visit (P=0.48) (Figure 4.5.; Table 4.3.). These differences were seen for both chocolate and sweet non-chocolate (both P<0.005) but not savoury high-calorie foods (P=0.13) (Table 4.3.).

There was a significantly greater appeal rating for the high-calorie (chocolate, sweet non-chocolate and savoury) and low-calorie foods relative to object pictures at both the Fasted Dummy and the Fasted Saline visit (P<0.001) (Figure 4.5.; Table 4.3.). There was no significant difference in the appeal of object relative to blurred pictures at either the Dummy Saline or the Fasted Saline visit (P=0.78) (Table 4.3.).

Figure 4.5. Picture appeal rating.



Appeal rating of pictures (high-calorie foods: white bar, low-calorie foods: striped bar, non-food related household objects: black bar, blurred pictures: dotted bar), Fasted Dummy vs. Fasted Saline visit, n=17 per group, data are expressed as mean ± SEM, *P<0.05.

Table 4.3. Picture appeal rating.

Picture Appeal Rating	**Fasted Dummy visit**	**Fasted Saline visit**
Absolute rating [a]		
High-calorie foods	4.01 ± 0.12	4.28 ± 0.11*
Low-calorie foods	3.91 ± 0.09	3.91 ± 0.11
Household objects	2.68 ± 0.15	2.62 ± 0.20
Blurred	2.20 ± 0.17	2.18 ± 0.20
Chocolate high-calorie foods	4.03 ± 0.13	4.30 ± 0.11***
Sweet non-chocolate high-calorie foods	3.99 ± 0.13	4.28 ± 0.11***
Savoury high-calorie foods	3.99 ± 0.16	4.21 ± 0.16
Difference in rating between [b]		
High-calorie foods & objects	1.33 ± 0.18	1.60 ± 0.23
Low-calorie foods & objects	1.23 ± 0.18	1.26 ± 0.25
Chocolate high-calorie foods & objects	1.35 ± 0.18	1.63 ± 0.19*
Sweet non-chocolate high-calorie foods & objects	1.32 ± 0.20	1.26 ± 0.24*
Savoury high-calorie foods & objects	1.31 ± 0.19	1.54 ± 0.28
High-calorie & low-calorie foods	0.09 ± 0.13	0.34 ± 0.12**
Chocolate high-calorie & low-calorie foods	0.12 ± 0.15	0.37 ± 0.13*
Sweet non-chocolate high-calorie & low-calorie foods	0.08 ± 0.13	0.36 ± 0.10***
Savoury high-calorie & low-calorie foods	0.07 ± 0.17	0.29 ± 0.18
Objects & blurred	0.47 ± 0.22	0.43 ± 0.20

Picture appeal rating [a]1=a lot, 2=a little, 3=neutral, 4=not really, 5=not at all; [b]1st category minus 2nd category, Fasted Dummy vs. Fasted Saline visit, n=17 per group, data are expressed as mean ± SEM, ***P<0.005, **P<0.01 *P<0.05.

4.2.5. Reaction time for appeal rating

There was a significant increased reaction time for appeal rating of all picture categories at the Fasted Dummy compared to the Fasted Saline visit (P<0.05) (Table 4.4.).

Table 4.4. Picture rating reaction time.

Picture Rating Reaction Time (msec)	**Fasted Dummy visit**	**Fasted Saline visit**
High-calorie foods	1395 ± 41	1226 ± 38**
Low-calorie foods	1398 ± 42	1250 ± 39*
Household objects	1399 ± 40	1227 ± 40**
Blurred	1127 ± 24	1008 ± 22*

Data shown as mean ± SEM, n=17 per group, *P<0.05, **P<0.01, ***P<0.001.

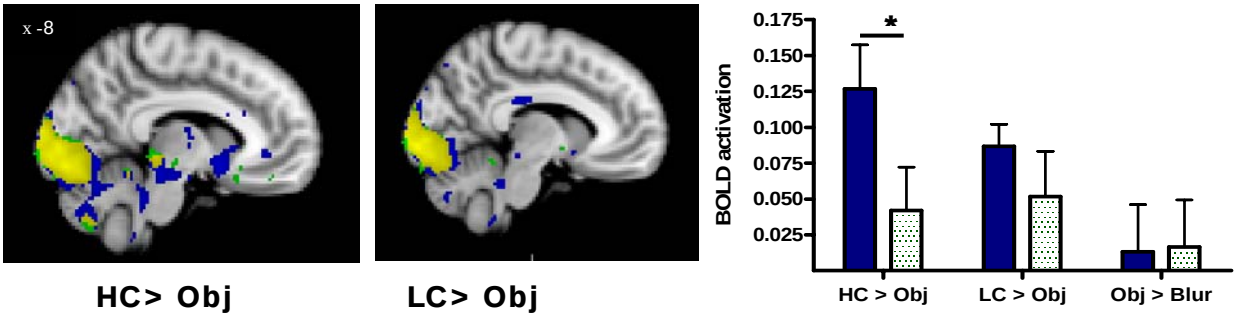
4.2.6. Brain activation

4.2.6.1. Region of interest (ROI) analysis

Activation in the left ventral striatum on viewing high-calorie foods vs. objects (P=0.03) at the Fasted Dummy visit was significantly greater compared to the Fasted Saline visit. This was not seen however for the right ventral striatum (P=1.0) or bilateral ventral striatum (p=0.31) nor for low-calorie foods vs. objects (P=0.16-0.81) (Figure 4.6., Table 4.5). Activation in the right ventral striatum (but not the left or bilateral ventral striatum (P=0.18-0.92)) when viewing household objects vs. blurred pictures (P=0.02) at the Fasted Dummy visit was significantly greater compared to the Fasted Saline visit (Table 4.5.).

There was no significant difference in activation in the insula (P=0.20-0.56), amygdala (P=0.30-0.96), or anterior OFC (P=0.23-1.0) on either side or bilaterally for high-calorie foods vs. objects, low-calorie foods vs. objects or objects vs. blurred pictures between the Fasted Dummy and Fasted Saline visit (Table 4.5.).

Figure 4.6. Ventral striatum activation to food pictures at fasted visits.



Left hand side: area of left ventral striatum showing activation for viewing high-calorie (HC) foods and low-calorie (LC) foods relative to objects (Obj) for the Fasted Dummy (dark blue clusters) and for the Fasted Saline visit (green clusters). Yellow colour: overlap of activation for both visits. Activation thresholded at FDR P<0.05, n=17. Co-ordinates are given in standard MNI space.

Right hand side: average activation of the left ventral striatum (contrast beta value) for high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) and objects relative to blurred (Blur) pictures between the Fasted Dummy (blue bar) and the Fasted Saline visit (green dotted bar). n=17 per group, data are expressed as mean ± SEM *P<0.05. ROI mask determined by overlap of Havard FSL atlas for nucleus accumbens with combined contrast Fasted Saline, Fed Saline, Fed Ghrelin for high-calorie foods relative to objects collapsed across visits FDR P<0.05.

Table 4.5. Magnitude of Activation within ROIs for fasted visits.

ROI	H	Fasted Dummy visits			Fasted Saline visit		
		High-calorie vs Object	Low-calorie vs Object	Object vs Blurred	High-calorie vs Object	Low-calorie vs Object	Object vs Blurred
Ventral striatum	L	0.127 ± 0.031	0.087 ± 0.015	0.013 ± 0.033	**0.042 ± 0.030***	0.052 ± 0.032	0.017 ± 0.033
	R	0.081 ± 0.03	0.031 ± 0.022	0.095 ± 0.036	0.084 ± 0.039	0.082 ± 0.031	0.011 ± 0.028*
	Bilateral	0.210 ± 0.050	0.120 ± 0.020	0.110 ± 0.060	0.130 ± 0.060	0.130 ± 0.060	0.030 ± 0.060
Amygdala	L	0.204 ± 0.056	0.059 ± 0.056	0.182 ± 0.056	0.170 ± 0.063	0.097 ± 0.06	0.121 ± 0.037
	R	0.202 ± 0.049	0.105 ± 0.047	0.157 ± 0.059	0.169 ± 0.039	0.109 ± 0.052	0.102 ± 0.037
	Bilateral	0.410 ± 0.100	0.160 ± 0.100	0.340 ± 0.110	0.340 ± 0.090	0.210 ± 0.100	0.220 ± 0.070
Insula	L	0.190 ± 0.039	0.131 ± 0.024	0.292 ± 0.038	0.150 ± 0.033	0.066 ± 0.050	0.227 ± 0.022
	R	0.173 ± 0.026	0.140 ± 0.024	-0.025 ± 0.228	0.123 ± 0.035	0.057 ± 0.048	0.020 ± 0.018
	Bilateral	0.360 ± 0.060	0.2720 ± 0.040	0.000 ± 0.060	0.270 ± 0.070	0.120 ± 0.100	0.050 ± 0.030
Anterior OFC	L	0.202 ± 0.053	0.130 ± 0.04	0.137 ± 0.042	0.262 ± 0.031	0.196 ± 0.063	0.139 ± 0.044
	R	0.122 ± 0.044	0.066 ± 0.036	0.159 ± 0.068	0.122 ± 0.06	0.070 ± 0.059	0.196 ± 0.052
	Bilateral	0.320 ± 0.090	0.260 ± 0.070	0.300 ± 0.100	0.380 ± 0.080	0.260 ± 0.110	0.330 ± 0.090

Average magnitude of activation within regions of interest (ROIs) for each hemisphere (H) at the Fasted Dummy visit and the Fasted Saline visit. Beta values extracted for each individual subject contrast estimates (vs. objects) for high-calorie and low-calorie foods and objects vs. blurred picture contrast: n=17 per group, data are expressed as mean ± SEM, *P<0.05 for Fasted Dummy visit vs. Fasted Saline visit.

4.2.6.2. Whole brain analysis

Additional brain areas were activated on viewing high-calorie and low-calorie foods relative to objects at the Fasted Dummy visit and the Fasted Saline visit using whole brain analysis (coordinates are given in Tables 4.6.-4.9.).

Compared to the first fasted fMRI study, less brain areas were activated when viewing high-calorie food pictures at the Fasted Dummy visit in the second study which can be due to the fact that the second study involved cannulation on a hospital ward. This might have afftected the stress level of the study participants. Also, the two fMRI studies used different softwares and atlases to analyse the data which may have biased the type of regions who are activated in each study. The second fMRI study used a different fMRI analysis software because is contains improved tools and is easier to use, especially for scripting purposes.

Notably was also the quantity of areas activated when comparing the Fasted Dummy with the Fasted Saline visit. For both contrast, viewing high-calorie and low-calorie foods vs. objects, at the Fasted Dummy visit more brain areas were activated. Especially when viewing low-calorie foods, half as many brain areas were activated on the Fasted Saline visit. Also the type of areas activated at each visit was slightly different for the two fasted visits. For example, the accumbens was only activated at the Fasted Dummy visit whereas at the Fasted Saline visit only the Parahippocampus and hippocampus were activated when viewing high-calorie and low-calorie foods vs. objects.

Table 4.6. Whole brain analysis at the Fasted Dummy visit for viewing high-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Occipital Pole	R	.82	13452	12	-92	0
Inferior Frontal Gyrus, pars triangularis	R	5.44	427	46	44	8
Insular Cortex	R	4.94	976	40	6	-14
Inferior Frontal Gyrus, pars triangularis	L	4.37	201	-42	38	8
Anterior Cingulate Gyrus	R	4.27	476	4	40	8
Inferior Temporal Gyrus, temporooccipital part	R	4.27	322	54	-46	-22
Precentral Gyrus	R	4.13	91	50	6	26
Supramarginal Gyrus, anterior division	R	4.09	34	52	-34	50
Lateral Occipital Cortex, superior division	L	3.99	252	-22	-66	44
Supramarginal Gyrus, anterior division	L	3.95	103	-46	-36	38
Orbitofrontal cortex	L	3.86	90	-26	34	-16
Frontal Pole	R	3.73	128	26	64	6
Insular Cortex	L	3.57	82	-32	16	10
Orbitofrontal Cortex	R	3.5	61	26	32	-20
Posterior Cingulate Gyrus	R	3.35	211	4	-28	26
Inferior Temporal Gyrus, temporooccipital part	L	3.35	32	-48	-46	-22
Postcentral Gyrus	L	3.28	5	-54	-16	46
Temporal Fusiform Cortex	R	3.27	15	30	-4	-40
Accumbens	R	3.18	30	10	18	0
Anterior Cingulate Gyrus	L	3.17	21	-10	24	22
Frontal Pole	L	3.09	23	-24	46	26
Precentral Gyrus	L	3.04	35	-44	-16	42
Inferior Frontal Gyrus, pars opercularis	R	3.04	14	48	18	30
Middle Frontal Gyrus	L	3.03	5	44	22	42
Middle Frontal Gyrus	R	2.81	6	38	6	54

Activation within whole brain analysis for high-calorie picture contrast (relative to objects) at the Fasted Dummy visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR $P<0.05$ and minimum 5 voxel cluster size.

Table 4.7. Whole brain analysis at the Fasted Dummy visit for viewing low-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Occipital Pole	L	7.05	9787	-14	-90	-10
Insular Cortex	R	4.72	649	38	6	-14
Occipital Fusiforum Gyrus	R	4.26	624	16	-74	40
Inferior Frontal Gyrus	R	5.13	462	46	42	8
Insular Cortex	L	4.89	443	-40	-4	2
Lateral Occipital Cortex	L	3.9	157	-24	-68	44
Precentral Gyrus	L	3.51	82	-40	-8	42
Inferior Frontal Gyrus	L	3.46	43	-36	34	10
Accumbens	L	3.08	40	-12	12	-8
Supramarginal Gyrus, anetrior division	L	3.34	31	-48	-36	36
Orbitofrontal Cortex	L	3.7	31	-28	36	-16
Caudate	R	3.39	29	10	22	4
Anterior Cingulate Gyrus	R	3.45	28	4	38	10
Thalamus	R	3.18	28	22	-30	-4
Inferior Temporal Gyrus	R	3.79	26	56	-48	-24
Precuneous Cortex	R	3.34	25	6	-74	56
Postcentral Gyrus	R	3.14	23	58	-14	24
Postcentral Gyrus	L	3.47	22	-50	-18	36
Precentral Gyrus	R	3.02	21	46	6	28
Frontal Pole	R	3.16	20	24	48	26
Orbitofrontal Cortex	R	3.17	19	26	36	-18
Middle Frontal Gyrus	R	3.2	12	38	6	54
Precentral Gyrus	L	2.9	11	-48	2	28
Amygdala	R	3.04	5	22	-2	-16

Activation within whole brain analysis for low-calorie picture contrast (relative to objects) at the Fasted Dummy visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR P<0.05 and minimum 5 voxel cluster size.

Table 4.8. Whole brain analysis at the Fasted Saline visit for viewing high-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Intracalcarine cortex	R	6.19	11206	12	-88	0
Insular Cortex	L	4.88	285	-38	-6	8
Oritofrontal Cortex	L	4.68	401	-26	36	-16
Parahippocampal Gyrus, posterior division	L	4.6	745	-20	-30	-6
Insular Cortex	R	4.55	191	38	8	-14
Lateral Occipital Cortex	L	3.97	40	-20	-62	42
Inferior Frontal Gyrus	R	3.89	18	50	42	8
Inferior Frontal Gyrus	L	3.77	59	-42	36	8
Amygdala	R	3.6	92	18	2	-14
Inferior Temporal Gyrus	R	3.5	96	50	-58	-12
Pallidum	R	3.48	23	16	2	-4
Anterior Cingulate Gyrus	R	3.41	69	0	40	-2
Precuneous Cortex	L	3.28	46	-18	-72	34
Postcentral Gyrus	L	3.26	25	-46	-18	38
Inferior Temporal Gyrus	L	3.25	18	-48	-46	-24
Parahippocampal Gyrus	R	3.19	15	18	6	-30
Amygdala	L	3.17	29	-18	2	-16
Frontal Pole	R	3.16	17	26	44	-18
Postcentral Gyrus	L	3.12	16	-62	-18	20
Precentral Gyrus	L	3.01	15	-50	4	24

Activation within whole brain analysis for high-calorie picture contrast (relative to objects) at the Fasted Saline visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR $P<0.05$ and minimum 5 voxel cluster size.

Table 4.9. Whole brain analysis at the Fasted Saline visit for viewing low-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Occipital Pole	R	6.41	6297	14	-100	8
Hippocampus	L	4.63	207	-22	-30	-6
Lateral Occipital Cortex	R	3.99	98	28	-66	46
Orbitofrontal Cortex	L	3.92	76	-26	34	-16
Thalamus	R	3.77	148	20	-28	-4
Superior Parietal Lobule	L	3.66	12	-30	-62	-46
Inferior Frontal Gyrus	R	3.49	51	42	44	10
Anterior Cingulate Gyrus	L	3.18	8	-10	28	-6
Insular Cortex	R	3.16	5	40	6	-12
Pallidum	L	3.14	13	-12	-2	-6
Thalamus	L	3.08	7	-12	-16	-4
Amygdala	R	3.07	5	16	0	-22

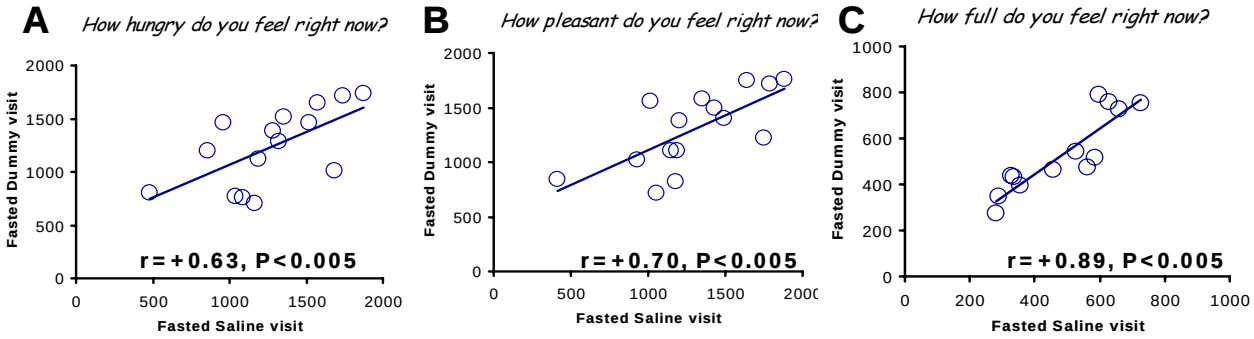
Activation within whole brain analysis for low-calorie picture contrast (relative to objects) at the Fasted Saline visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR P<0.05 and minimum 5 voxel cluster size.

4.2.7. Correlations between Fasted visits

4.2.7.1. Appetite visual analogue scores

Hunger, pleasantness-to-eat and fullness VAS AUC ratings were positively correlated between Fasted Dummy and Fasted Saline visits (r=+0.63-0.89, P<0.005) (Figure 4.7.).

Figure 4.7. Correlations of appetite VAS ratings between fasted visits.

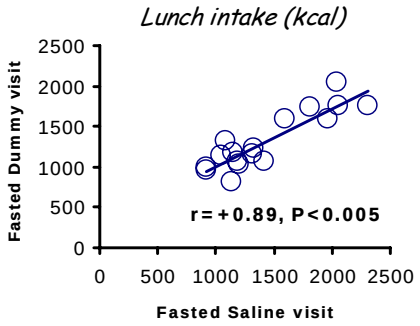


Correlations of appetite VAS ratings (AUC): (A) hunger (n=15), (B) pleasantness to eat (n=15) and (C) fullness (n=13) between the Fasted Dummy and the Fasted Saline visit. Some subjects have been excluded from the analysis because of incomplete data sets. *r* represents Pearson correlation coefficient.

4.2.7.2. Lunch intake

Lunch intake correlated positively between the Fasted Dummy and the Fasted Saline visits (r=+0.89, P<0.005) (Figure 4.8.).

Figure 4.8. Correlation of *ad libitum* lunch intake between fasted visits.

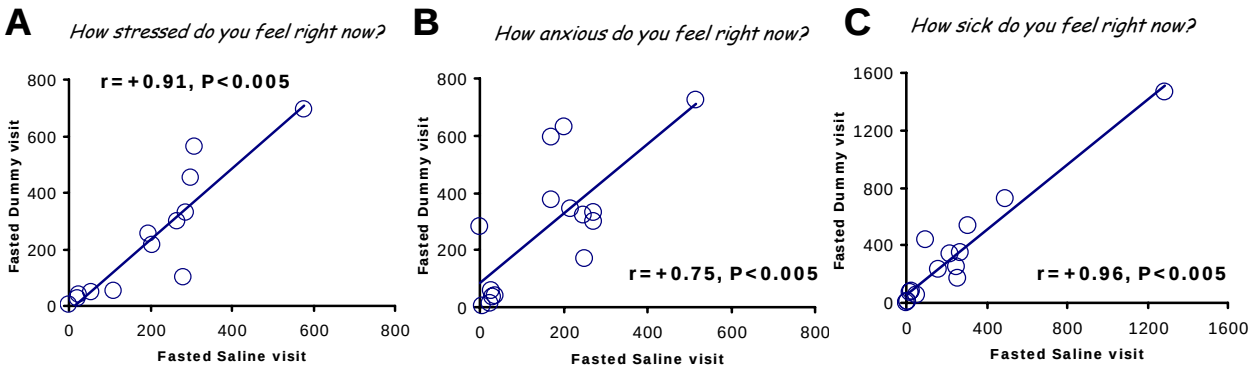


Correlation of *ad libitum* lunch intake between the Fasted Dummy and Fasted Saline visit. n=17 per group, *r* represents Pearson correlation coefficient.

4.2.7.3. Stress ratings

Stress, anxiety and sickness ratings correlated positively between the Fasted Dummy and the Fasted Saline visit (r=+0.70-0.96, P<0.005) (Figure 4.9.).

Figure 4.9. Correlations of VAS stress ratings between fasted visits.

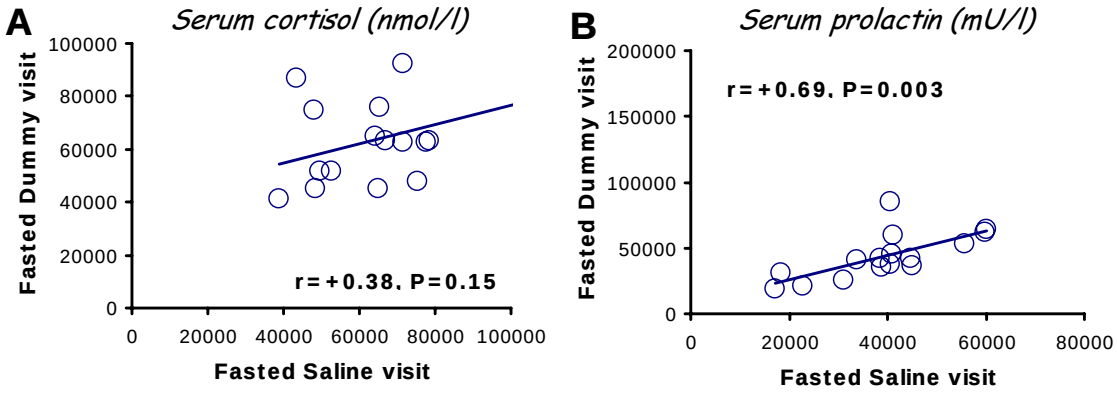


Correlations of VAS stress ratings (AUC): (A) stress (n=13), (B) anxiety (n=15) and (C) sickness (n=15) between the Fasted Dummy and the Fasted Saline visit. Some subjects have been excluded from the analysis because of incomplete data set. *r* represents Pearson correlation coefficient.

4.2.7.4. Serum stress hormones

Serum prolactin levels at the Fasted Dummy visit correlated positively with levels at the Fasted Saline visit (r=+0.69, P=0.003) (Figure 4.10.). However, there was no significant correlation for the serum cortisol levels between the fasted visits (r=+0.38, P=0.15) (Figure 4.10.).

Figure 4.10. Correlations of stress hormone levels between fasted visits.

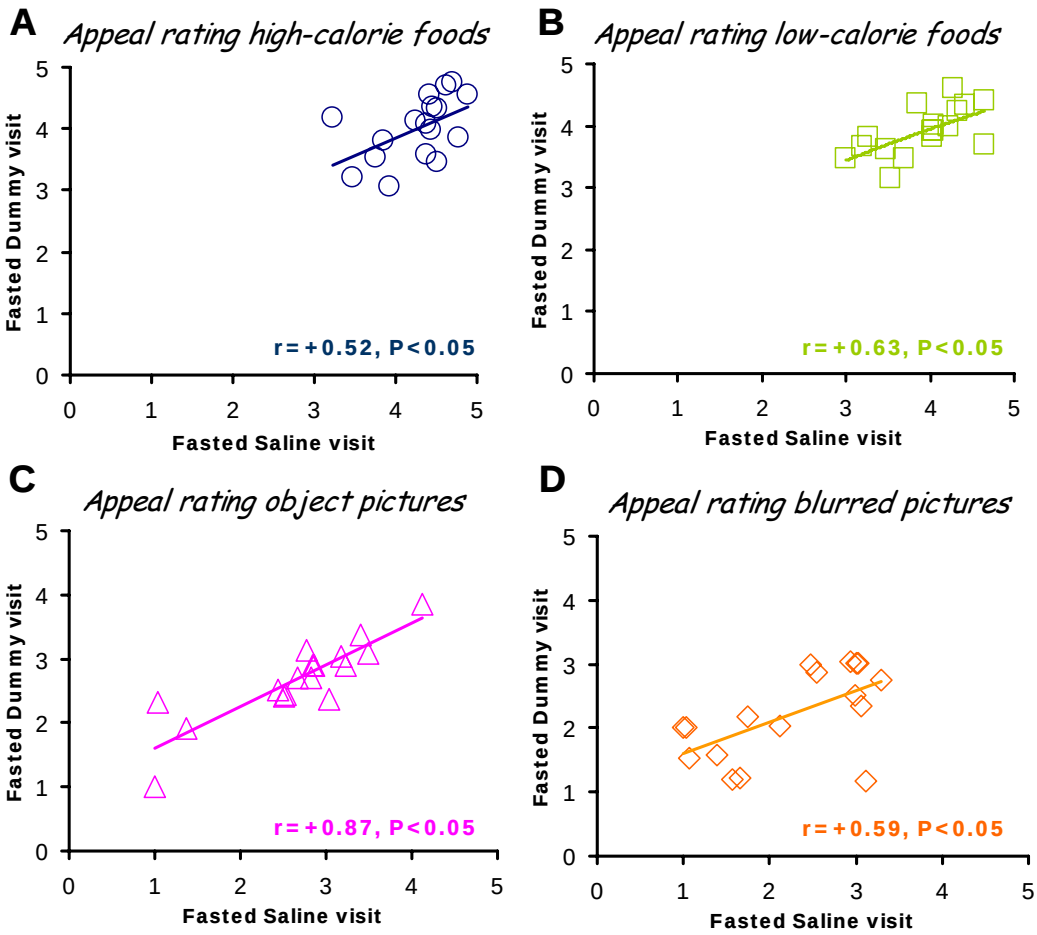


Correlations of serum stress hormone levels (AUC) for (A) cortisol (n=16) and (B) prolactin (n=16) between the Fasted Dummy and the Fasted Saline visit. Some subjects have been excluded from the analysis because of incomplete data set. *r* represents Pearson correlation coefficient.

4.2.7.5. Appeal rating

Appeal ratings for high-calorie and low-calorie food, object and blurred pictures correlated positively between the fasted visits (r=+0.52-0.87, P<0.05) (Figure 4.11.).

Figure 4.11. Correlations of appeal rating scores between fasted visits.

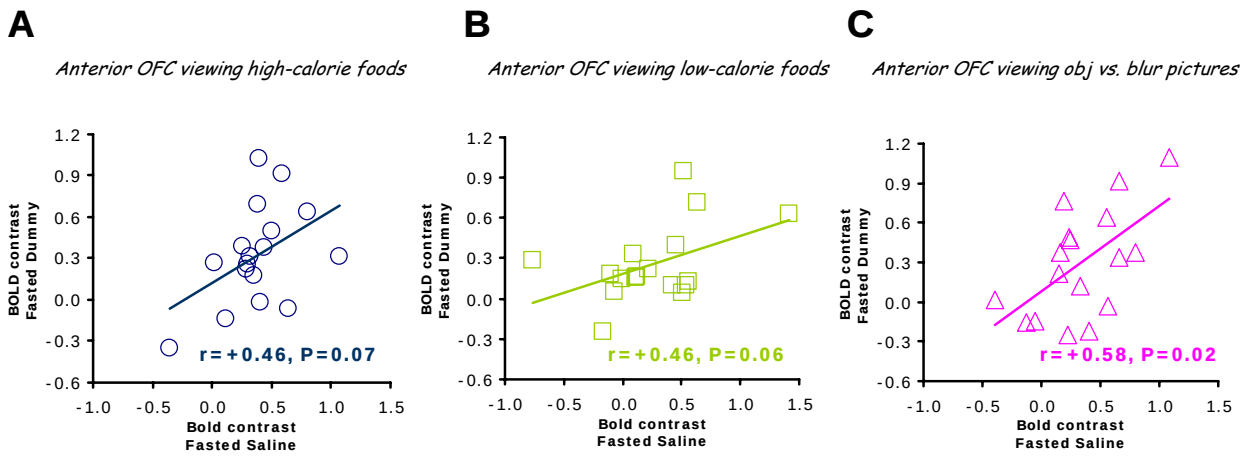


Correlations of appeal rating scores for (A) high-calorie foods (blue circles), (B) low-calorie foods (green squares), (C) objects (pink triangles) and (D) blurred pictures (orange squares) between the Fasted Dummy and the Fasted Saline visit. n=17, *r* represents Pearson correlation coefficient.

4.2.7.6. Brain activation

There was a trend towards a positive correlation of the activation of the anterior OFC between the fasted visits when viewing high-calorie foods vs. object pictures (left: r=+0.47, P=0.06, right: r=+0.41, P=0.11, bilateral: r=+0.46, P=0.07), low-calorie foods vs. object pictures (left: r=+0.47, P=0.06, right: r=+0.28, P=0.29; bilateral: r=+0.46, P=0.06) and a significant correlation for objects vs. blurred pictures (left: r=+0.51, P=0.04, right: r=+0.61, P=0.009; bilateral: r=+0.58, P=0.02) (Figure 4.12.). None of the activation for any of the picture contrasts in the other ROIs correlated between the two fasted visits.

Figure 4.12. Correlation of anterior OFC activation between fasted visits.



Correlation of activation (contrast beta value) of the anterior OFC (average bilateral) between the Fasted Dummy and the Fasted Saline visit on viewing (A) high-calorie foods over objects (blue circles), (B) low-calorie foods over objects (green squares) and (C) objects over blurred (pink triangles) pictures. n=17, *r* represents Pearson correlation coefficient.

4.2.8. Summary reproducibility of eating behaviour studies

This chapter examined the reproducibility of appetite fMRI studies by comparing two identical study fMRI visits in which one was the Fasted Dummy visit and one the Fasted Saline visit. Appetite studies have shown that stress can alter food intake and responses in brain reward systems (Adam and Epel, 2007; Chandarana et al., 2009; Blundell et al., 2010; Born et al., 2010).

There was significantly lower *ad libitum* lunch intake, and significantly higher anxiety and sickness ratings at the Fasted Dummy visit compared to the Fasted Saline visit although the stress ratings and stress hormone levels were not significantly different between the two visits. There was also a trend towards greater fullness, lower rating of prospective pleasantness-to-eat and actual pleasantness of lunch at the Fasted Dummy visit. High-calorie foods were significant less appealing at the Fasted Dummy visit compared to the Fasted Saline visit, resulting in high-calorie foods being no more appealing than low-calorie foods at the Fasted Dummy visit (unlike at the Fasted Saline visit). Therefore pleasantness and food appeal visit were reduced and anxiety increased at the Fasted Dummy compared to the Fasted Saline visit. This confirms that it might be important to include a dummy visit to acclimatise volunteers to the study environment.

Interestingly, all appetite and stress / anxiety measures (excluding serum cortisol levels) correlated positively between the Fasted Dummy and the Fasted Saline visit.

Activation in the ventral striatum was significantly higher at the Fasted Dummy visit compared to the Fasted Saline visit when viewing high-calorie foods vs. objects. This may be be explained by habituation of the ventral striatum activation to viewing high-calorie foods on subsequent visits which will be discussed in more detail in chapter 6.2. There was no significant positive correlation of activation in any ROI between the fasted visits but there was a trend towards positive correlation of anterior OFC activation between the Fasted Dummy and the Fasted Saline visit. Therefore, the test-retest reliability of the fMRI activation to food and non-food pictures was not as robust with the exception of the anterior OFC activation. Problems with fMRI reproducibilty between visits have been found in other studies and will be discussed in section 6.4.

Chapter 5

RESULTS

GHRELIN fMRI STUDY

5.1. General subject's characteristics

In this chapter, the Fasted Saline visit has been compared with the Fed Saline visit (5.2.), and the Fed Saline visit has been compared with the Fed Ghrelin visit by using the following measurements: VAS appetite and stress ratings, fMRI hunger rating, blood appetite and stress hormones, lunch intake, tastiness and pleasantness, dietary records, fMRI appeal rating and fMRI BOLD signal. For the Fasted/ Fed Saline comparison this chapter also analysed relationships between brain reward activations and appetite measures. Study characteristics and general comparisons (e.g. food picture ratings) are shown below (Table 5.1., Figure 5.1.). There was a significant difference of the percentage carbohydrate intake at supper prior to the study day between the Fasted and Fed Saline visit with increased intake on the Fed Saline day ($P=0.006$). Time fasting since supper ($P<0.001$) was significantly longer on the Fasted Saline visit. The days of menstrual cycle were significantly less at the Fed Ghrelin visit compared to the Fed Saline visit ($P=0.049$). There were no significant differences in any other characteristic.

Table 5.1. Subject characteristics.

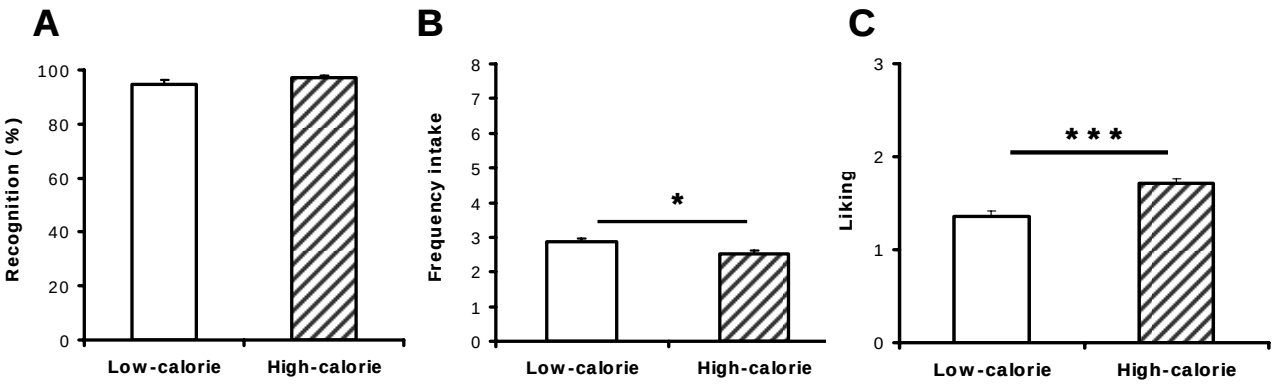
	Fasted Saline	Fed Saline	Fed Ghrelin
N	17	17	17
Gender			
Male	13	13	13
Female	4	4	4
Age (*years*)	24.9 ± 1.6	24.9 ± 1.6	25.0 ± 1.6
Height (*m*)	1.80 ± 0.0	1.80 ± 0.0	1.80 ± 0.0
Weight (k*g*)	76.3 ± 3.6	76.4 ± 3.7	76.5 ± 3.7
BMI ($kg/m^{2)}$	24.1 ± 0.7	24.2 ± 0.7	24.2 ± 0.7
Body fat (*%*)	17.1 ± 1.6	17.9 ± 1.8	17.6 ± 1.5
LBM (*kg*)	64.6 ± 3.3	61.1 ± 4.0	61.0 ± 4.2
Day of menstrual cycle *(females)*	5.2 ± 1.2	8.8 ± 1.3	4.6 ± 1.4*
Time since last visit *(days)*	18.0 ± 9.0	17.0 ± 7.2	24.5 ± 6.9
Food intake at supper prior to study			
Energy *(kcal)*	1012 ± 179	1103 ± 188	980 ± 173
Protein *(%)*	21 ± 2	21 ± 3	18 ± 2
Fat *(%)*	40 ± 5	34 ± 5	33 ± 4
Carbohydrate *(%)*	38 ± 5**	45 ± 5	49 ± 4
Food intake whole day prior to study			
Energy *(kcal)*	2645 ± 397	2488 ± 273	2209 ± 218
Protein *(%)*	17 ± 1	17 ± 1	18 ± 1
Fat *(%)*	38 ± 4	32 ± 2	35 ± 2
Carbohydrate *(%)*	45 ± 3	52 ± 3	46 ± 3
Time fasting since supper at t=0 (hrs)	17.1 ± 0.2***	14.6 ± 0.2	14.8 ± 0.3
Time sleep (hrs)	7.0 ± 0.2	7.3 ± 0.2	7.3 ± 0.4
Time t=0 to injection	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1
Time injection to first food fMRI scan *(hrs)*	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
Time injection to lunch *(hrs)*	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0
Questionnaires			
Positive Affect	30.6 ± 1.3	32.6 ± 1.4	31.4 ± 1.4
Negative Affect	15.7 ± 1.7	13.5 ± 0.8	15.4 ± 1.3
Absolute Head motion *(mm)*	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1

n=17 per group, Mean ± SEM, *P<0.05, **P<0.01, ***P<0.005 vs Fed Saline (paired t-test between Fasted Saline vs. Fed Saline and Fed Saline vs Fed Ghrelin visit).

5.1.1. Picture ratings at end of study

Ninety-five percent of the low-calorie foods and 97 percent of the high-calorie foods were correctly identified by the subjects (P=0.24) (Figure 5a). On average they liked the high-calorie foods more than the low-calorie foods (P<0.001) (Figure 5a). The differences were seen for each type of high-calorie foods: chocolate (P<0.001), sweet non-chocolate (P<0.001) and savoury (P=0.02). However, on average they ate the low-calorie foods more often than high-calorie foods (P=0.003) (Figure 5.1.).

Figure 5.1. Food picture ratings.



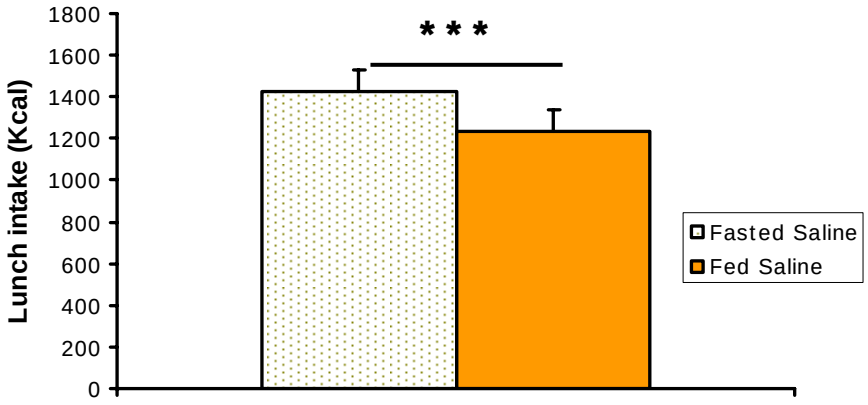
Food picture ratings. (A) Recognition, (B) liking and (C) frequency of consumption of food pictures (high-calorie: white bar; low-calorie: striped bar), n = 17 per group, Data expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.005.

5.2. Fasted Saline visit versus Fed Saline visit

5.2.1. Appetite visual analogue scores and food intake

At the Fasted Saline visit, ratings for hunger (P<0.001), pleasantness to eat (P<0.001), volume able to eat (P<0.001) were significantly greater than at the Fed Saline visit while fullness was significantly lower (P<0.001) (Figure 5.3.). There was a 15% increase in energy intake at lunch (P=0.002) at the Fasted Saline visit compared to the Fed Saline visit (Figure 5.2.). There was no significant difference in lunch tastiness (P=0.40) between the two visits but there was a tendency for lunch pleasantness to be greater on the Fasted Saline visit (P=0.06) (Table 5.2.).

Figure 5.2. *Ad libitum* lunch intake.



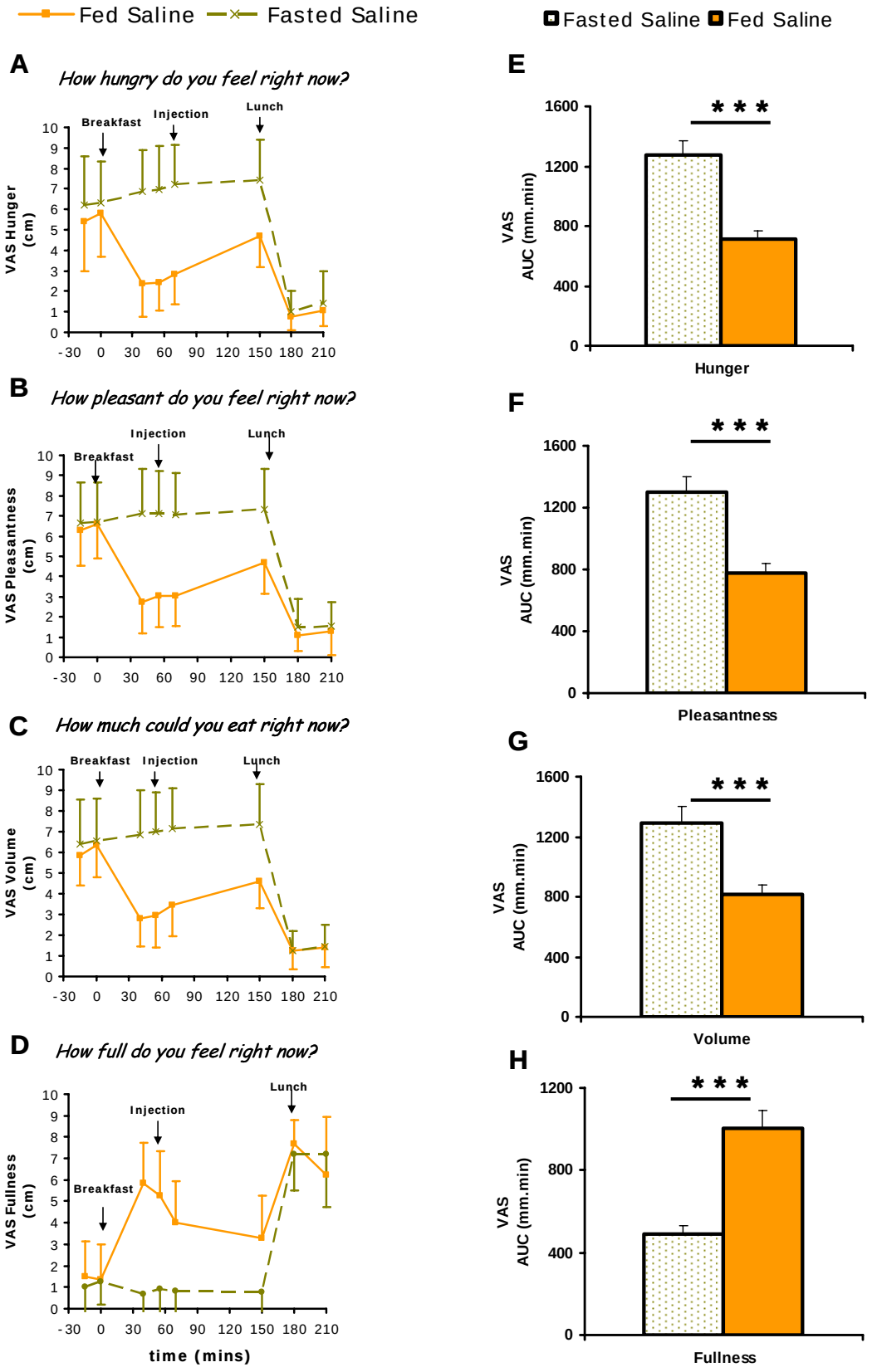
Ad libitum lunch intake for each visit separately (Fasted Saline, green dotted bar, Fed Saline, orange bar), n=17 per group, data are expressed as mean ± SEM, ***P<0.0055.

Table 5.2. *Ad libitum* lunch intake.

	Fasted Saline	Fed Saline
Lunch intake		
Total lunch *(kcal)*	1428 ± 101	1237 ± 103***
Lunch tastiness	6.4 ± 0.3	5.8 ± 0.4
Lunch pleasantness	6.8 ± 0.3	6.1 ± 0.3

n=17 per group, data shown as mean ± SEM, Fasted Saline vs. Fed Saline visit, ***P<0.005.

Figure 5.3. VAS appetite scores.

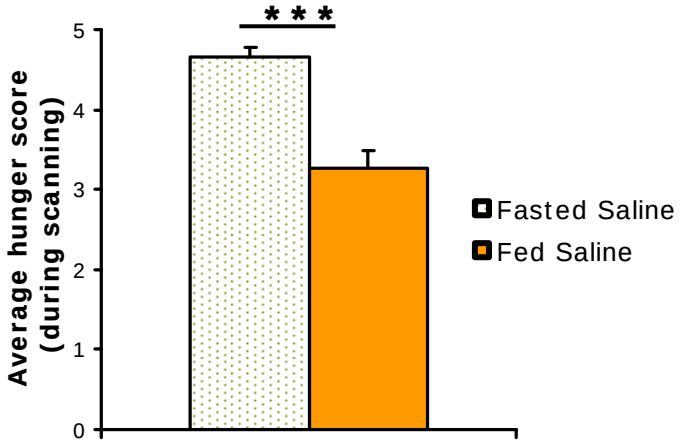


Absolute VAS scores for hunger, pleasantness to eat, volume able to eat and fullness ratings for each visit separately (Fasted Saline, green dotted bar and Fed Saline, orange bar), (A-D) time course of VAS scores, (E-H) mean AUC for each score, n=15 per group (2 subjects have been excluded from the analysis because of incomplete data), data are expressed as mean ± SEM, ***P<0.005.

5.2.2. Hunger scores during scanning

The average hunger rating during scanning was significantly higher on the Fasted Saline than the Fed Saline visit (P<0.001) (Figure 5.4.).

Figure 5.4. Hunger rating during scanning.

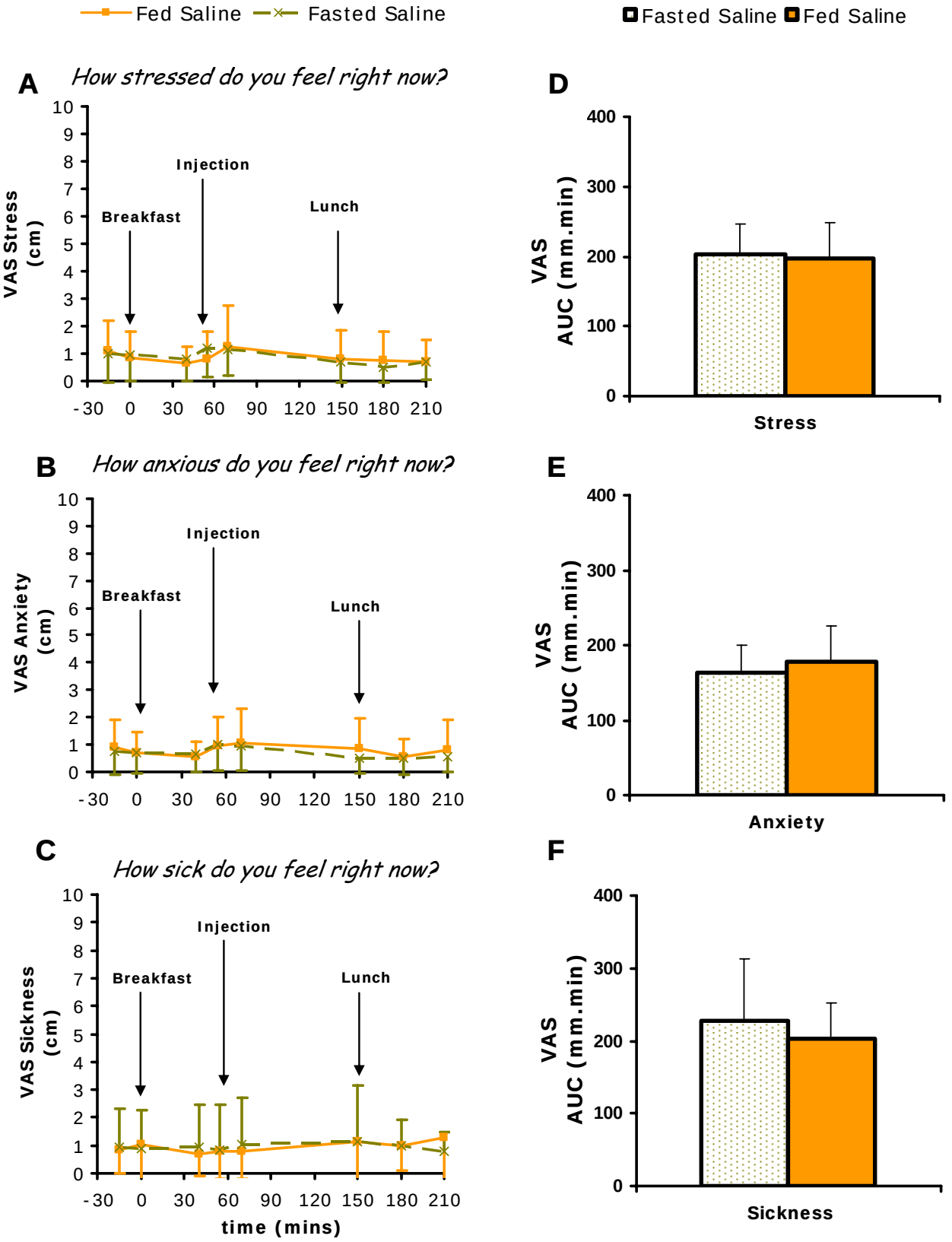


Average hunger score during scanning for each visit separately (Fasted Saline, green dotted bar; Fed Saline, orange bar), n=17 per group. Data are expressed as mean ± SEM, ***P<0.005.

5.2.3. Stress response and serum stress levels

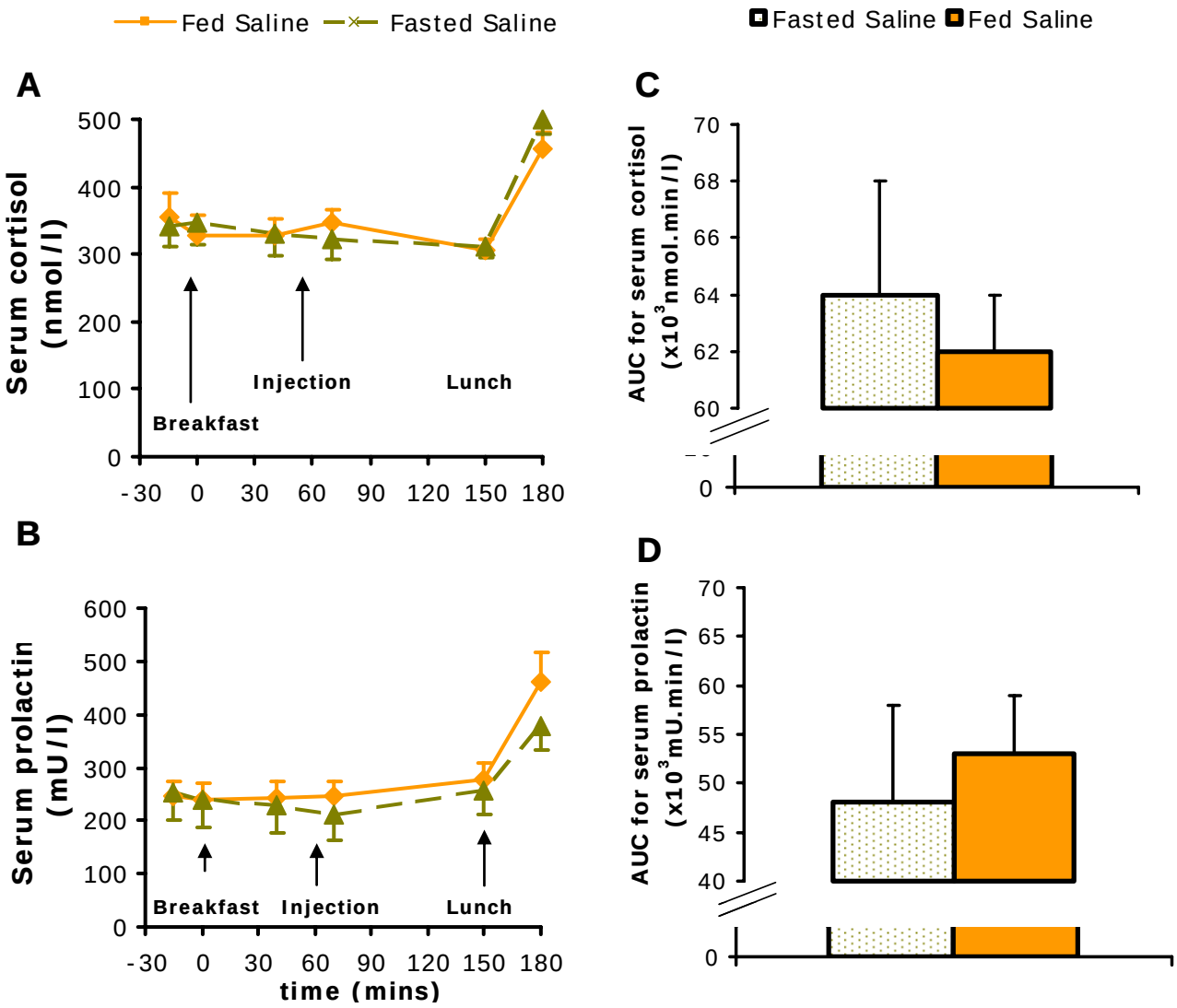
There was no significant difference in VAS stress (P=0.79), anxiety (P=0.59) or sickness (P=0.60) ratings between the Fasted Saline and the Fed Saline visit (Figure 5.5.). Serum cortisol (P=0.71) and prolactin (P=0.34) levels were also not significantly different between the two visits (Figure 5.6.).

Figure 5.5. VAS stress scores.



Absolute VAS scores for stress, anxiety and sickness ratings for each visit separately (Fasted Saline, green dotted bar and Fed Saline visit, orange bar), (A-C) time course of stress scores, (D-F) mean AUC for each score, n=15 per group (2 subjects have been excluded from the analysis because of incomplete data set). Data expressed as mean ± SEM, *P<0.05.

Figure 5.6. Cortisol and prolactin serum levels.

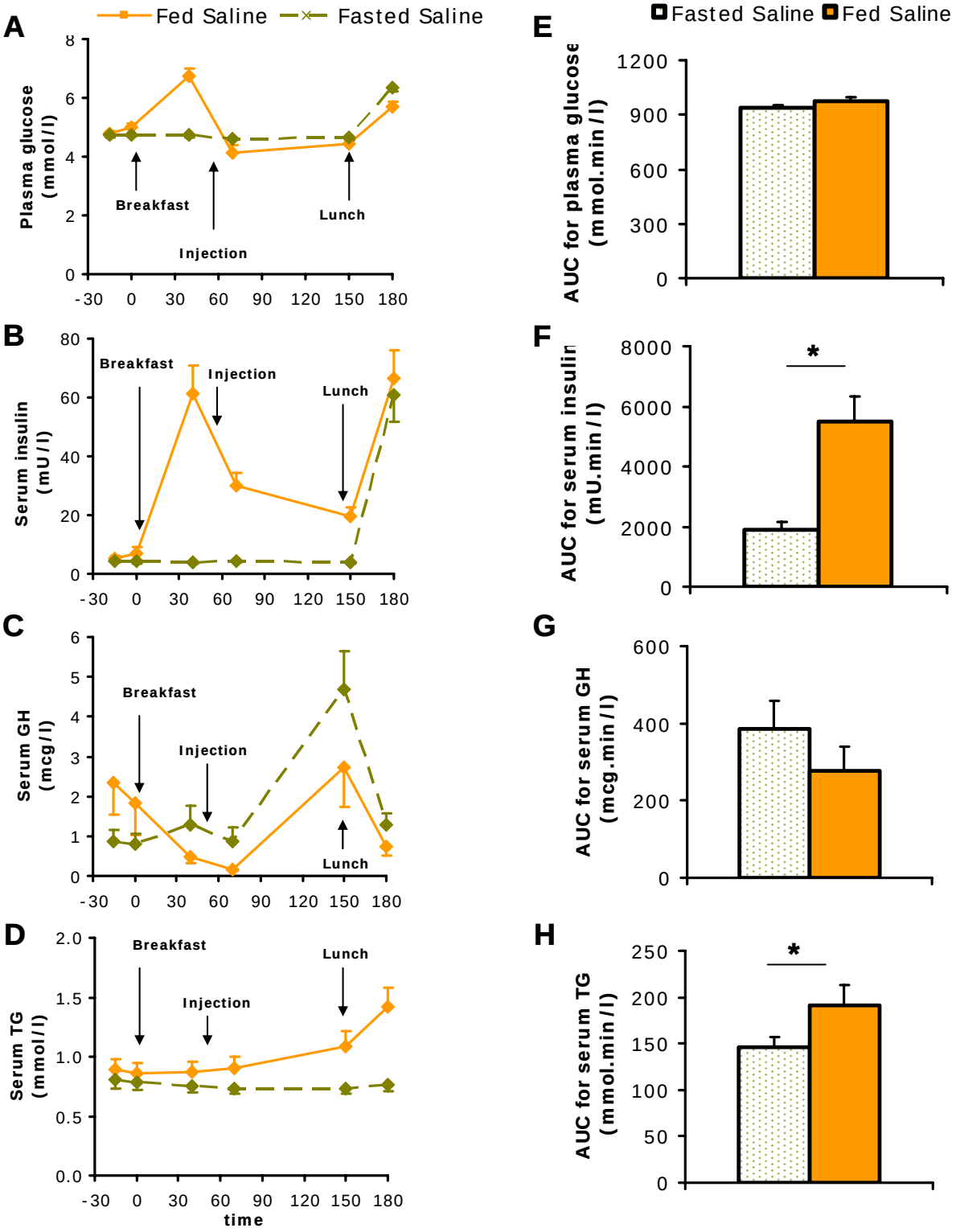


Cortisol and prolactin serum levels for each visit separately (Fasted Saline, green dotted bar and Fed Saline visit, orange bar), (A, B) time course of stress hormones, (C, D) AUC for each serum stress hormone, n=17 per group, data are expressed as mean ± SEM, *P<0.05.

5.2.4. Metabolic parameters

Serum insulin and triglycerides were significant greater at the Fed Saline visit compared to the Fasted Saline visit due to post-prandial increases after eating breakfast (P<0.05). There was no significant difference in serum growth hormone (P=0.15) between the visits but there was a tendency for plasma glucose to be higher on the Fed Saline visit as due to levels rising after breakfast (P=0.08) (Figure 5.7.).

Figure 5.7. Metabolic parameters.

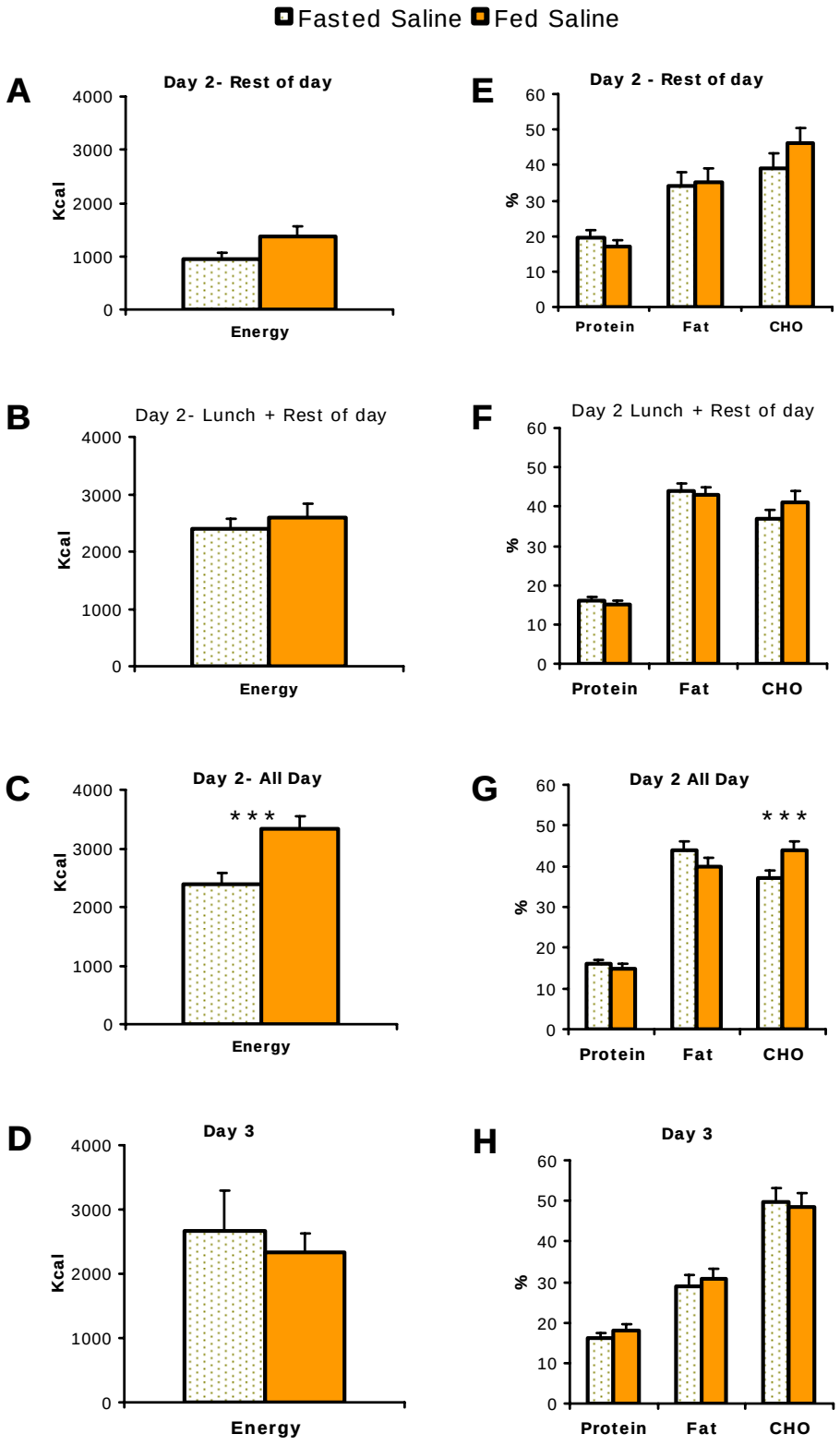


Glucose, insulin, growth hormone (GH) and tryglyceride (TG) levels for each visit separately (Fasted Saline visit, green dotted bar and Fed Saline visit, orange bar), (A-D) time course of metabolic parameters, (E-H) AUC for each metabolic parameter, n=16 per group (1 subject has been excluded from the analysis because of incomplete data set), data expressed as mean ± SEM, *P<0.05.

5.2.5. Dietary records

There were no significant differences in energy intake nor macronutrient composition over the rest of the study day after lunch, nor when lunch and the rest of study day were combined, nor the day after the study visit, between the Fasted Saline and Fed Saline visits ($P=0.29$-0.87). However when including breakfast, total energy intake over the study day and % carbohydrate consumed were both significantly greater at the Fed Saline visit compared to the Fasted Saline visit (both $P<0.005$) (Figure 5.8.).

Figure 5.8. Food intake on study day and day after study.

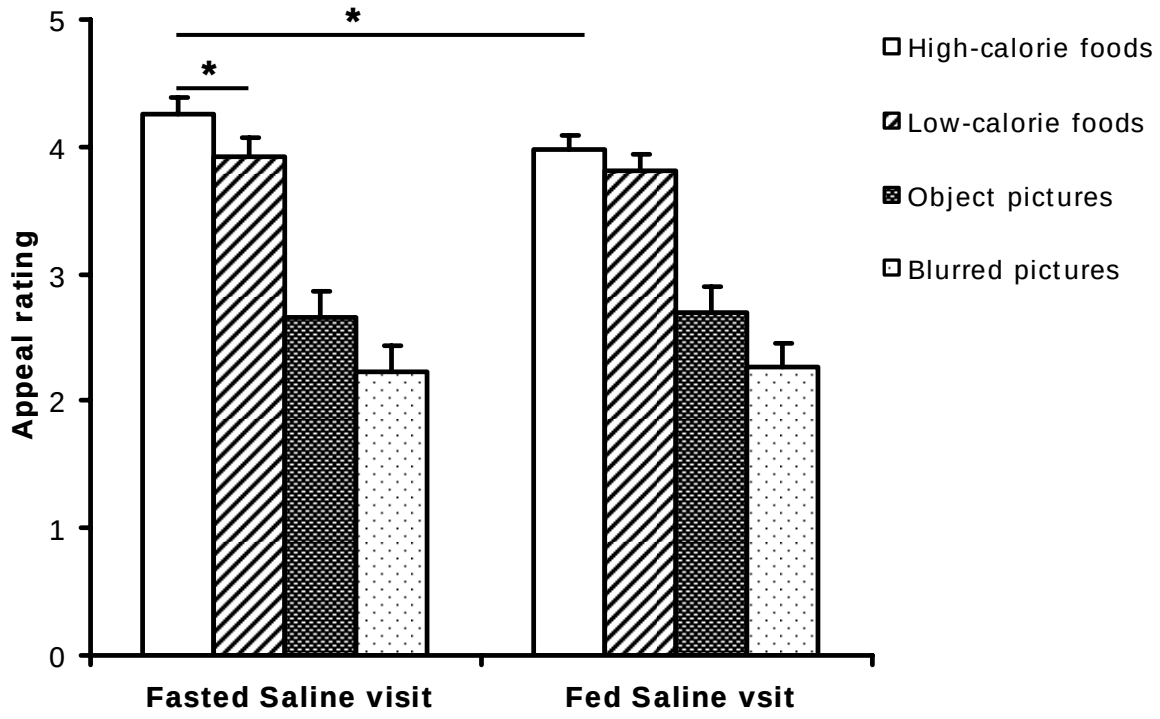


Intake of energy (A-D) and macronutrients (E-G) over the rest of the study day (day 2), the rest of the study day combined with lunch (day2), the whole day of the study day (day 2), and one day after the study day (day 3), comparison between Fasted Saline visit (green dotted bar) and Fed Saline visit (orange bar), n=13 per group (4 subjects have been excluded from the analysis because of incomplete data set). Data are expressed as mean ± SEM. ***P<0.005.

5.2.6. Food picture appeal rating

High-calorie foods (and for both sweet and savoury sub-categories) were more appealing at the Fasted Saline visit compared to the Fed Saline visit ($P<0.05$), but not low-calorie foods, object or blurred pictures ($P=0.25$-0.86) (Figure 5.9., Table 5.3.). High-calorie foods were more appealing than low-calorie foods at the Fasted Saline ($P<0.05$) but not at the Fed Saline visit ($P=0.30$) (Figure 5.9., Table 5.3.). The significance of this was dependent on the type of high-calorie food: being significant for chocolate or sweet non-chocolate ($P<0.05$) but not savoury high-calorie foods ($P=0.13$) (Table 5.3.). All categories of high-calorie foods and low-calorie foods were more appealing than both object and blurred pictures at both visits ($P<0.001$) (Figure 5.9., Table 5.3.). There was no significant difference in the appeal of object relative to blurred pictures at the Fasted Saline compared to the Fed Saline visit ($P=0.94$) (Table 5.3.).

Figure 5.9. Picture appeal rating.



Appeal rating of pictures (high-calorie foods: white bar, low-calorie foods: striped bar, non-food related household objects: black bar, blurred pictures: dotted bar), Fasted Saline vs. Fed Saline visit, n=17 per group. Data are expressen as mean ± SEM, *P<0.05.

Table 5.3. Picture appeal rating.

Picture Appeal Rating	**Fasted Saline visit**	**Fed Saline visit**
Absolute rating [a]		
High-calorie foods	4.28 ± 0.11	3.95 ± 0.12*
Low-calorie foods	3.91 ± 0.11	3.76 ± 0.15
Household objects	2.62 ± 0.20	2.64 ± 0.19
Blurred	2.18 ± 0.20	2.20 ± 0.19
Chocolate high-calorie foods	4.30 ± 0.11	4.06 ± 0.12*
Sweet non-chocolate high-calorie foods	4.28 ± 0.11	4.00 ± 0.14*
Savoury high-calorie foods	4.21 ± 0.16	3.90 ± 0.17*
Difference in rating between [b]		
High-calorie foods & objects	1.60 ± 0.23	1.29 ± 0.24**
Low-calorie foods & objects	1.26 ± 0.25	1.12 ± 0.26
Chocolate high-calorie foods & objects	1.63 ± 0.19	1.36 ± 0.21*
Sweet non-chocolate high-calorie foods & objects	1.26 ± 0.24	1.30 ± 0.25**
Savoury high-calorie foods & objects	1.54 ± 0.28	1.20 ± 0.28*
High-calorie & low-calorie foods	0.34 ± 0.12	0.17 ± 0.16
Chocolate high-calorie & low-calorie foods	0.37 ± 0.13	0.24 ± 0.17
Sweet non-chocolate high-calorie & low-calorie foods	0.36 ± 0.10	0.18 ± 0.14
Savoury high-calorie & low-calorie foods	0.29 ± 0.18	0.08 ± 0.21
Objects & blurred	0.43 ± 0.20	0.44 ± 0.18

[a] Picture appeal rating (1=a lot, 2=a little, 3=neutral, 4=not really, 5=not at all; [b] 1st category minus 2nd category), Fasted Saline vs. Fed Saline, n=17 per group, data are expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.005.

5.2.7. Reaction time for appeal rating

There was no significant difference in the reaction time for appeal rating of any of the picture categories between the Fasted and Fed Saline visit (Table 5.4.), suggesting the absence of non-specific effect of visit type on the picture viewing and rating task.

Table 5.4. Picture rating reaction time.

Picture Rating Reaction Time (msec)	**Fasted Saline visit**	**Fed Saline visit**
High-calorie foods	1226 ± 38	1330 ± 38
Low-calorie foods	1250 ± 39	1332 ± 40
Household objects	1227 ± 40	1349 ± 39
Blurred	1008 ± 22	1098 ± 25

Data shown as mean ± SEM, n=17 per group.

5.2.8. Brain activation

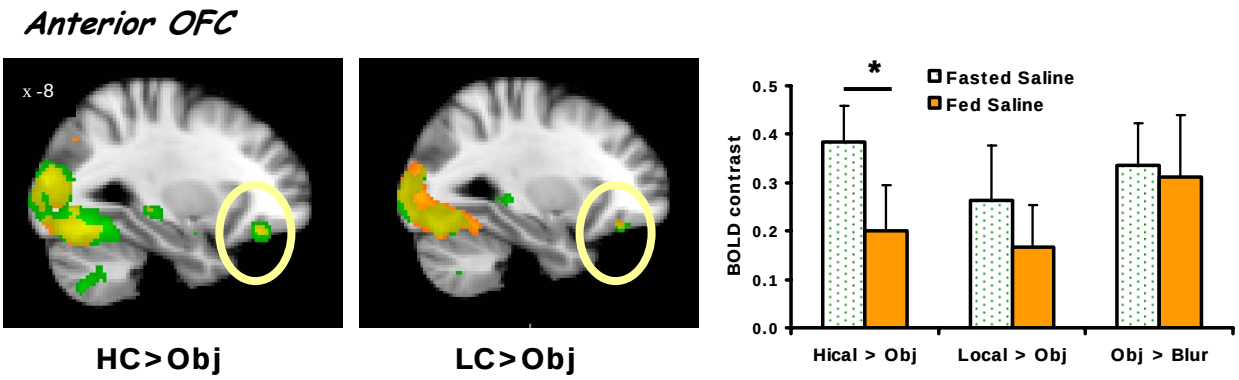
5.2.8.1. Region of interest (ROI) analysis

Activation in the left (P=0.008) and bilateral (P=0.04) anterior OFC on viewing high-calorie foods vs. objects at the Fasted Saline visit was significantly greater compared to the Fed Saline visit. This was not seen however for the right anterior OFC (P=0.31) nor for low-calorie foods vs. objects (P=0.38-0.88) (Figure 5.10., Table 5.5.).

There was no significant difference in activation in the ventral striatum, insula and amygdala on either side or bilaterally when viewing high-calorie foods vs. objects or low-calorie foods vs. objects between the Fasted Saline and Fed Saline visits (P=0.13-0.97) (Table 5.5.).

Activation in the ventral striatum, insula, amygdala and anterior OFC when viewing household objects vs. blurred pictures was not significant different between the Fasted Saline and Fed Saline visits (P=0.5-0.91) (Table 5.5.).

Figure 5.10. Brain activation for Fasted Saline and Fed Saline visits.



Left hand side: area of anterior OFC showing activation for viewing high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) for the Fasted Saline (green clusters) and for the Fed Saline visit (orange clusters). Yellow colour: overlap of activation for both visits. Activation thresholded at FDR P<0.05, n=17. Co-ordinates are given in standard MNI space.

Right hand side: average activation in the bilateral anterior OFC (contrast beta value) for high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) and objects relative to blurred (Blur) pictures between the Fasted Saline (green dotted bar) and Fed Saline visits (orange bar). n=17 per group, data expressed as mean activation ± SEM, *P<0.05. ROI mask determined by anterior OFC cluster (y>74) with combined contrast Fasted Saline, Fed Saline, Fed Ghrelin for high-calorie food > object contrast collapsed across visits thresholded at FDR P<0.05.

Table 5.5. Magnitude of Activation within ROIs for Fasted Saline and Fed Saline visit.

ROI	H	Fasted Saline visits			Fed Saline visit		
		High-calorie vs Object	Low-calorie vs Object	Object vs Blurred	High-calorie vs Object	Low-calorie vs Object	Object vs Blurred
Ventral striatum	L	0.042 ± 0.030	0.052 ± 0.032	0.017 ± 0.033	0.052 ± 0.048	0.013 ± 0.044	0.004 ± 0.044
	R	0.084 ± 0.039	0.082 ± 0.031	0.011 ± 0.028*	0.067 ± 0.032	0.015 ± 0.037	0.003 ± 0.047
	Bilateral	0.130 ± 0.060	0.130 ± 0.060	0.030 ± 0.060	0.120 ± 0.060	0.030 ± 0.070	0.010 ± 0.080
Amygdala	L	0.170 ± 0.063	0.097 ± 0.06	0.121 ± 0.037	0.163 ± 0.051	0.063 ± 0.046	0.087 ± 0.061
	R	0.169 ± 0.039	0.109 ± 0.052	0.102 ± 0.037	0.155 ± 0.062	0.082 ± 0.053	0.055 ± 0.071
	Bilateral	0.340 ± 0.090	0.210 ± 0.100	0.220 ± 0.070	0.320 ± 0.110	0.140 ± 0.090	0.140 ± 0.130
Insula	L	0.150 ± 0.033	0.066 ± 0.050	0.227 ± 0.022	0.130 ± 0.041	0.091 ± 0.034	0.047 ± 0.043
	R	0.123 ± 0.035	0.057 ± 0.048	0.020 ± 0.018	0.124 ± 0.038	0.110 ± 0.036	-0.013 ± 0.047
	Bilateral	0.270 ± 0.070	0.120 ± 0.100	0.050 ± 0.030	0.250 ± 0.080	0.200 ± 0.070	0.030 ± 0.090
Anterior OFC	L	0.262 ± 0.031	0.196 ± 0.063	0.139 ± 0.044	**0.134 ± 0.051****	0.139 ± 0.051	0.144 ± 0.060
	R	0.122 ± 0.06	0.070 ± 0.059	0.196 ± 0.052	0.066 ± 0.048	0.059 ± 0.049	0.168 ± 0.073
	Bilateral	0.380 ± 0.080	0.260 ± 0.110	0.330 ± 0.090	**0.200 ± 0.090***	0.170 ± 0.090	0.310 ± 0.130

Average magnitude of activation within regions of interest (ROIs) for each hemisphere (H) at the Fasted Saline and the Fed Saline visit. Beta values extracted for each individual subject contrast estimates (vs. objects) for high-calorie and low-calorie foods and objects vs. blurred picture contrast: n=17 per group, data are expressed as mean SEM, *P<0.05, **P<0.01 for Fasted Saline vs. Fed Saline visit.

5.2.8.2. Whole brain analysis

Additional brain areas were activated on viewing high-calorie foods relative to objects at the Fasted Saline visit (Table 4.8., 4.9.) and the Fed Saline visit (Table 5.6., 5.7.) using whole brain analysis.

A similar number of brain regions where activated at the Fasted and Fed Saline visit. Also the type of regions activated where similar for the high-and low-calorie food contrast (vs. objects). However, the parahippocampus and hippocampus were only activated at the Fasted Saline visit but not at the Fed Saline visit.

Table 5.6. Whole brain analysis at the Fed Saline visit for viewing high-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Occipital pole	L	5.96	-8	-92	-6	-8
Precuneous Cortex	R	4.55	28	-62	42	28
Insula Cortex	R	4.45	36	6	-16	36
Orbitofrontal Cortex	L	4.41	-26	36	-16	-26
Inferior Frontal Gyrus, pars triangularis	R	4.02	40	40	6	40
Insula Cortex	L	4.01	-38	4	-14	-38
Thalamus	L	3.81	-20	-30	-4	-20
Precuneous Cortex	L	3.68	-24	-68	44	-24
Superior Parietal Lobe	L	3.57	-32	-48	44	-32
Amygdala	R	3.51	16	0	-14	16
Inferior Frontal Gyrus, pars triangularis	L	3.3	-42	42	10	-42
Precentral Gyrus	R	3.22	50	6	22	50
Postcentral Gyrus	R	3.22	64	-16	28	64
Orbitofrontal Cortex	R	3.19	26	34	-12	26
Anterior Cingulate Gyrus	L	3.17	-4	-6	26	-4
Anterior Supramarginal Gyrus	L	3.12	-46	-32	34	-46

Activation within whole brain analysis for high-calorie picture contrast (relative to objects) at the Fed Saline visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR $P<0.05$ and minimum 5 voxel cluster size.

Table 5.7. Whole brain analysis at the Fed Saline visit for viewing low-calorie foods (vs.objects).

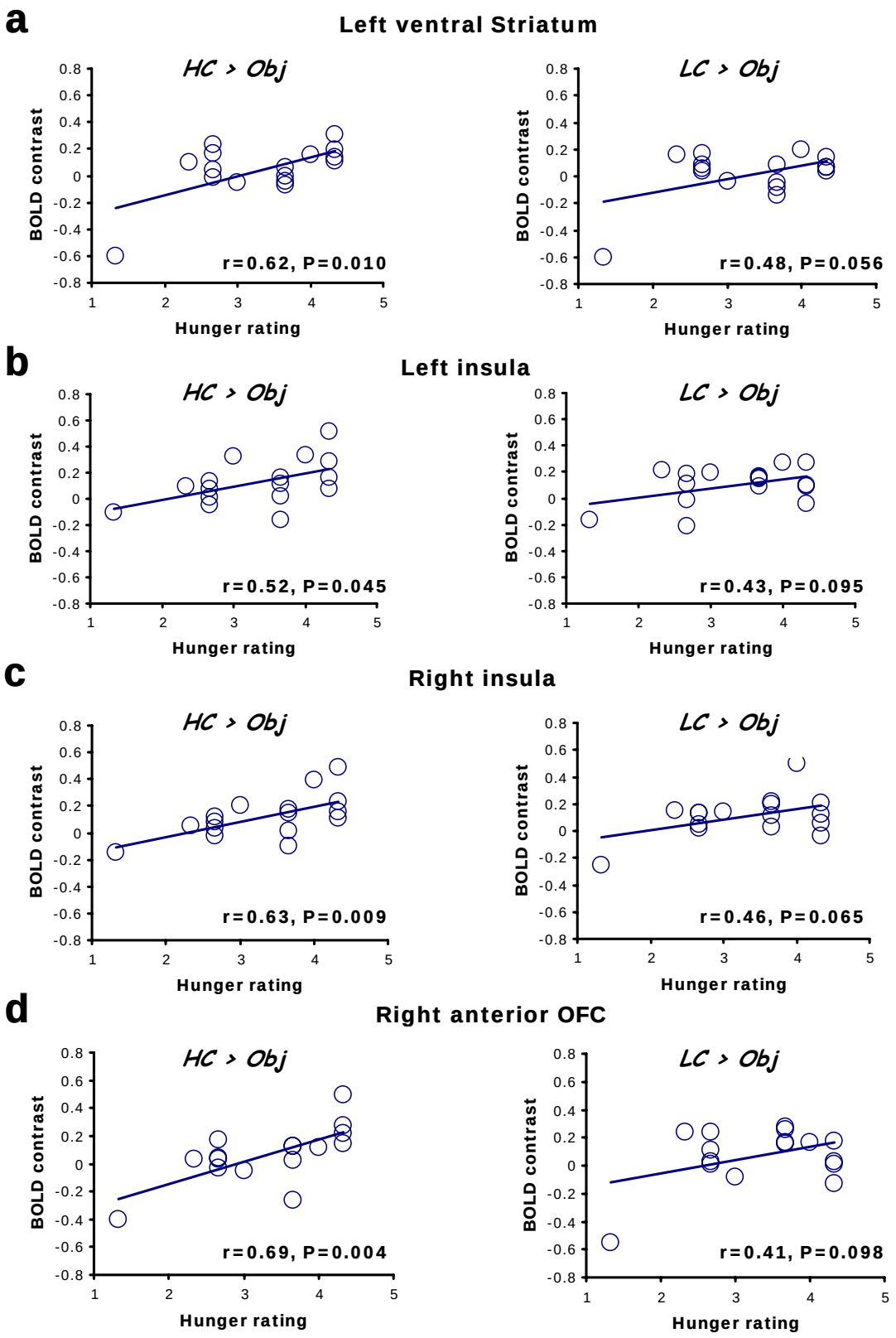
Region	H	*Z*	v	x	y	z
Occipital Pole	L	6.8	7284	-8	-94	-6
Insula Cortex	R	4.56	109	40	-2	-2
Inferior Frontal Gyrus	R	3.87	101	44	42	6
Orbitofrontal Cortex	L	4.42	77	-26	36	-16
Lateral Occipital Cortex, superior división	R	4.13	64	28	-60	42
Insula Cortex	L	4.03	38	-36	2	-12
Postcentral Gyrus	R	3.66	28	62	-14	26
Amygdala	R	3.23	8	18	0	-12
Intracalcarine Cortex	L	3.09	5	-22	-78	10
Thalamus	L	3.19	5	-22	-30	-2
Orbitofrontal Cortex	R	3.05	5	28	20	-8

Activation within whole brain analysis for low-calorie picture contrast (relative to objects) at the Fed Saline visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR P<0.05 and minimum 5 voxel cluster size.

5.2.9. Relationship between brain activation and hunger rating

At the Fasted Saline visit, activation in each ROI for each contrast did not correlate with hunger rating in the scanner (r=-0.34-0.44, P=0.08-0.19). By contrast, at the Fed Saline visit, average hunger rating in the scanner positively correlated with activation in the left ventral striatum (P=0.01), right anterior OFC (P=0.004) and left (P=0.045) and right (P=0.009) insula when viewing high-calorie foods vs. objects. Average hunger rating in the scanner also tended to positively correlate with activation in these ROIs when viewing low-calorie foods vs. objects (P=0.06-0.1). There were no significant correlations for activation when viewing objects vs. blurred pictures (r=-0.45-0.09, P>0.05) at the Fed Saline visit (Figure 5.11.). There was no positive correlation between any other ROI and the average hunger rating in the scanner for all contrasts (P>0.05).

Figure 5.11. Relationship between brain activation and hunger ratings at the Fed Saline visit.



Relationships between activation (contrast beta value) within left ventral striatum (a), left insula (b), right insula (c) and right anterior OFC (d) on viewing high-calorie (HC) foods (vs. objects (Obj) (left graphs), and low-calorie (LC) foods (vs. objects) (right graphs) and hunger rating in the scanner at the Fed Saline visit. n=16 (one subject missing due to incomplete data set), *r* represents Pearson correlation coefficient.

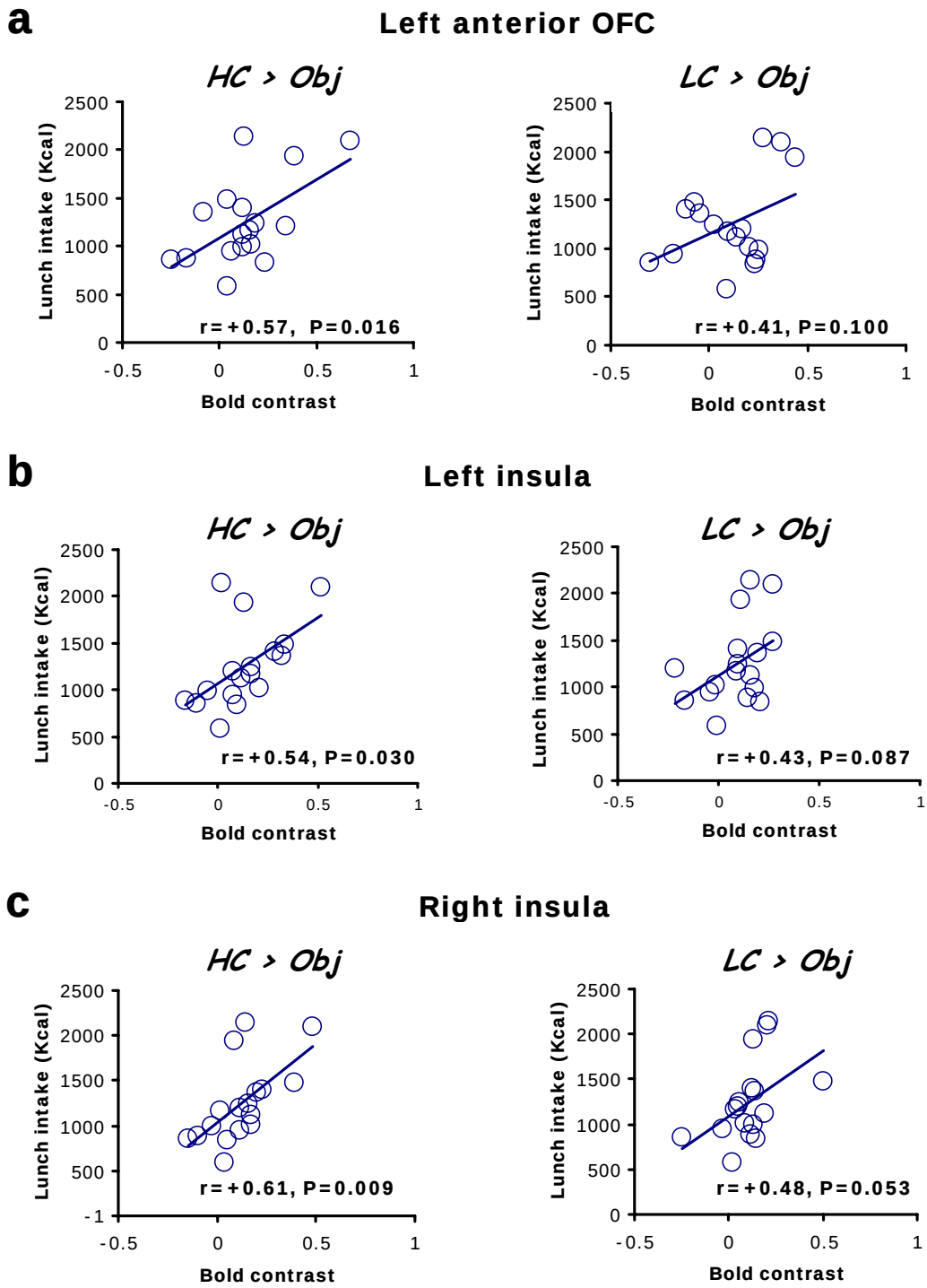
5.2.10. Relationship between brain activation and lunch intake

At the Fasted Saline visit, activation in the right insula when viewing high-calorie foods (vs. objects) positively correlated with *ad libitum* lunch intake (P=0.03) (Figure 5.13.). None of the other ROIs for any of the contrasts correlated with lunch intake at the Fasted Saline visit (r=-0.33-0.39, P=0.12-0.98).

At the Fed Saline visit, activation in the left anterior OFC (P=0.016), left (P=0.03) and right (P=0.009) insula when viewing high-calorie foods vs. objects positively correlated with *ad libitum* lunch intake (Figure 5.12.). There was no positive correlation between activation in any ROI on viewing low-calorie foods vs. objects (P=0.21-0.98) and lunch intake at the Fed Saline visit other than a tendency for the left (P=0.09) and right (P=0.05) insula to be positively correlated with lunch intake. There was no correlation between activation in any ROI on viewing objects vs. blurred pictures and lunch intake at the Fed Saline visit (P=0.19-0.81).

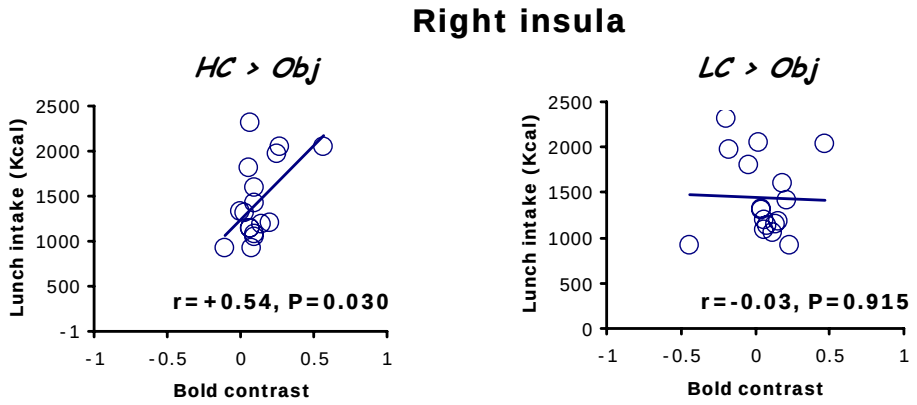
Interestingly, there was no positive correlation between VAS hunger (P=0.33 and 0.46), volume able to eat (P=0.17 and 0.29) nor pleasantness to eat (P=0.25 and 0.55) ratings just before lunch (time point +150 mins) and *ad libitum* lunch at either the Fasted Saline (Figure 5.14.) or the Fed Saline visit (Figure 5.15.).

Figure 5.12. Relationship between brain activation and lunch intake at the Fed Saline visit.



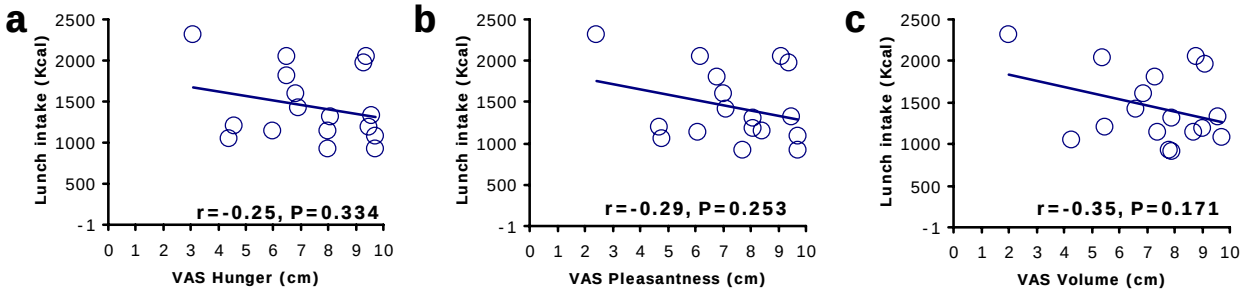
Relationship between activation (contrast beta value) within (a) left anterior OFC, (b) left insula and (c) right insula on viewing high-calorie (HC) foods (vs. objects (Obj)) (left graphs), and low-calorie (LC) foods (vs. objects) (right graphs) and *ad libitum* lunch intake at the Fed Saline visit. n=17, *r* represents Pearson correlation coefficient.

Figure 5.13. Relationship between brain activation and lunch intake at the Fasted Saline visit.



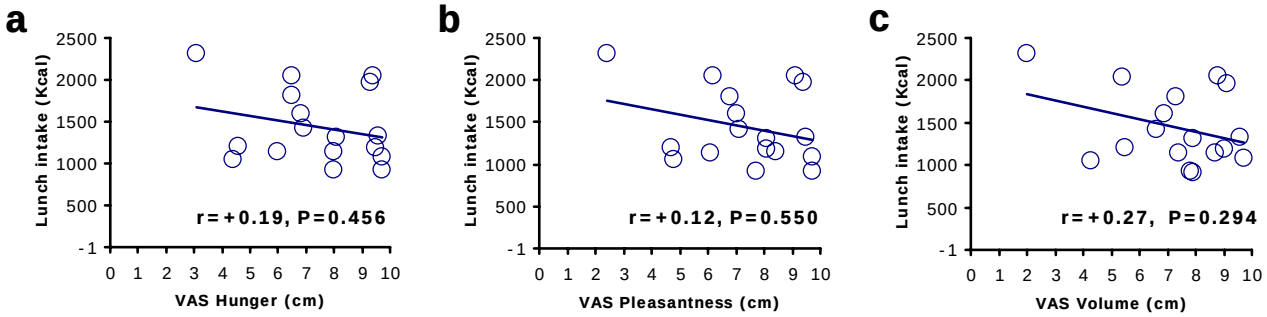
Relationship between activation (contrast beta value) within (a) right insula on viewing high-calorie (HC) foods (vs. objects (Obj)) (left graph), and low-calorie (LC) foods (vs. objects) (right graph) and *ad libitum* lunch intake at the Fasted Saline visit. n=17, *r* represents Pearson correlation coefficient.

Figure 5.14. Relationship between VAS appetite scores and lunch intake at the Fasted Saline visit.



Relationship between VAS (a) hunger-, (b) volume- and (c) pleasantness to eat ratings and *ad libitum* lunch intake at the Fasted Saline visit. n=17, *r* represents Pearson correlation coefficient.

Figure 5.15. Relationship between VAS appetite scores and lunch intake at the Fed Saline visit.



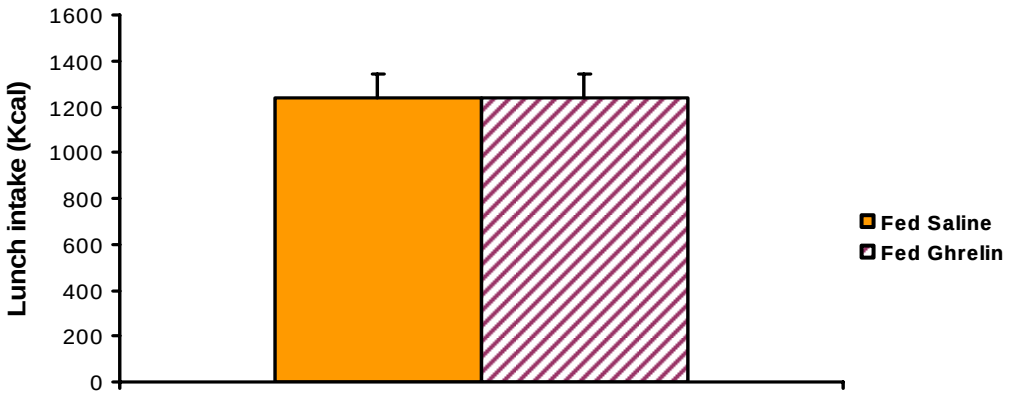
Relationship between VAS (a) hunger-, (b) volume- and (c) pleasantness to eat ratings and *ad libitum* lunch intake at the Fed Saline visit. n=17, *r* represents Pearson correlation coefficient.

5.3. Fed Saline visit versus Fed Ghrelin visit

5.3.1. Appetite visual analogue scores and food intake

Using the Area under curve (AUC), there was no significant difference in hunger (P=0.60), pleasantness (P=0.53), volume able to eat (P=0.89) and fullness (P=0.55) between the Fed Saline and the Fed Ghrelin visit (Figure 5.16.). There was also no significant difference for *ad libitum* energy intake (P=0.98), lunch tastiness (P=0.53) and pleasantness (P=0.49) between the two visits (Figure 5.17., Table 5.8.). Ghrelin was injected at t = +55 minutes, therefore the above measures were also analysed for time point t = +55, +70 and +150 minutes only, using a paired t-test. Results show that there were no significant differences in hunger (P=0.2-0.42), pleasantness (P=0.6-0.97), volume able to eat (P=0.4-0.88) and fullness (P=0.19-0.73) between the Fed Saline and the Fed Ghrelin visit (Figure 5.16.).

Figure 5.17. *Ad libitum* lunch intake.



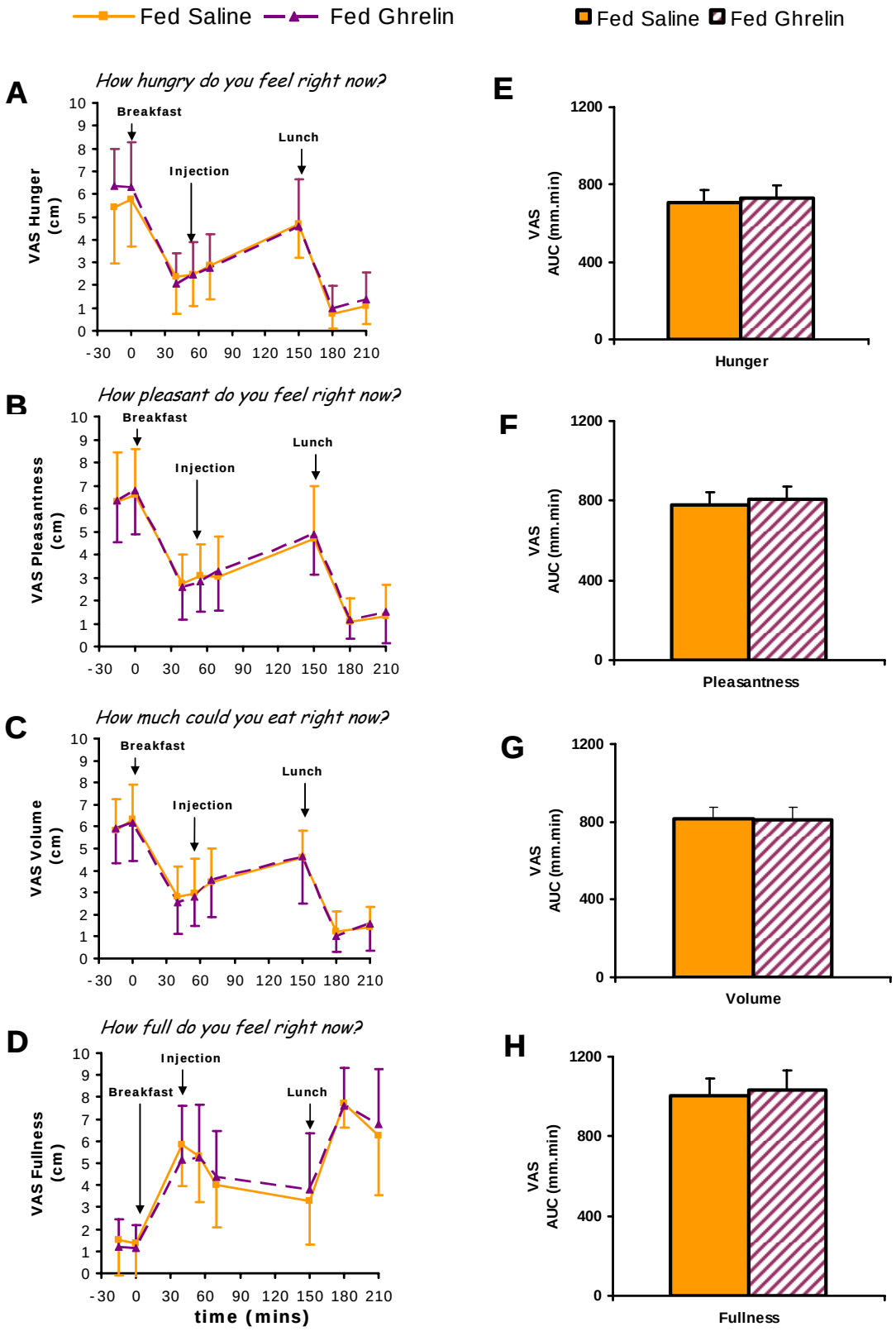
Ad libitum lunch intake for each visit separately (Fasted Saline, green dotted bar, Fed Ghrelin, orange bar), n=17 per group, data are expressed as mean ± SEM, P*<0.05.

Table 5.8. *Ad libitum* lunch intake.

	Fed Saline	Fed Ghrelin
Lunch intake		
Total lunch *in Kcal*	1237 ± 103	1235 ± 104
Lunch tastiness	5.8 ± 0.4	5.8 ± 0.3
Lunch pleasantness	6.1 ± 0.3	5.8 ± 0.3

n=17 per group, data shown as mean ± SEM, Fed Saline vs. Fed Ghrelin visit, *P<0.05.

Figure 5.16. VAS appetite scores.

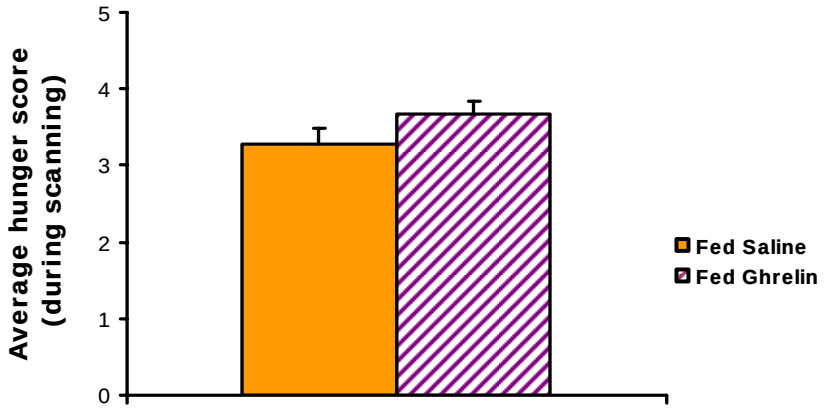


Absolute VAS scores for hunger, pleasantness to eat, volume able to eat and fullness ratings for each visit separately (Fed Saline, orange bar; Fed Ghrelin, purple spriped bar), (A-D) time course of VAS scores, (E-H) mean AUC for each score, n=15 per group (2 subjects have been excluded from the analysis because of incomplete data set), data are expressed as mean ± SEM, *P<0.05.

5.3.2. Hunger scores during MRI scanning

The average hunger rating during MRI scanning did not significantly differ between the Fed Saline and Fed Ghrelin visit but there was a tendency for hunger ratings to be greater at the Fed Ghrelin visit (P=0.10) (Figure 5.18.).

Figure 5.18. Hunger rating during scanning.

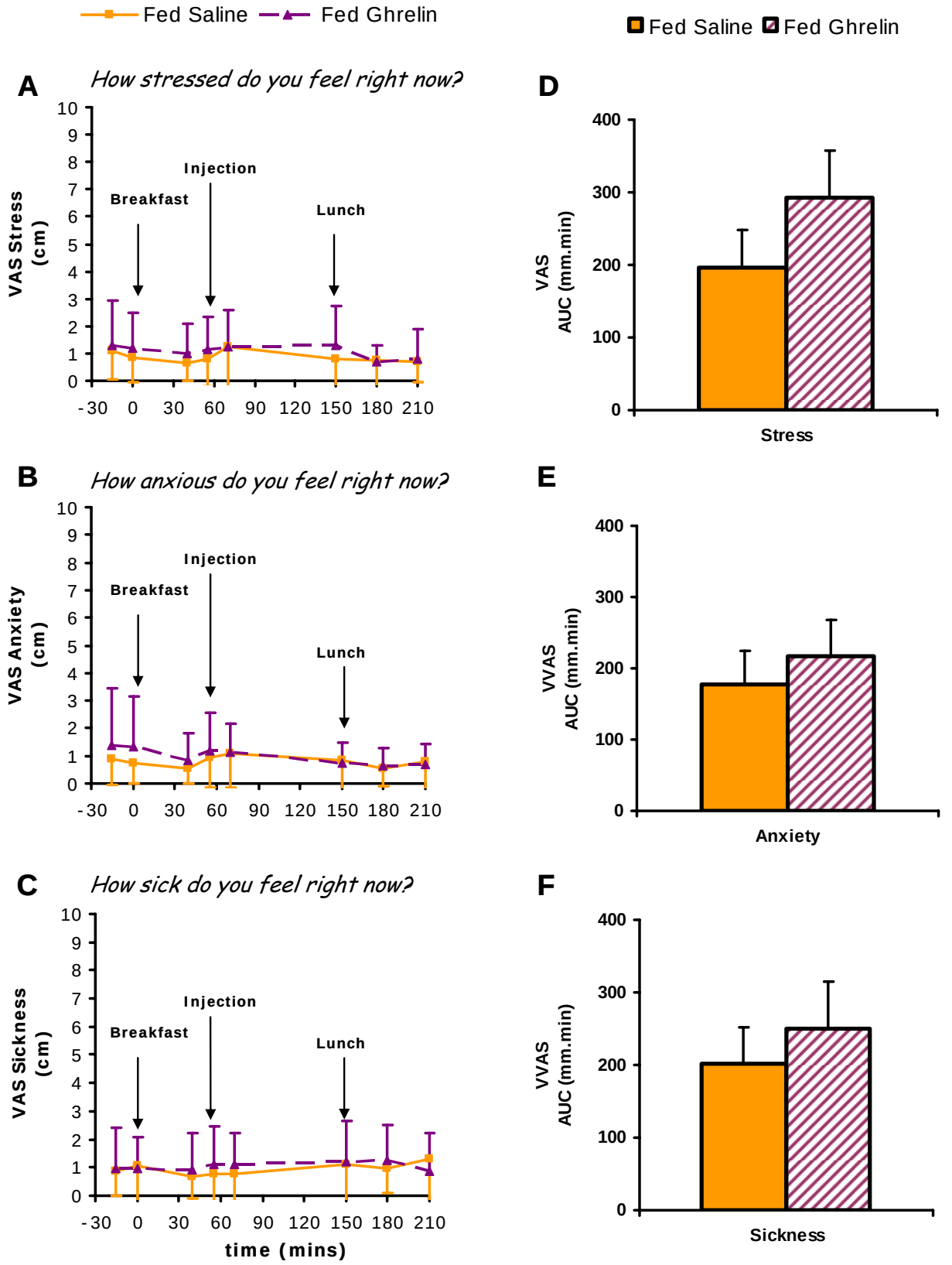


Average hunger scores during scanning for each visit separately (Fed Saline, orange bar; Fed Ghrelin, purple striped bar), n=17 per group, data are expressed as mean ± SEM, *P<0.05.

5.3.3. Stress ratings and serum stress levels

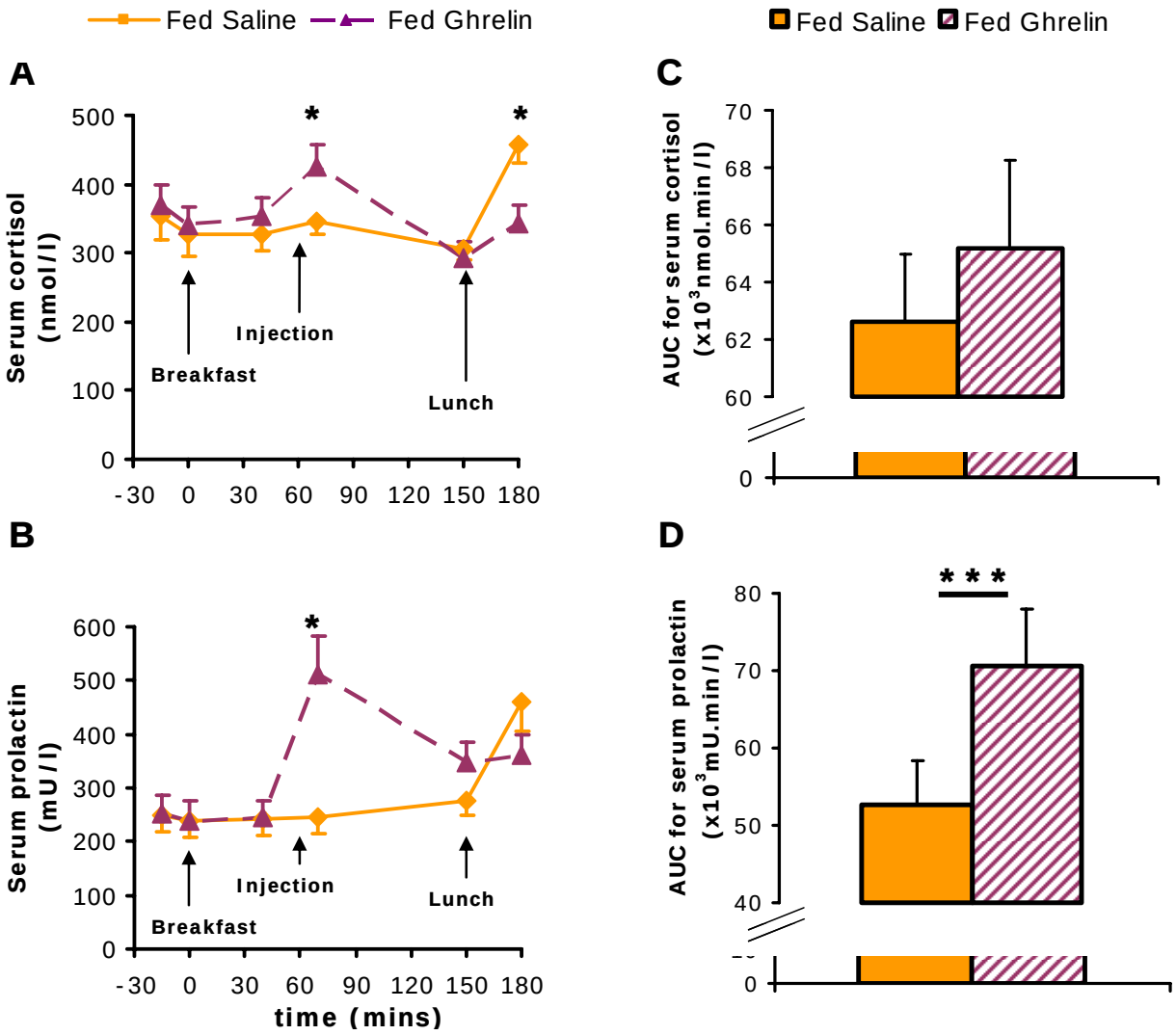
There was no significant difference in stress (P=0.15), anxiety (P=0.52) and sickness (P=0.24) AUC ratings between the Fed Saline and the Fed Ghrelin visit nor at the t= +55, t= +70 and +150 mins time points (P=0.17-0.92) (Figure 5.19.). AUC serum cortisol levels were also not significantly different between the two visits (P=0.50) although levels were significantly greater after ghrelin injection compared to injection of saline at t= +70mins, (P=0.02), but not at t= +150 mins (P=0.25) (Figure 5.20.). However, serum cortisol levels increased significantly at t = +180mins (P=0.005) on the ghrelin compared to saline injection day. AUC serum prolactin levels were significantly greater on the Fed Ghrelin visit compared to the Fed Saline visit (P<0.001) and at both t= +70 and +150 mins (P=0.002-0.01) (Figure 5.20.).

Figure 5.19. VAS stress scores.



Absolute VAS scores for stress, anxiety and sickness ratings for each visit separately (Fed Saline, orange bar and Fed Ghrelin visit, purple striped bar), (A-C) time course of stress scores, (D-F) mean AUC for each score, n=15 per group (2 subjects have been excluded from the analysis because of incomplete data set), data are expressed as mean ± SEM, *P<0.05.

Figure 5.20. Cortisol and prolactin serum levels.

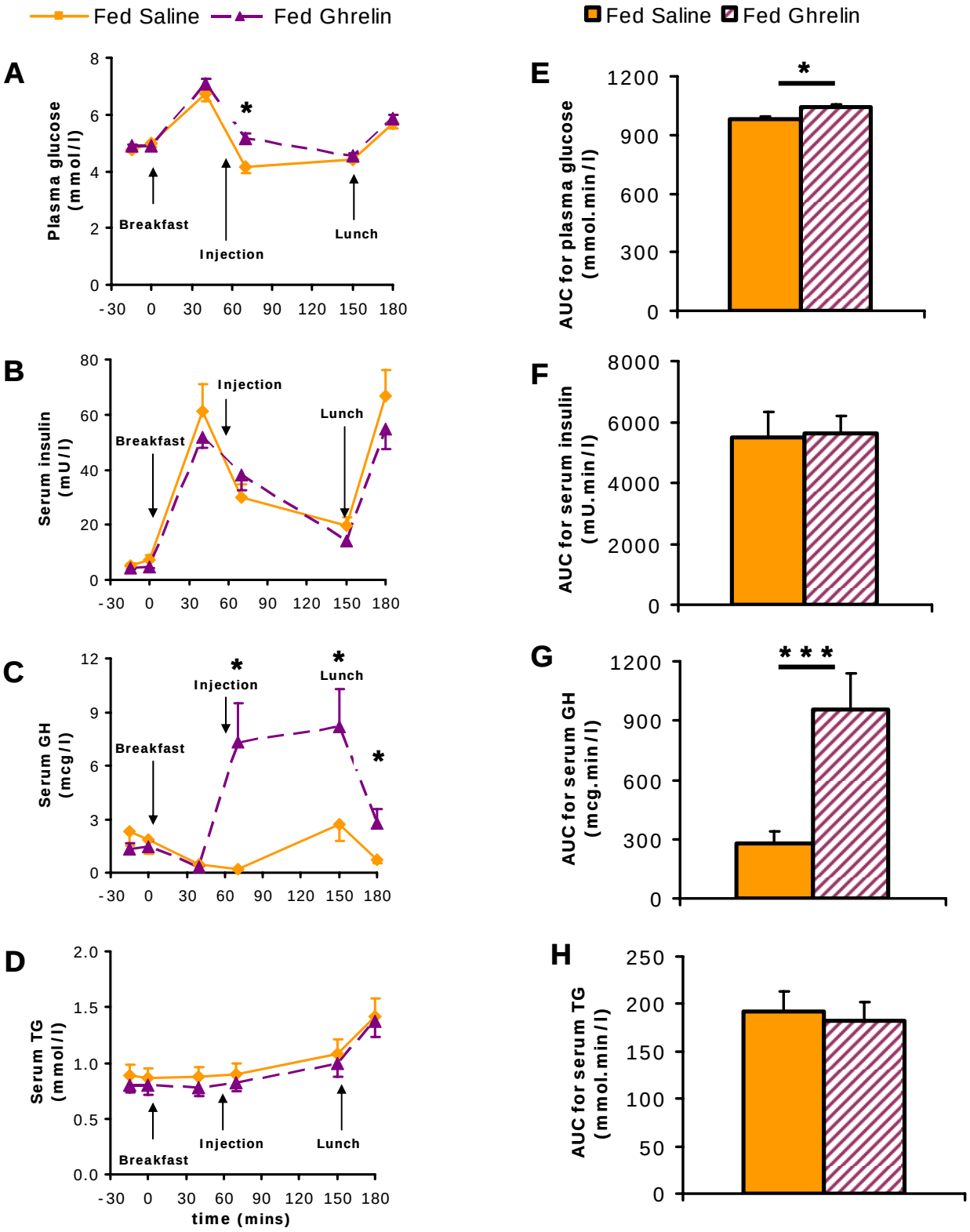


Serum cortisol and prolactin levels for each visit separately (Fed Saline, orange bar and Fed Ghrelin visit, purple striped bar), (A, B) time course of stress hormones, (C, D) mean AUC for each serum stress hormone, n=17 per group, data are expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.005.

5.3.4. Metabolic parameters

AUC plasma glucose (P=0.02) and serum growth hormone (P=0.002) levels were significantly increased at the Fed Ghrelin visit compared to the Fed Saline visit and at t= +70 (P=0.008-0.02) and +150 minutes time points (P=0.02), and for growth hormone at t = +180 minutes (P=0.02). There were no significant differences in AUC serum insulin (P=0.79) and triglyceride (P=0.44) levels between the two visits, nor at t= +70 and +150 minutes (P=0.06-0.45), except for insulin which showed a tendency to be significantly decreased at t= +70 minutes after injection of ghrelin (P=0.06) (Figure 5.21.).

Figure 5.21. Metabolic parameters.

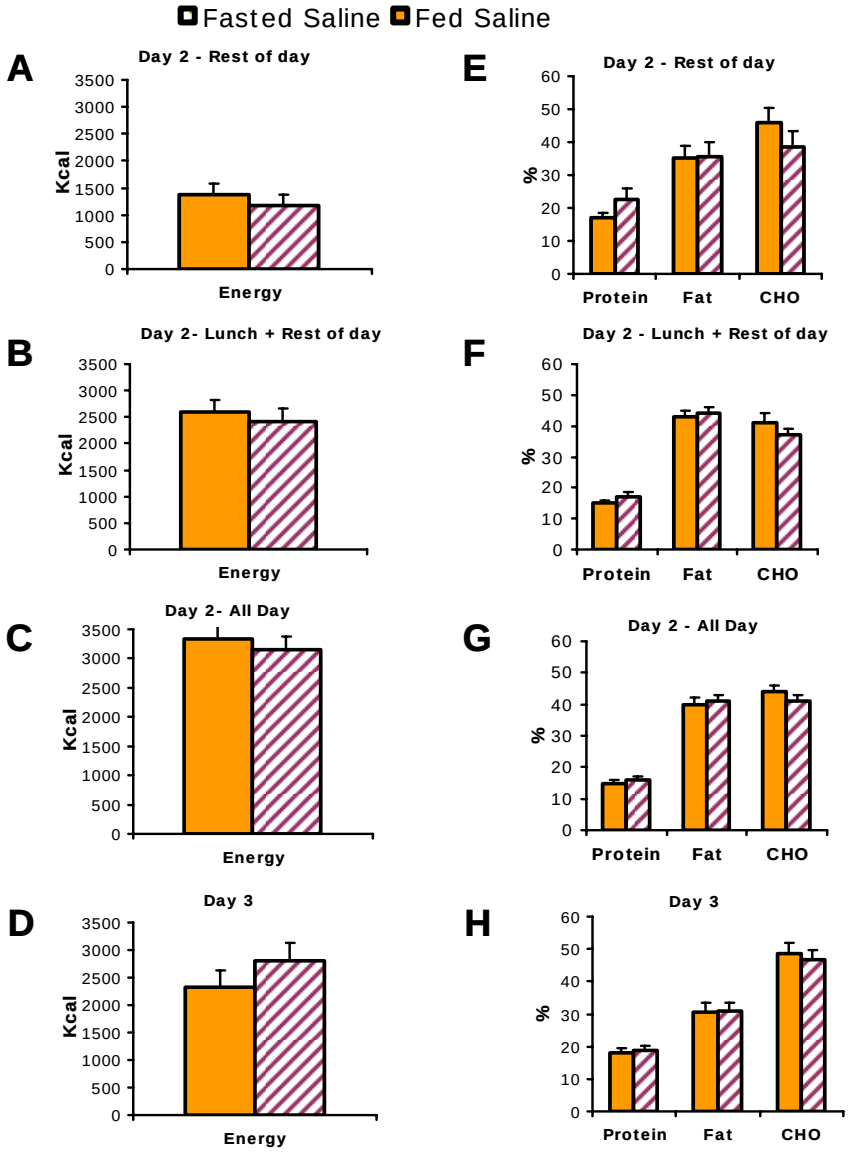


Glucose, insulin, growth hormone (GH) and tryglyceride (TG) levels for each visit separately (Fed Saline visit, orange bar and Fed Ghrelin visit, purple striped bar), (A-D) time course of metabolic parameters, (E-H) mean AUC for each metabolic parameter, n=16 per group (2 subjects have been excluded from the analysis because of incomplete data set), data are expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.005.

5.3.5. Three-day-dietary records

There were no significant differences in energy intake nor macronutrient composition over the rest of the study day after lunch, nor when breakfast and lunch and the rest of study day were combined, nor when lunch and the rest of the study day were combined, nor the day after the study visit, between the Fed Saline and Fed Ghrelin visits (P=0.23-0.56) (Figure 5.22.).

Figure 5.22. Food intake on study day and day after study.



Intake of energy (A-D) and macronutrients (E-G) over the rest of the study day (day 2), the rest of the study day combined with lunch (day2), the whole day of the study day (day 2), and one day after the study day (day 3), comparison between Fed Saline visit (green dotted bar) and Fed Ghrelin visit (orange bar), n=13 per group (4 subjects have been excluded from the analysis because of incomplete data set). Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.005.

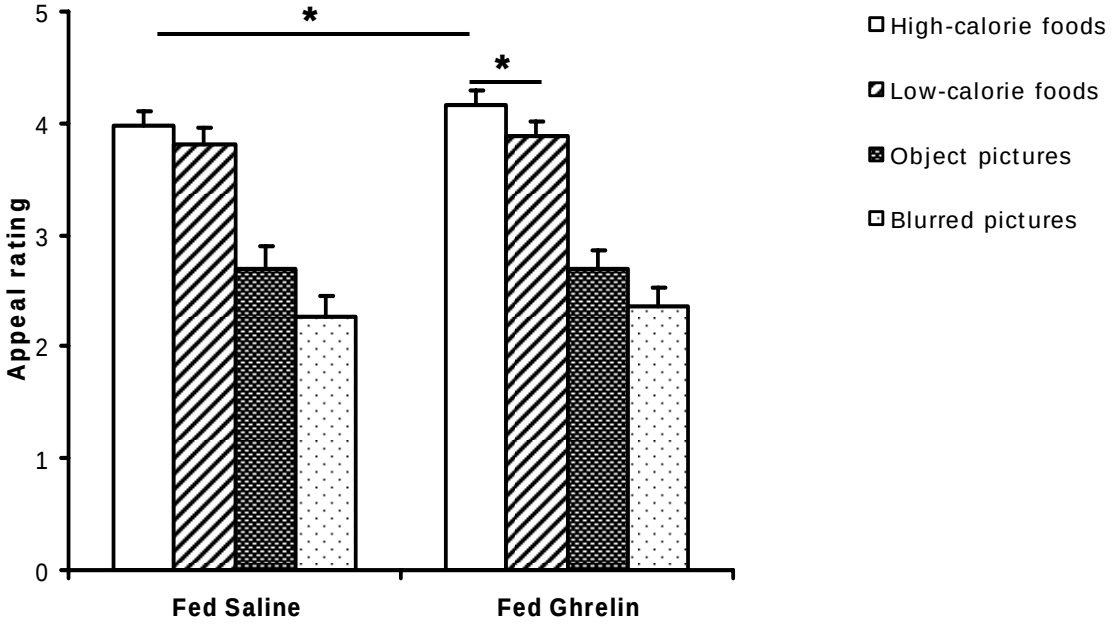
5.3.6. Food picture appeal rating

High-calorie foods were more appealing at the Fed Ghrelin visit compared to the Fed Saline visit (P=0.02) but not low-calorie food, object and blurred pictures (P=0.5-0.9) (Figure 5.23., Table 5.9.). This was significant for the chocolate high-calorie food sub-category (P=0.01) but not the sweet non-chocolate or savoury high-calorie foods (P=0.07-0.17).

High-calorie foods were more appealing than low-calorie foods at the Fed Ghrelin visit (P<0.05) but not at the Fed Saline visit (P=0.30) (Figure 5.23., Table 5.9.). These differences at the Fed Ghrelin visit were dependent on the type of high-calorie food in the picture since differences were significant for chocolate and sweet non-chocolate (P<0.05) but not savoury high-calorie foods (P=0.50) (Table 5.9.).

All categories of high-calorie foods and low-calorie foods were more appealing than both object and blurred pictures at both visits (P<0.001) (Figure 5.23., Table 5.9.). There was no significant difference in the appeal of object relative to blurred pictures at the Fed Saline visit compared to the Fed Ghrelin visit (P=0.37) (Table 5.9.).

Figure 5.23. Picture appeal rating.



Appeal rating of pictures (high-calorie foods: white bar, low-calorie foods: striped bar, non-food related household objects: black bar, blurred pictures: dotted bar), Fed Saline vs. Fed Ghrelin, n=17 per group, data are expressed as mean ± SEM, *P<0.05.

Table 5.9. Picture appeal rating.

Picture Appeal Rating	**Fed Saline visit**	**Fed Ghrelin**
Absolute rating [a]		
High-calorie foods	3.95 ± 0.12	4.15 ± 0.12*
Low-calorie foods	3.76 ± 0.15	3.85 ± 0.13
Household objects	2.64 ± 0.19	2.65 ± 0.17
Blurred	2.20 ± 0.19	2.30 ± 0.17
Chocolate high-calorie foods	4.06 ± 0.12	4.32 ± 0.13*
Sweet non-chocolate high-calorie foods	4.00 ± 0.14	4.18 ± 0.13
Savoury high-calorie foods	3.90 ± 0.17	4.00 ± 0.17
Difference in rating between [b]		
High-calorie foods & objects	1.29 ± 0.24	1.47 ± 0.23
Low-calorie foods & objects	1.12 ± 0.26	1.19 ± 0.25
Chocolate high-calorie foods & objects	1.36 ± 0.21	1.62 ± 0.23*
Sweet non-chocolate high-calorie foods & objects	1.30 ± 0.25	1.48 ± 0.24
Savoury high-calorie foods & objects	1.20 ± 0.28	1.31 ± 0.25
High-calorie & low-calorie foods	0.17 ± 0.16	0.28 ± 0.13
Chocolate high-calorie & low-calorie foods	0.24 ± 0.17	0.43 ± 0.14
Sweet non-chocolate high-calorie & low-calorie foods	0.18 ± 0.14	0.29 ± 0.12
Savoury high-calorie & low-calorie foods	0.08 ± 0.21	0.12 ± 0.17
Objects & blurred	0.44 ± 0.18	0.34 ± 0.20

Picture appeal rating ([a]1=a lot, 2=a little, 3=neutral, 4=not really, 5=not at all; [b]1st category minus 2nd category), Fed Saline vs. Fed Ghrelin, n=17 per group, data are expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.005.

5.3.7. Reaction time for appeal rating

The reaction time for appeal rating of both food categories and object pictures at the Fed Ghrelin visit was significantly shorter compared to the Fed Saline visit (P<0.05), but not for rating blurred pictures (P=0.09) (Table 5.10.).

Table 5.10. Picture rating reaction time.

Picture Rating Reaction Time (msec)	**Fed Saline visit**	**Fed Ghrelin visit**
High-calorie foods	1330 ± 38	1220 ± 38**
Low-calorie foods	1332 ± 40	1209 ± 38*
Household objects	1349 ± 39	1238 ± 40***
Blurred	1098 ± 25	1022 ± 22

Data shown as mean ± SEM, n=17 per group, Fed Saline vs. Fed GHrelin visit: *P<0.05, **P<0.01, ***P<0.005.

5.3.8. Brain activation

5.3.8.1. Region of interest (ROI) analysis

Activation in the right (P=0.04) and bilateral (P=0.04) anterior OFC on viewing high-calorie foods vs. objects at the Fed Ghrelin visit was significantly greater compared to the Fed Saline visit. There was a tendency for activation in left anterior OFC (P=0.06) on viewing high-calorie foods vs. objects, and also for low-calorie foods vs. objects in the left, right and bilateral anterior OFC (P=0.07-0.11), to be greater at the Fed Ghrelin than Fed Saline visit (Figure 5.24., Table 5.11.).

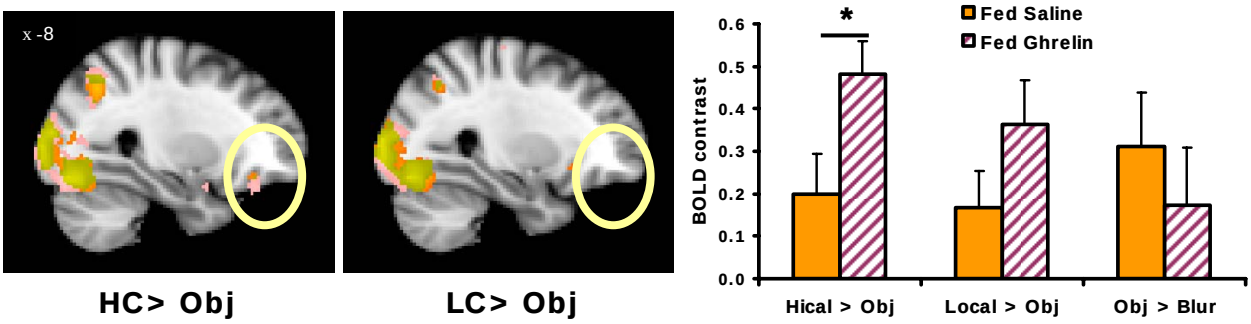
There was no significant difference in activation in the ventral striatum, insula and amygdala on either side or bilaterally when viewing high-calorie foods vs. objects or low-calorie foods vs. objects between the Fed Saline and the Fed Ghrelin visit (P=0.14-0.98) (Table 5.11).

Activation in the ventral striatum, insula, amygdala and anterior OFC when viewing objects vs. blurred pictures was not significant different between the Fed Saline and Fed Ghrelin visits (P=0.12-0.89) (Table 5.11.).

When observing statistical activation maps for individual visits, there was evidence of significant activation in the left ventral striatum at the Fed Ghrelin visit when viewing high-calorie foods vs. objects which was not apparent at the Fasted Saline and Fed Saline visits nor on viewing low-calorie foods vs. objects. However such comparisons can be misleading because of activation for some visits and contrasts being at just sub-threshold levels.

Nevertheless there was a weak tendency for activation in the left ventral striatum on viewing high-calorie foods vs. objects to be greater at the Fed Ghrelin visit compared to the Fed Saline visit (P=0.14). As discussed in Chapter 6.2. this had the effect of tending to reverse the decrease in ventral striatum activation to high-calorie foods seen after the initial Fasted Dummy visit (Figure 5.25. A-D).

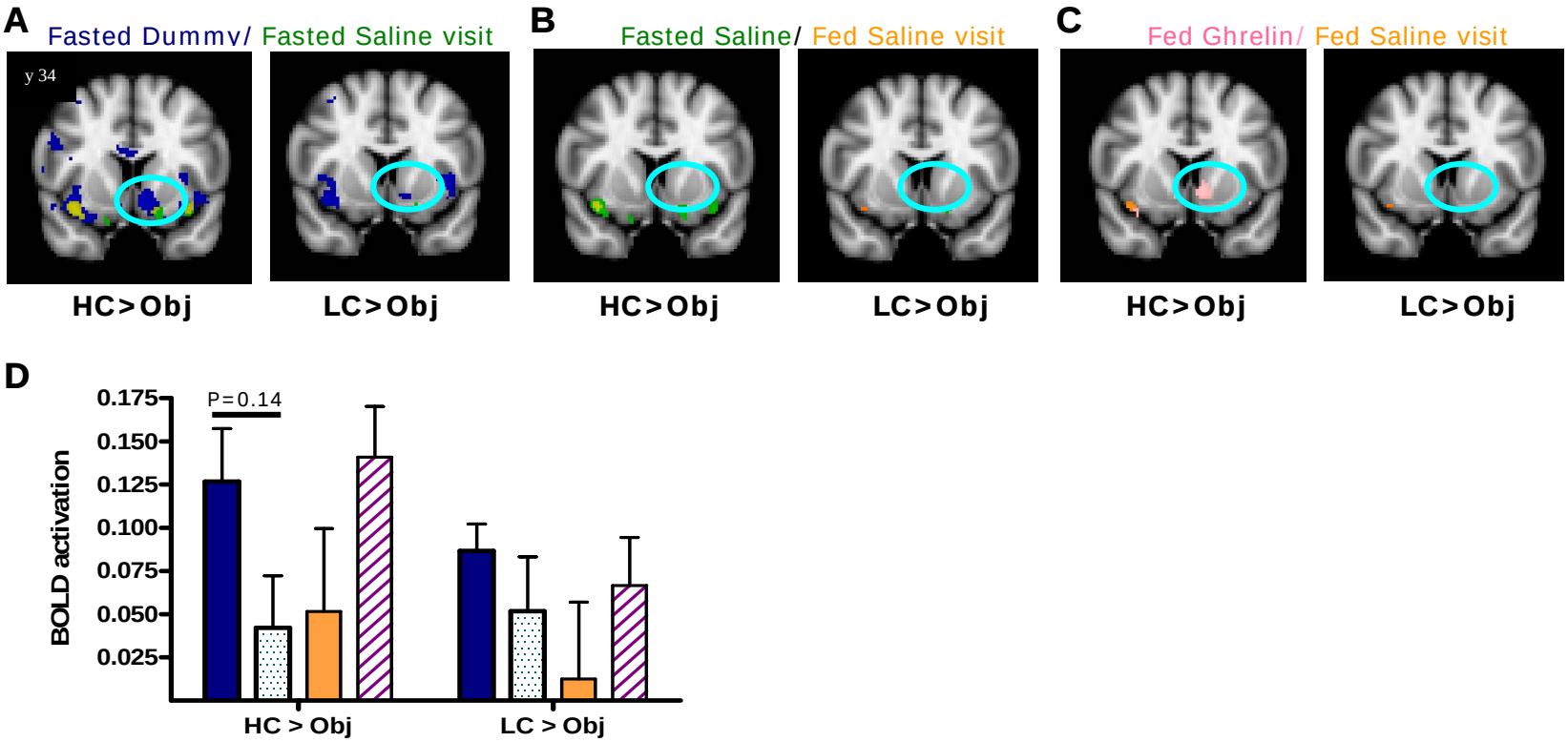
Figure 5.24. Anterior OFC activation for Fed Saline and Fed Ghrelin visit.



Left hand side: area of anterior OFC showing activation for viewing high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) for the Fed Saline (orange clusters) and for the Fed Ghrelin visit (pink clusters). Yellow colour: overlap of activation for both visits. Activation thresholded at FDR P<0.05, n=17. Co-ordinates are given in standard MNI space.

Right hand side: average activation in the bilateral anterior OFC (contrast beta value) for high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) and objects relative to blurred (Blur) pictures between the Fed Saline (orange bar) and the Fed Ghrelin visit (striped purple bar). n=17 per group, data expressed as mean activation ± SEM, *P<0.05. ROI mask determined by anterior OFC cluster (y>74) from combined contrast Fasted Saline, Fed Saline, Fed Ghrelin for high-calorie food > object contrast collapsed across visits thresholded at FDR P<0.05.

Figure 5.25. Ventral striatum activation for Fed Saline and Fed Ghrelin visit.



Top figure: area of ventral striatum showing activation for viewing high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) for the (A) Fasted Dummy visit (dark blue cluster) and Fasted Saline visit (green cluster), (B) Fasted Saline visit (green cluster) and Fed Saline visit (orange cluster) and (C) for the Fed Ghrelin visit (pink clusters) and the Fed Saline visit (orange cluster). Yellow colour: overlap of activation for both visits. Activation thresholded at FDR P<0.05, n=17. Co-ordinates are given in standard MNI space.

Bottom figure: average activation in the left ventral striatum (contrast beta value) for high-calorie (HC) and low-calorie (LC) foods relative to objects (Obj) and objects relative to blurred (Blur) pictures between the Fasted Dummy (dark blue bar), Fasted Saline (dotted green bar), Fed Saline (orange bar), and Fed Ghrelin (striped purple bar) visits. n=17 per group, data expressed as mean activation ± SEM, *P<0.05. ROI mask determined by overlap of Harvard FSL atlas for nucleus accumbens with combined contrast Fasted Saline, Fed Saline, Fed Ghrelin for high-calorie foods > object contrast collapsed across visits FDR P<0.05.

Table 5.11. Magnitude of Activation within ROIs for Fed Saline and Fed Ghrelin visit.

ROI	H	Fed Saline visit			Fed Ghrelin visit		
		High-calorie vs Object	Low-calorie vs Object	Object vs Blurred	High-calorie vs Object	Low-calorie vs Object	Object vs Blurred
Ventral striatum	L	0.052 ± 0.0448	0.013 ± 0.044	0.028 ± 0.040	0.141 ± 0.029	0.067 ± 0.028	0.062 ± 0.041
	R	0.067 ± 0.032	0.015 ± 0.037	0.003 ± 0.047	0.108 ± 0.019	0.065 ± 0.027	-0.005 ± 0.035
	Bilateral	0.120 ± 0.060	0.030 ± 0.070	0.010 ± 0.080	0.250 ± 0.040	0.130 ± 0.050	-0.070 ± 0.050
Amygdala	L	0.163 ± 0.051	0.063 ± 0.046	0.087 ± 0.061	0.168 ± 0.037	0.133 ± 0.053	0.126 ± 0.053
	R	0.155 ± 0.062	0.082 ± 0.053	0.055 ± 0.071	0.187 ± 0.052	0.111 ± 0.050	0.141 ± 0.053
	Bilateral	0.320 ± 0.110	0.140 ± 0.090	0.140 ± 0.130	0.350 ± 0.080	0.240 ± 0.100	0.270 ± 0.100
Insula	L	0.130 ± 0.041	0.091 ± 0.034	0.047 ± 0.043	0.116 ± 0.030	0.065 ± 0.030	0.074 ± 0.038
	R	0.124 ± 0.038	0.110 ± 0.036	-0.013 ± 0.047	0.125 ± 0.028	0.085 ± 0.033	0.030 ± 0.030
	Bilateral	0.250 ± 0.080	0.200 ± 0.070	0.030 ± 0.090	0.240 ± 0.050	0.150 ± 0.060	0.100 ± 0.060
Anterior OFC	L	0.134 ± 0.051	0.107 ± 0.048	0.144 ± 0.06	0.290 ± 0.044	0.201 ± 0.050	0.078 ± 0.073
	R	0.066 ± 0.048	0.059 ± 0.049	0.168 ± 0.073	**0.191 ± 0.045***	0.162 ± 0.061	0.096 ± 0.067
	Bilateral	0.200 ± 0.090	0.170 ± 0.090	0.310 ± 0.130	**0.480 ± 0.080***	0.360 ± 0.100	0.170 ± 0.140

Average magnitude of activation within each regions of interest (ROI) for each hemisphere (H) at the Fed Saline visit and the Fed Ghrelin visit. Beta values extracted for each individual subject contrast estimates (vs. objects) for high-calorie and low-calorie foods (n=17 per group) and objects vs. blurred picture contrast: *P<0.05 for Fed Saline visit vs. Fed Ghrelin visit.

5.3.8.2. Whole brain analysis

Additional brain areas were activated on viewing high-calorie foods relative to objects at the Fed Saline visit (Table 5.6., 5.7.) and the Fed Ghrelin (Table 5.12. and 5.13.) visit using whole brain analysis.

The number of activated brain areas when viewing particularly high-calorie foods was increased at the Fed Ghrelin visit but not at the Fed Saline visit. Especially the hippocampus, parahippocamous and accumbens are only activated on the Fed Ghrelin visit but not at the Fed Saline visit.

When comparing all four study visits the results show activation especially in the accumbens only at the Fasted Dummy visit and Fed Ghrelin visit, in the parahippocampus and hippocampus only at the Fasted Saline and Fed Ghrelin visit and in the amygdala only at the Fasted Saline and Fed Saline visit. The number of brain regions was notably high at the Fasted Dummy and the Fed Ghrelin visit and low at the Fasted and Fed Saline visit for both contrasts (H>O, L>O). In general, the number of areas activated when viewing low-calorie foods compared to when viewing high-calorie foods was reduced for all visits. All areas had activation in the visual cortices (Occipital cortex and intracalcarine cortex).

Table 5.12. Whole brain analysis at the Fed Ghrelin visit for viewing high-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Occipital pole	L	6.32	8257	-14	-94	-2
Orbitofrontal Cortex	L	4.64	201	-26	34	-16
Insula Cortex	L	4.19	105	-36	-4	4
Parahippocampus Gyrus	L	4.13	129	-18	-2	-20
Accumbens	L	4.1	243	-8	8	-4
Hippocampus	L	4.01	205	-26	-30	-4
Lateral Occipital Cortex	R	4.04	144	30	-60	50
Orbitofrontal Cortex	R	3.97	70	26	36	-20
Insula Cortex	R	3.9	139	42	-4	0
Parahippocampus Gyrus	R	3.79	134	14	-30	-8
Inferior Frontal Gyrus	L	3.69	50	-42	38	8
Supramarginal Gyrus, anterior division	L	3.68	30	-62	-22	34
Paracingulate Gyrus	L	3.53	41	-6	48	-8
Anterior Cingulate Gyrus	R	3.47	62	2	40	0
Postcentral gyrus	L	3.41	51	-46	-18	42
Posterior Cingulate Gyrus	L	3.33	82	-2	-34	30
Accumbens	R	3.36	40	8	16	-2
Inferior Frontal Gyrus	R	3.6	39	44	40	8
Thalamus	R	3.43	33	12	-2	8
Precentral Gyrus	R	3.3	19	46	4	20
Pallidum	R	3.28	16	16	-4	-2
Lateral Occipital Cortex	L	3.18	23	-46	-62	-8
Superior Frontal Gyrus	L	3.15	14	-16	-8	56
Parietal Operculum	L	3.07	8	-38	-38	28
Subcallosal Cortex	R	3.05	7	2	20	2

Activation within whole brain analysis for high-calorie picture contrast (relative to objects) at the Fed Ghrelin visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR $P<0.05$ and minimum 5 voxel cluster size.

Table 5.13. Whole brain analysis at the Fed Ghrelin visit for viewing low-calorie foods (vs.objects).

Region	H	*Z*	v	x	y	z
Occipital Pole	R	6.99	7700	12	-90	-2
Orbitofrontal Cortex	L	4.27	121	-24	36	-16
Insula Cortex	R	4.38	81	40	-4	4
Insula Cortex	L	4.37	67	-38	-6	8
Postcentral Gyrus	L	3.67	39	-64	-16	22
Orbitofrontal Cortex	R	3.44	38	20	34	-24
Posterior Cingulate Gyrus	L	3.19	31	-4	-36	26
Lateral Occipital Cortex, superior division	R	3.41	29	28	-62	46
Supramarginal Gyrus	L	3.28	15	-48	-36	34
Precentral Gyrus	L	3.41	13	-52	-12	40
Hippocampus	R	3.52	13	24	-28	-6
Precentral Gyrus	R	3.35	10	48	-12	54
Inferior Frontal Gyrus	L	3.09	5	-50	6	22

Activation within whole brain analysis for low-calorie picture contrast (relative to objects) at the Fed Ghrelin visit (n=17 per group). Results represent co-ordinates of the peak voxel (x, y, z in MNI space (in mm)) of the cluster for each hemisphere (H), number of voxels within cluster (v, 2x2x2mm). Threshold correction for multiple corrections: FDR P<0.05 and minimum 5 voxel cluster size.

5.4. Summary results ghrelin fMRI study

VAS hunger, pleasantness to eat, volume able to eat were significantly greater and fullness ratings significantly lower at the Fasted Saline visit compared to the Fed Saline visit, but not at the Fed Ghrelin visit compared to the Fed Saline visit. Hunger rating during MRI scanning was significantly greater at the Fasted Saline visit compared to the Fed Saline visit, and there was a tendency for hunger rating during scanning to be greater at the Fed Ghrelin visit compared to the Fed Saline visit.

Ad libitum lunch intake was 15% greater at the Fasted Saline visit compared to the Fed Saline visit and there was a tendency for lunch pleasantness to be greater at the Fasted Saline visit, but there was no significant difference in lunch tastiness between the two visits. However there was no significant difference for *at libitum* lunch intake, tastiness and pleasantness between the Fed Saline and the Fed Ghrelin visits.

Analysis of VAS stress ratings revealed that there were no significant differences in stress, anxiety and sickness, between the Fasted and Fed Saline, and the Fed Saline and Fed Ghrelin visits although there was a tendency of stress and sickness to be greater after ghrelin injection at the Fed Ghrelin visit. Serum prolactin and cortisol levels were significantly elevated after the injection at the Fed Ghrelin visit compared to the Fed Saline visit, but not at the Fasted Saline compared to Fed Saline visit.

Analysis of metabolic parameters revealed that serum insulin and triglyceride levels but not plasma glucose and serum growth hormone levels were increased at the Fed Saline visit compared to the Fasted Saline visit. At the Fed Ghrelin visit plasma glucose and serum growth hormone levels but not serum insulin and triglyceride levels were significantly higher compared to the Fed Saline visit.

High-calorie foods were more appealing than low-calorie foods at the Fasted Saline and Fed Ghrelin visits but not at the Fed Saline visit. Chocolate and sweet non-chocolate but not savoury high-calorie foods were more appealing than low-calorie foods at the Fasted Saline and Fed Ghrelin visits, but not the Fed Saline visit. High-calorie foods and low-calorie foods were more appealing relative to object pictures at each of the three visits. There was no significant difference in the appeal of object relative to blurred pictures between any of the three visits. Interestingly, the reaction time for appeal rating of both food categories and object pictures at the Fed Ghrelin visit was significantly shorter compared to the Fed Saline visit but not for rating blurred pictures. These differences in reaction time for appeal rating were not seen between the Fasted and Fed Saline visit.

At both the Fasted Saline and Fed Ghrelin visits, anterior OFC activation on viewing high-calorie foods was significantly greater than the Fed Saline visit. There was a trend for the anterior OFC activation to low-calorie foods to be greater at the Fed Ghrelin than Fed Saline visit, but this was not seen for the Fasted Saline compared to the Fed Saline visit. There was no significant difference in activation in any other ROI for any other picture contrast between any of the three visits.

There was a weak trend for activation in the ventral striatum on viewing high-calorie foods to be greater at the Fed Ghrelin visit than the Fed Saline visit. This trend was was not apparent for the Fasted Saline compared to the Fed Saline nor for the low-calorie foods. Interestingly this ventral striatum activation to high-calorie foods was also apparent at the Fasted Dummy visit but was less apparent at the Fasted Saline visit (see discussion 6.3.2.).

This study was thus able to demonstrate that fasting and ghrelin both increased the appeal of high-calorie over low-calorie foods and increased anterior OFC activation to high-calorie foods. The effects on OFC activation to low-calorie foods were less marked, with only a trend seen for ghrelin administration.

At the Fed Saline visits, individual activation in the ventral striatum, anterior OFC and insula when viewing high-calorie foods positively correlated with individual hunger ratings in the scanner. The correlations with activation to low-calorie foods showed similar trends. No such correlations were seen at the Fasted Saline visits.

At the Fed Saline visit, activation in the anterior OFC, and insula when viewing high-calorie foods correlated positively with *ad libitum* lunch intake with a trend for activation to low-calorie foods. At the Fasted Saline visit, only activation in the insula when viewing high-calorie foods positively correlated with *ad libitum* lunch intake with no trend seen for low-calorie foods. By contrast, there was no positive correlation between VAS appetite ratings just before lunch and *ad libitum lunch* at either the Fed Saline or Fasted Saline visits.

Chapter 6

DISCUSSION

CONCLUSION

FUTURE WORK

6.1. Discussion for Fed–Fasted fMRI study and Fasted Saline vs. Fed Saline comparison from ghrelin fMRI study

6.1.1. The main findings of the fed-fasted fMRI are:

(i) Greater hunger, pleasantness to eat, volume able to eat and less fullness when fasted compared to when fed.

(ii) Food picture appeal bias towards high-calorie foods when fasted.

(iii) Ventral striatum, amygdala, insula, medial and lateral OFC show greater activation to high-calorie compared to low-calorie foods, when fasting but not when fed.

(iv) Individual differences in appeal of high-calorie foods positively correlated with individual differences in hunger and pleasantness to eat between fasted and fed visits.

(v) Individual differences in appeal bias to high-calorie over low-calorie foods positively correlated with differences in activation in both the medial OFC and lateral OFC for the high-calorie vs. low-calorie contrast between fasted and fed visits.

6.1.2. The main findings of the Fasted Saline vs. Fed Saline comparison from the ghrelin study are:

(i) Greater hunger, pleasantness to eat, volume able to eat and less fullness at the Fasted Saline visit compared to the Fed Saline visit.

(ii) *Ad libitum* lunch intake was increased by 15% at the Fasted Saline visit compared to the Fed Saline visit and there was a tendency for lunch pleasantness (but not tastiness) to be greater on the Fasted Saline visit.

(iii) High-calorie foods were more appealing at the Fasted Saline visit compared to the Fed Saline visit but not low-calorie foods.

(iv) Activation in the anterior OFC to high-calorie foods was significantly increased at the Fasted Saline compared to the Fed Saline visit but not low-calorie foods.

(v) At the Fed Saline visit, but not the Fasted Saline visit, hunger rating positively correlated with activation in the ventral striatum, anterior OFC and insula to high-calorie foods with a similar trend for low-calorie foods.

(vi) At the Fed Saline visit, activation in the insula and anterior OFC when viewing high-calorie foods correlated with lunch intake with a similar trend for low-calorie foods.

(vii) At the Fasted Saline visit, activation in the insula when viewing high-calorie foods positively correlated with lunch intake, but not for low-calorie foods.

(viii) There was no positive correlation between VAS appetite scores (just before lunch) and lunch intake at either fed or fasted visits.

6.1.3. Effect of nutritional state on food appeal

In the two fMRI studies, hunger and pleasantness were increased and fullness was decreased at the fasted visits compared to the fed visits.

It is suggested that subjects' palatability of food is altered in the fed, compared with the fasting state (Berridge, 1991). The two fMRI studies found that high-calorie foods were more appealing than low-calorie foods at the fasted visit but not at the fed visit. These results were further supported by data from the first fed/fasted study when individual differences in the appeal of high-calorie foods between the fed and fasted state also correlated with the individual differences in hunger and pleasantness to eat.

The second Fasted / Fed Saline study also showed that *ad libitum* lunch intake at the Fasted Saline visit was significantly increased compared to the Fed Saline visit and that there was a tendency for lunch pleasantness to be greater at the Fasted Saline visit. These findings confirm that nutritional state (fasting, e.g. not eating breakfast) impacts on the appeal and pleasantness of food (Cabanac, 1971; Uher et al., 2006; Stoeckel et al., 2007).

Cross-sectional studies have shown connections between lack of breakfast consumption, and increased daily fat intake, subsequent consumption of high fat snacks, and more binge eating disorder in children and adults (Rampersaud et al., 2005; Masheb and Grilo, 2006; Niemeier et al., 2006; van der Heijden et al., 2007; Dubois et al., 2008;

Marin-Guerrero et al., 2008; Timlin et al., 2008). Missing breakfast is positively related to greater weight gain in normal-weight adolescents (Berkey et al., 2003; Timlin et al., 2008) and during the transition between adolescence to early adulthood (Rampersaud et al., 2005; Niemeier et al., 2006; van der Heijden et al., 2007). Eating breakfast is linked to healthier food choices such as more vegetables and milk, fewer soft drinks, and a lower intake of french fries in children and adults (Rampersaud et al., 2005; van der Heijden et al., 2007; Dubois et al., 2008). Thus, the findings from these studies suggest a potential brain reward mechanism influencing food preference by which fasting may lead to weight gain. Indeed, fasting leads to increased impulsive snacking and fat intake in obese individuals in a caloric restriction weight-reduction programme (Schlundt et al., 1992). The results of these studies however would therefore support the advice for regular consumption of a healthy breakfast (Rampersaud et al., 2005).

The effect of skipping breakfast on subsequent intake of fatty, calorie-rich foods and impulsive snacking depends on the usual breakfast habits (Schlundt et al., 1992). The majority of the subjects of the two studies ate breakfast regularly. However, there was a difference in the type of breakfast subjects were given in the two fMRI studies. At the first fed-fasted fMRI study they were instructed to eat a filling breakfast of their own choice whereas in the Fasted / Fed Saline fMRI study they were given a fixed breakfast. Being able to choose a breakfast such as in the first fMRI study will lead people to eat what they like, whereas in the second study the breakfast was probably not the most favourite food for all the volunteers. Furthermore even though the calorie content of breakfast for the second study was chosen to be the same as the average for the first study, an identical amount of calories was given for for men and women and for subjects of different size. Thus, the food type and volume of the breakfast relative to body habits were different in the two studies. This may explain why differences between appetite ratings between the fasted and fed visit were greater at the first fed-fasted study compared to the second Fasted / Fed Saline study. These differences in appetite ratings between the two studies were paralleled in the food picture appeal ratings. Both studies showed that food picture

appeal is biased towards high-calorie foods when not eating breakfast. However, the differences of appeal ratings were greater between the fed and fasted visit in the first fed-fasted study where subject had a filling breakfast of their own choice.

This could explain why although skipping breakfast in the second fMRI study led to greater food intake at lunch it did not lead to any overall reduction in energy intake or macronutrient content between the Fasted and Fed Saline visit over the whole study day. Another explanation is that the test lunch contained only one savoury high-calorie food which may have impaired the ability to separate differences in food intake between the visits. This assumption could be underpinned with the appeal rating data which showed that although high-calorie foods were more appealing than low-calorie foods at the Fasted Saline visit this significance was dependent on the type of high-calorie food: being significant for chocolate or sweet non-chocolate but not savoury high-calorie foods.

In future studies it would be interesting to vary the breakfast size according to the subjects dietary needs calculated using resting energy expenditure which adjusts for age, gender and weight, and to give a test lunch buffet consisting of a mixture of sweet and savoury low-calorie and high-calorie foods.

6.1.4. Effect of food type on brain reward systems

The sensory properties of foods play a very important role in the way people select their food and how much they eat (Sorensen et al., 2003; Stoeckel et al., 2008). The findings of the first fed-fasted study showed that viewing pictures of high-calorie over low-calorie foods increased activation in the ventral striatum, amygdala, anterior insula, and medial and lateral OFC. The second Fasted/ Fed Saline study showed that viewing pictures of high-calorie foods over objects increased activation in the anterior OFC.

These findings agree with earlier PET and BOLD imaging research in non-obese healthy volunteers, that compared to non-food-pictures, visual food stimuli induce greater activity in the amygdala, ACC, insula, OFC, DLPFC and striatum (Gordon et al., 2000, LaBar et al., 2001, Morris and Dolan, 2001, Wang et al., 2004, Holsen et al., 2005, Simmons et al., 2005, St-Onge et al., 2005, Porubska et al., 2006, Fuhrer et al., 2008,

Schur et al., 2009). Activity is also greater in the ventral striatum, OFC, amygdala and insula when viewing appetizing compared with bland or disgusting foods (Beaver et al., 2006, Calder et al., 2007). Compared to low-calorie food pictures, viewing high-calorie food stimuli induces greater activity in the insula, medial OFC, medial and dorsolateral PFC (Gordon et al., 2000, Killgore et al., 2003, Siep et al., 2009). Activity is also greater in the amygdala and OFC when chosing highly preferred foods from a menu (Hinton et al., 2004).

6.1.5. Combined effect of nutritional state and food type on brain reward systems

Nutritional state (fasting) impacts on food reward and increases the appeal of food (Cabanac, 1971; Uher et al., 2006; Stoeckel et al., 2007). Animal and human studies have demonstrated that food restriction increases the rewarding properties of drugs of misuse (Palmiter, 2007). The sensation of reward is also influenced by energy status, as the subject's palatability of food is altered in the fasted compared to the fed state (Berridge, 1991).

The novelty of the two fed/ fasted studies was that an interaction has been shown between nutritional state and food category (high-calorie foods) on the activation of brain reward systems. When fasted (first fed-fasted study), activation in the ventral striatum, amygdala, anterior insula, and medial and lateral OFC was increased on viewing pictures of high-calorie over low-calorie foods. However, the second Fasted / Fed Saline study showed that viewing pictures of high-calorie foods over objects only increased activation in the anterior OFC. These differences in activations in the two fMRI studies could be justified by various factors which are explained in the following section ('Activation in the ventral striatum'). The results however agree with previous imaging studies that have looked at individual components of nutritional state and food category. They show that in a fasted state, the calorie content of food pictures modulates the activation of brain reward systems such as the in the insula, OFC, ventral striatum and amygdala when viewing high-calorie over low-calorie foods in lean subjects (Frank et al., 2010, Gordon et

al., 2000, Killgore et al., 2003, Siep et al., 2009), when viewing appetizing over disgusting (or bland) foods in lean subjects (Beaver et al., 2006, Calder et. al. 2007) and when choosing highly preferred foods from a menu in obese subjects (Hinton et al., 2004, Piech et al., 2009).

6.1.6. Activation in the ventral striatum

In the fed-fasted fMRI study, activation in this brain region was increased when fasted (compared to when fed) on viewing high-calorie foods which is in agreement with previous research (Beaver et al., 2006; Rolls and McCabe, 2007; Stoeckel et al., 2008; Stoeckel et al., 2009). This may be due to the ventral striatum playing an incentive motivational role in food intake and activation of this reward system enables behavioural responses necessary for obtaining a goal object, aimed at anticipated food receipt (Berridge, 1996, Kelley, 2004). There was however no activation in the second Fasted/ Fed Saline fMRI study when viewing high-calorie foods at the Fasted Saline visit which may be explained by various reasons:

(i) The breakfast was equal for men and women whereas in the first study subjects where instructed to have a filling breakfast of their choice. This resulted in the differences in VAS appetite ratings and appeal scores between the fasted and fed visit being greater in the first study compared to the second study.

(ii) In the second study, the Fasted and Fed Saline visit were randomised together with the Fed Ghrelin visit to either the 2nd, 3rd or 4th study visit. Viewing the food pictures for the 3rd or 4th time (compared to the fed-fasted study) may reduce the novelty and motivation to receive the high-calorie food pictures and therefore decrease activation in the ventral striatum. Indeed, the second study has shown that activation in the ventral striatum was decreased between the Fasted Dummy and Fasted Saline visit. These findings are supported by recent research showing that contextual novelty

enhances neural responses underlying reward representation in the ventral striatum (Guitart-Masip et al., 2010).

(iii) In the second study, a different atlas (as part of the new fMRI analysis software) was used to draw the regions of interest such as the ventral striatum. In the first study the automated anatomical labeling atalas (AAL) as part of SPM 5 software was used and in the second study, the Havard atlas as part of the FSL software. This could lead to differences in the anatomical location of each ROI.

(iv) Finally, there were differences between the studies in how the relevant voxels in the ROIs were defined. The first study looked at differences in activation at peak voxel (defined as most significant difference in activation when viewing high-calorie over low-calorie foods at the fasted visit), whereas the second study looked at the differences in average activation within those voxels in the whole ROI that were activated on average across 3 visits for high-calorie foods compared to objects. This alternative method using union fMRI was chosen because there were voxels whose activity was greater for high-calorie than low-calorie foods at both the Fed Saline and Fasted Saline visits, while in the first study there were only significant voxels at the fasted visit.

The second fMRI study found that individual hunger ratings positively correlated with activation in the ventral striatum when viewing high-calorie foods but only at the Fed Saline visit, with a similar trend for low-calorie foods. This may be explained by a threshold effect when fasting with a non-linear relationship between activation and hunger once a certain hunger level is reached when fasted. Below this level, varying degrees of satiation in the fed state may can still correlate with activation in the ventral striatum. This hypothesis is supported by results from the first fMRI study when individual differences in hunger positively correlated with individual differences in appeal bias towards high-calorie foods in the fed state but not in the fasted state.

6.1.7. Activation in the insula cortex

In the first fMRI study, activation in the insula was significantly increased when viewing high-calorie foods only in the fasted state. This is in accordance with previous studies which showed that fasting increased insula activation when viewing food relative to non-food pictures (Gordon et al., 2000; St-Onge et al., 2005; Schur et al., 2009; Siep et al., 2009), high-calorie over low-calorie foods (Frank et al., 2010), viewing and smelling food (Wang et al., 2004; Wang et al., 2009) and after choosing high-incentive over low-incentive foods written on a menu (Hinton et al., 2004).

The second fMRI study showed that individual hunger ratings are positively correlated with activation in the insula when viewing high-calorie foods only at the Fed Saline visit with a similar trend for low-calorie foods. This disagrees with a previous study which demonstrated that subjective ratings of hunger when fasted for at least 5 hours correlated with activation in the insula to food pictures (Porubska et al., 2006). As discussed for the ventral striatum above these differences may be related to threshold effects and the duration of fasting.

The insula cortex has been suggested to play an important role in taste processing and belongs to the “visceral stimulation network” (Welzl et al., 2001; Porubska et al., 2006; James et al., 2009). Previous studies suggested that representations of experience with food (colour, taste, flavour, reward value) are generated in the insula (de Araujo et al., 2003; Haase et al., 2009a). Bender and colleagues demonstrated that the insula is activated to taste versus tasteless stimuli irrespective of taste (sweet, sour and salty) (Bender et al., 2009). Thus, this region of the insula is responsible in encoding taste irrespective of taste demand (Bender et al., 2009). Haase et al. confirmed insula activation in response to sweet, sour and salty taste in both hungry and satiety states (Haase et al., 2009a). However, none of these studies commented on the calorie content of the food.

The findings of the second fMRI study are the first to show that activation in the insula when viewing high-calorie foods positively correlated with *ad libitum* lunch intake at both the Fasted and Fed Saline visits, with a trend for activation to low-calorie foods at

the Fed Saline visit only. As with the hunger rating correlations discussed above, this demonstrates that when fasted, correlations with appetite measures are only seen for activation to high-calorie foods, while when fed they are seen for high-calorie and a tendency for low-calorie foods. This again supports the interaction between nutritional state and biases towards high-calorie foods (see section below entitled “Activation in the OFC”).

6.1.8. Activation in the amygdala

In the first, but not second fMRI study there was increased activation in the amygdala when viewing high-calorie over low-calorie foods on the fasted visit. This is in agreement with earlier neuroimaging research showing that fasting (vs. post-prandial state) activates the amygdala when viewing food over non-food pictures (LaBar et al., 2001; Morris and Dolan, 2001; Holsen et al., 2005; Fuhrer et al., 2008; Schur et al., 2009; Siep et al., 2009), when choosing high-incentive over low-incentive foods written on a menu (Hinton et al., 2004) or when imagining and chosing attractive written dishes from a restaurant menu (Piech et al., 2009). The amygdala has been implicated in emotional processing particularly responses of positive and negative affect, reward processing, and behaviour (Pessoa and Ungerleider, 2004). Suggested reasons for the lack of differences in activation in the amygdala between nutritional states in the second fMRI study are similar to these discussed above for the ventral striatum, principally the different preload (breakfast), the novelty effect of the Fasted Dummy visit, the different anatomical labeling atalas and the different statistical fMRI analysis approach.

6.1.9. Activation in the orbitofrontal cortex (OFC)

In the two fMRI studies, activation in the OFC was significantly increased when viewing high-calorie foods in the fasted compared to the fed state. In the first fed/fasted fMRI study, activation in the medial and lateral OFC was significantly increased on viewing high-calorie over low-calorie foods in the fasted but not the fed state. These findings of an interaction between activity in brain reward systems with food type and nutritional state is

in agreement with a previous $H_2{}^{15}O$ PET study, which showed that fasting (vs. post-prandial state) activates the medial OFC when choosing high-incentive over low-incentive foods written on a menu (Hinton et al., 2004). Other fMRI studies have also demonstrated that compared to low-calorie food pictures, viewing high-calorie food stimuli induces a greater activation in the OFC (Gordon et al., 2000; Killgore et al., 2003; Goldstone et al., 2009; Siep et al., 2009; Frank et al., 2010). In normal weight subjects in a fasted hungry state, visual food versus non-food stimuli are reported to produce greater activation in the OFC, using functional imaging techniques (Holsen et al., 2005). However, the findings of this research study are the first to show that fasting also enhances the subjective appeal of high-calorie more than low-calorie foods, and the change in appeal bias towards high-calorie foods is positively correlated with medial and lateral OFC activation (Goldstone et al., 2009). This suggests a role for the OFC in the representation of food reward value.

Furthermore the second fMRI study revealed that hunger ratings were positively correlated with activation in the OFC when viewing high-calorie foods in the fed state with a similar trend for low-calorie foods, but not in the fasted state. However, appeal rating differences between the Fasted Saline and Fed Saline visit did not correlate with differences in activation in the OFC in the second study. As discussed earlier (‘Activation in the ventral striatum (i)), this could be explained by smaller changes in appetite and food appeal between the fasted and fed visits due to the fixed breakfast given in the second study, compared to the first study (Goldstone et al., 2009).

A large meta-analysis of the existing neuroimaging data has shown that activity in the OFC is related to the monitoring and learning of reward value of reinforcers (Kringelbach & Rolls, 2004). New research suggests that specific brain reward areas are divided into specific functional subregions. The reward value of a reinforcer is assigned in more anterior parts of the OFC, where it can be modulated by hunger and other internal states, and can be used to influence subsequent behaviour, stored for monitoring, learning and memory, and made available for subjective hedonic experience (Kringelbach et al., 2003; Kringelbach and Rolls, 2004; Kringelbach, 2005).

In the second study, anterior OFC activation to high-calorie foods (but not low-calorie foods) positively correlated with *ad libitum* lunch intake in the fed state *only*. As described for the insula and ventral striatum, this may be explained by a threshold effect in the fasted state. Furthermore one previous fMRI study has examined the relationship between non-task related BOLD activation and subsequent caloric intake (lunch) in the fasted state under hormonal manipulation (Batterham et al., 2007). This study showed that after infusion of saline (low PYY levels) resting activity in the hypothalamus correlated with subsequent caloric intake. On the PYY infusion day, activation in the OFC predicted subsequent caloric intake. Therefore, in the presence of PYY, a postprandial satiety factor, brain activity predicting caloric intake appeared to switch from a homeostatic area (hypothalamus) to a hedonic area (OFC). My results are entirely consistent with this since correlations of OFC activation and lunch intake were only seen in the fed state in which PYY levels would be expected to be higher compared to the fasted state.

An interesting finding of this study was that there was no positive correlation between VAS appetite ratings just before lunch and *ad libitum* lunch intake at the Fed Saline or Fasted Saline visits. As this study showed positive correlations of insula and anterior OFC activation and *ad libitum* lunch intake, this is the first study to show that brain activity when viewing food pictures may be a better predictor of subsequent food intake then appetite ratings (Blundell et al., 2010). One possible explanation could be that subjects use different VAS rating strategies while BOLD activation is a less subjective measure to compare between subjects.

6.1.10. Control tasks

The assessment of activity during control tasks (viewing object vs. blurred pictures) suggests that the results of the two fed/fasted studies are unlikely to be due to any non-specific changes in BOLD fMRI signal of nutritional state as there was no significant differences in activation in brain reward systems when viewing objects over blurred pictures (Noseworthy et al., 2003). Visual cortices (occipital cortex and lateral geniculate nucleus) showed also no significant difference in activation on viewing a 4Hz flashing

checkerboard between fed and fasted states in the first fed-fasted study. The analysis of the AMV task of the second Fasted-Fed Saline study will hopefully confirm these non-specific changes in brain activation.

Previously, caffeine has been shown to have the potential to modify fMRI results, as the BOLD signal is a function of both neural activity and changes in vascular tone through coupling of neural activity to the haemodynamic response. Therefore any manipulation that alters vascular tone can alter BOLD responses. For example, caffeine blocks adenosine receptors, resulting in vasoconstriction. A previous study demonstrated that in high caffeine users BOLD signal changes are increased after visual stimulation compared to low caffeine users (Laurienti et al., 2002). The same group of researchers further showed that acute caffeine intake reduces cerebral perfusion and increase BOLD signal intensity compared to placebo in response to passive visual (flashing checkerboard) and passive auditory (bursts of white noise) stimuli (Laurienti et al., 2003).

Therefore any pharmacological or nutritional manipulation has the potential to non-specifically alter BOLD responses and so control tasks should be included as standard.

6.1.11. Study limitations

There are several limitations that should be taken into account when making any conclusions from these two studies about the effects of nutritional state on brain responses to food pictures:

1. A relatively small numbers of subjects leading to possibility of type I errors, especially given the number of comparisons done. For this reason the number of ROIs was kept limited. However confirmation of the findings will need to be made with a larger cohort.
2. The sample size has been determined using previous publications. Without *prior* power calculations the probability of type II error is increased. The conclusions therefore remain preliminary until they are re-examined in further study populations with larger subject numbers.
3. Different pre-loads were given between the 2 studies - a filling breakfast of the subject's own choice vs. a fixed breakfast. In order to compare brain activity between

subjects it is important to have the same macronutrient composition consumed by each of them.

4. The second study attempted to control for food intake on the night prior to each study visit. They were instructed to have the same type and amount of food for supper at 8pm the night prior to each study day. However, in the second study participants consumed more carbohydrates at the Fed Saline visit compared to the Fasted Saline visit. The first study did not control for these variations which could influence food intake on the study days.

5. The results from these studies may not be directly relevant to overweight/ obese subjects as there may be differential effects on food reward systems in obese subjects. Increased BMI has been correlated with decreased ability to inhibit food intake (Myslobodsky, 2003; Nederkoorn et al., 2006a; Lowe et al., 2009; Nederkoorn et al., 2009) and with reduced satiety (le Roux et al., 2006). Obese and postobese subjects have been found to have altered activity at rest in brain reward systems after eating a meal (DelParigi et al., 2004; Le et al., 2006; Le et al., 2007). Obese subjects have further exaggerated activation of brain reward systems when viewing high-calorie vs. low-calorie foods (Rothemund et al., 2007; Stoeckel et al., 2008; Martin et al., 2009). There was no significant correlation between BMI and activation for the high-calorie vs. low-calorie food contrast when fasted in any ROI in the first fed/fasted fMRI study. This may be explained by the homogenous group of the study subjects with an average BMI of 22.1 ± 0.5 kg/m^2 (range 18.2-27.1) who were all non-obese or overweight on BMI criteria. Therefore it was not possible to investigate the effect of BMI on activation in brain reward systems. This can be addressed in future studies by comparing non-obese and obese subjects.

6. This studies used a mixed-block fMRI picture paradigm which was not designed to differentiate whether different categories of high-calorie foods produced differential activation in reward systems. Although there was a suggestion that sweet high-calorie foods tended to be of greater appeal than savoury high-calorie foods when fasted, the study was not designed or powered to examine this in detail. The heterogeneous ethnicity in this study could also bias the preference and reward for certain food types. Future

studies can address this specifically using an event-related fMRI design or blocks separating sweet and savoury high-calorie foods with sufficient numbers of different high-calorie pictures and using a homogenous ethinicty group used to a western diet. Previous taste fMRI studies have distinguished between tasting sweet, sour, salty and tastless solutions (Bender et al., 2009; Haase et al., 2009; Spetter et al., 2010) but differential effects of sweet vs. savoury food pictures on brain activation have yet to be reported.

7. In order to improve the results when running appetite fMRI studies it may be important to implement an inital dummy visit to acclimatise (stress, anxiety) subjects to the MRI scanning and the fMRI protocol, although many subjects in the first study had previously experienced MRI scans (Chandarana et al., 2009). A Fasted Dummy visit was applied in the second fMRI study. The results and discussion of the impact of such Fasted Dummy visit will be shown in section 6.4.

8. The fist study did not control for the day of menstrual cycle although there is evidence for variation in appetite and fMRI brain response to rewarding stimuli over the menstrual cycle (days 2-10 after day of first bleed) in women (Dreher et al., 2007; Van Vugt, 2009, Frank et al., 2010). The second fMRI study controlled for menstrual cycle.

9. The first fMRI study did not collect field maps (due to delayed sequence development), a method to correct for geometric distortions in brain reward areas such as the medial OFC located at the bottom edge of the frontal cortex.

6.1.12. Conclusion of effects of nutritional state on brain responses to food pictures

The two studies have highlighted brain areas involved in appetite reward circuitry whose activity may vary depending on nutritional state and type of food stimulus. The results demonstrate that in healthy subjects, fasting increases activity in brain reward systems when viewing high-calorie compared to low-calorie foods, including ventral striatum, amygdala, insula, OFC, increases appeal of and preference for high-calorie foods. Furthermore activity in these brain regions, especially to high-calorie foods when fed, is predicted by hunger and predicts subsequent food intake.

An increase in activation in brain reward systems when fasting while viewing high-calorie foods may be explained by a change in neuroendocrine factors. Endocannabinoid, serotonergic, opioid, orexin and dopaminergic pathways change with nutritional state and may be responsible for the changing activity in brain reward systems in response to appetizing visual food cues (Cardinal et al., 2002; Kringelbach and Rolls, 2004; Volkow and Wise, 2005; Palmiter, 2007; Berthoud and Morrison, 2008). As discussed in the Introduction, imaging studies in humans have provided evidence of dopamine involvement in the motivational properties of food intake (Volkow and Wise, 2005). The mechanism by which nutritional state and food stimulate dopamine signaling is unclear. A new concept has been put forward that suggest hormones such as leptin, insulin and ghrelin directly affect the dopamine reward pathways. These hormones activate receptors on VTA dopamine neurons, thereby either stimulate (ghrelin) or inhibit (leptin and insulin) dopamine signaling in the NAc. The NAc (ventral striatum) receives and projects onto the VTA, insula and OFC (Verhagen and Engelen, 2006; Fields et al., 2007; Van Vugt, 2009).

Leptin shows a direct inhibitory effect on dopaminergic neurons projecting to the ventral striatum in rodents (Cota et al., 2006). Functional MRI studies using visual food have shown that obese humans with a very rare condition of leptin deficiency, leptin replacement reduces hunger, 'liking' of food as well as brain activation in the insula and ventral striatum (Baicy et al., 2007; Farooqi et al., 2007). However, although I did not meaure leptin levels in the first study, and have yet to assay the samples in the second study, it is unlikely that plasma leptin levels changed in the current studies as the time between eating breakfast and imaging was relatively short (1.2 ± 0.1hrs) (Korbonits et al., 1997; Hinton et al., 2004).

In humans, gastric distension and other anorexigenic gut hormones such as PYY and insulin that are released after eating a meal may also mediate the effects on brain reward systems seen in this study (Anthony et al., 2006; Batterham et al., 2007; Wang et al., 2008; Guthoff et al., 2009).

The stomach-derived orexigenic hormone ghrelin is a prime candidate since its plasma levels increase with fasting and descrease after eating a meal. Ghrelin appears to

modulate the response to food cues of a neural network involved in reward and the regulation of feeding (Malik et al., 2008). How ghrelin acts on the brain is not known, but several potential mechanisms have been indentified as previously described (See section 1.4.7. in Introduction). However, it remains unclear whether ghrelin may have a selective effect on viewing high-calorie vs. low-calorie food pictures during negative energy balance such as observed in the present study.

The next Section (discussion of Ghrelin fMRI Study) will address the hypothesis that changes in ghrelin may be responsible for the effects of fasting on food reward. This study examined whether ghrelin administration to fed healthy volunteers is able to mimic fasting to increase activation in brain reward systems when viewing high-calorie foods.

6.2. Discussion Fed Ghrelin vs. Fed Saline fMRI study

6.2.1. The main findings of this study are as follows:

(i) There was no significant difference in VAS appetite scores and lunch intake between the Fed Ghrelin and Fed Saline visit but there was a tendency for hunger ratings to be greater at the Fed Ghrelin visit.

(ii) High-calorie foods were more appealing than low-calorie foods at the Fed Ghrelin but not Fed Saline visit. High-calorie foods (but not low-calorie foods) were significantly more appealing at the Fed Ghrelin visit compared to the Fed Saline visit (as with the Fasted Saline visit).

(iii) Activation in the anterior OFC on viewing high-calorie foods at the Fed Ghrelin visit was significantly greater compared to the Fed Saline visit (as with the Fasted Saline visit).

(iv) There was a slight tendency for greater activation in the ventral striatum to high-calorie foods at the Fed Ghrelin compared to the Fed Saline visit.

(v) Plasma glucose and serum GH levels were significantly increased at the Fed Ghrelin visit compared to the Fed Saline visit.

(vi) After ghrelin injection serum cortisol and prolactin levels were significantly increased compared to injection of saline, but there was no difference in anxiety or stress VAS ratings.

6.2.2. Effect of ghrelin on appetite ratings and lunch intake

Between the Fed Ghrelin and the Fed Saline visits there were no significant differences in VAS appetite ratings (hunger, fullness, pleasantness and volume able to eat) or *ad libitum* lunch intake. This could be due to various reasons:

1. The relatively small number of subjects (n=17) participating in this study, although the only fMRI study which administered ghrelin used only 20 subjects for their analysis (Malik et al., 2008). Previous ghrelin administration studies used 16 or less patients (Druce et al., 2006; Ashby et al., 2009).
2. Some of the patients described a burning pain while giving the ghrelin or saline injections which lasted for about 30 seconds. This could lead to increased stress and therefore decreased hunger. However, the VAS stress ratings showed no significant differences between the Fed Saline and Fed Ghrelin visits. Prolactin and cortisol levels increased after injection of ghrelin which is in agreement with a previous study (Tong et al., 2010). A previous study showed that increased appetite and cravings for foods high in sugar and fat is caused by high cortisol levels (Epel et al., 2001).
3. Ghrelin may not have been absorbed well in some subjects as ghrelin was injected subcutaneously. This would disagree with previous studies in healthy volunteers, obese patients and dialysis patients where a subcutaneous injection of ghrelin increased ghrelin plasma levels, increased appetite, and increased energy intake at the subsequent meal (Druce et al., 2006; Ashby et al., 2009). Although ghrelin levels have yet to be measured for my study, pilot measurements from 3 other subjects given the same dose of ghrelin from the same batch, has shown levels of 300-1800 pg/mL 15 mins after subcutaneous injection (Milliplex assay, Millipore Corporation, St. Charles, MO, USA) suggesting achievement of high levels of active ghrelin. Furthermore serum

GH levels rose after ghrelin injection confirming bioactivity of the ghrelin injection (Wren et al., 2001).

4. The effect of ghrelin had worn off by lunch. In this study, there was a long gap between ghrelin injection and lunch (95 minutes) to enable the scanning to take place. In contrast to the results of this study, Neary et al. demonstrated that *only* 30 minutes after a subcutaneous injection of ghrelin, energy intake is increased by 28 percent in cancer patients with anorexia (Neary et al., 2004). Druce et al. showed that subcutaneous ghrelin increases food intake in obese as well as lean healthy subjects by 20 percent *only* 30 minutes after injection (Druce et al., 2005; Druce et al., 2006). Other studies demonstrated that ghrelin injection to malnourished dialysis patients increases food intake by 25 percent after *only* 30 minutes of ghrelin injection (Wynne et al., 2005b; Ashby et al., 2009). Previous researchers have shown a decline in total and acylated ghrelin 15 to 75 minutes after subcutaneous injections (Neary et al., 2004; Druce et al., 2005; Wynne et al., 2005b). Although pilot studies in 3 subjects from my study have shown acylated ghrelin levels 75-450 pg/mL, which are still elevated above pre-injection levels (<50 pg/mL), 95 mins after subcutaneous injection, it may not be the absolute ghrelin levels but the direction of change of ghrelin levels that may be important. Possibly high levels of desacyl ghrelin due to breakdown of acylated ghrelin may antagonize the orexigenic effect of acyl ghrelin.
5. The absent effect of ghrelin to increase lunch intake could have also been caused by showing food pictures in the scanner abolishing the difference between the Fed Saline and Fed Ghrelin visit due to the impact of 'sensory-specific satiety' (SSS). This SSS causes a dropping in ratings of pleasantness relative to foods that have not been eaten when a food is repeatedly viewed, smelled or consumed (Rolls et al., 1981, Kringelbach et al., 2003, O'Doherty et al., 2000, Small et al., 2001).
6. Recent research suggests that ghrelin enhances sweet food consumption and preference regardless of its caloric content in rodents (Disse et al., 2010). This study used savoury pasta or curry for lunch without any sweet component and the nature of the study meal might play an important role when investigating ghrelin's ability to

increase food intake. This is supported by the food appeal data in the MRI scanner showing that while high-calorie food pictures were more appealing relative to low-calorie food pictures at the Fed Ghrelin but not at the Fed Saline visit, the effects was greatest for sweet chocolate or sweet non-chocolate foods compared to savoury high-calorie foods. In a previous study where they showed 27 percent increase in energy intake after injection of ghrelin in healthy volunteers, researchers used Madeira cake or cinnamon and raisin loaf as the *ad libitum* meal (Druce et al., 2005). Another feeding study used sweet brioche rolls as the *ad libitum* meal in malnourished dialysis patients (Wynne et al., 2005b). This may help to explain why my ghrelin fMRI study showed no difference in food intake, as ghrelin might enhance the intake of sweet and not savoury foods. Although some of the previous ghrelin studies on food intake used savoury test meals as in this ghrelin fMRI study (Neary et al., 2004), these studies used an intravenous infusion rather than a subcutanous injection of ghrelin, fasted rather then fed subjects, or patients with cachexia rather than healthy volunteers (Neary et al., 2004; Ashby et al., 2009). In future studies, it would be useful to employ either a different test lunch such as cake as in previous studies or a buffet meal where volunteers can choose between high-calorie sweet, high-calorie savoury, low-calorie sweet and low-calorie savoury foods.

6.2.3. Effect of ghrelin on brain reward systems

At the Fed Ghrelin visit, there was a significant increase in activation of the anterior OFC on viewing high-calorie foods, compared to the Fed Saline visit. As previously described in more detail (see Section 30 in Introduction) circulating ghrelin may act indirectly on brain reward systems through the hypothalamus (Saper et al., 2002; Kelley, 2004) or also act directly on dopamine neurons in the VTA (Abizaid et al., 2006, Zigman et al., 2006) (Cummings et al., 2007). Ghrelin can act on the VTA via orexin-producing neurons in the lateral hypothalamus (LH). Ghrelin-induced feeding is suppressed in orexin knockout mice (Toshinai et al., 2003). Orexin neurons can project to brain reward centres especially VTA

(Chemelli et al., 1999). Recent studies have shown that orexin is involved in motivated behaviour for drugs of abuse as well as natural rewards (Cason et al., 2010).

Malik and colleagues demonstrated that intravenous ghrelin administration to healthy volunteers leads to increased activation to food (vs. non-food) pictures in the amygdala, OFC, anterior insula, and striatum and hippocampus using fMRI (Malik et al., 2008). My study did not show any activation in the insula and amygdala. This may be due to several differences between my study and that of Malik et al.:

1. Inclusion of male subjects only in the study by Malik et al. Previous neuroimaging data suggested that the two genders respond differently in their brain reward activation to food stimuli (Del Parigi, 2002; Smeets et al., 2006; Wang et al., 2009; Frank et al., 2010).
2. Malik et al. showed no measurements of stress hormone levels compared to my ghrelin fMRI study, where stress hormone levels were not significant different between fasted visits. However, as described in more detail in Section 6.4., anxiety and stress are suggested to alter feeding behavior (Adam and Epel, 2007; Chandarana et al., 2009). In this ghrelin fMRI study but not in the Malik study each participant had one Fasted Dummy visit to acclimatize to the hospital environment and to the fMRI protocol. Indeed, VAS anxiety and sickness rating at the Fasted Dummy visit were significant increased compared to the Fasted Saline visit. The Malik study showed borderline significant increases for irritable and nausea feelings in the ghrelin relative to the control condition. In order to explore the possibility that the results in the group that received ghrelin were due to ‘order’ effects, Malik et al. subsequently recruited an additional eight subjects who underwent the same paradigm with the exception that they only received normal saline rather than ghrelin. In this group of subjects there was no significant difference in food hedonic ratings and no increased activation in brain reward systems. In the control group were also increases of borderline significance in the subjective ratings for irritable and

bored in the second relative to the first block of images but nausea levels did not change.

3. There was a difference between the two studies in the route of ghrelin administration: the Malik study used an intravenous ghrelin injection rather then a subcutaneous ghrelin injection in my study. Therefore the effect on food intake and appetite ratings might be different between the two studies as subcuatnous injection might cause extra stress and therefore influence appetite. After ghrelin injection serum cortisol and prolactin levels were significantly increased compared to injection of saline, but there was no difference in anxiety or stress VAS ratings.
4. As they left a three hour interval between giving breakfast and fMRI scanning, the effect of the meal may have been attenuated after this period and therefore the effect of the fed state may be less marked (subjects were less satiated) which could lead to increased appetite at the time of scanning in the Malik study.
5. The researchers in the Malik study have no control of the subjects being awake in the MRI scanner as no active behavioural task (passive picture viewing) was performed such as the appeal rating of the pictures like in this study. However, in the Mailk study approximately 30 min later after the scanning, subjects were shown all 45 food images that they had viewed in the scanner intermixed with 26 novel food images and were asked to state whether or not they had seen each image while in the scanner. This recognition/ memory task was performed to ensure that subjects were paying attention to the images during the scan.
6. The Malik study further showed food pictures only but did not distinguish between the hedonic value of foods such as high- and low-calorie foods. Therefore the effect of differences between food versus non-food might be greater than the effect of high-or low-calorie pictures versus objects.
7. Different methods for determining ROIs for magnitiude of activation were performed in two studies. Mailk et al. created t-statistical maps to compare

BOLD signal in response to food vs. Non-food pictures at a specific voxel in the brain for the t-test contrast for ghrelin > saline visit (difference in post- vs pre-injection scan to control for differences in baseline on each study day to improve the ability to detect differences between study days). They then extracted the magnitude of activation of significant peak activated voxels. In my ghrelin fMRI study, significant activated voxels in the brain were determined when viewing high-calorie foods > objects averaged across all three study visits (Fasted Saline, Fed Saline and Fed Ghrelin) were determined and ROIs were defined using an anatomical labeling atlas. The average activation in in each ROI was then extracted to calculate the magnitude of activation between each contrast to compere between visits. Future analysis should look at a direct t-test comparison of whole brain activation data between ghrelin and saline fed visits (with appropriate correction for multiple comparisons) from my study.

8. The Malik study did not apply field maps to correct for OFC signal compression or drop out compared to my ghrelin fMRI study.
9. In disagreement with this study, Malik found a difference in activation in the amygdala when given ghrelin which could be explained by the different approach to correct for study 'order' as they calculated the difference in activation pre-and post-injection of ghrelin (see above 7.). The test/ re-test results of Section 4.2.7.6. (Chapter 4) showed no significant correlations between fasted visits (Fasted Dummy vs. Fasted Saline) in any ROI expecpt with a tendency for the anterior OFC. This could clarify that there was no significant difference in the amygdala activation between the Fed Ghrelin and Saline visit.

6.2.4. Effect of ghrelin on the anterior OFC

In agreement with the Mailk study (Malik et al., 2008), my ghrelin fMRI study showed greater anterior OFC activation when viewing high-calorie foods at the Fed Ghrelin compared to Fed Saline visit. There was no significant difference in activation in this area

when viewing low-calorie foods. These results were similar to that seen in the comparison between Fasted Saline and Fed Saline visits. A study by Malik and colleagues found that effects of ghrelin on the OFC response were correlated with self-rated hunger ratings (Malik et al., 2008). This was not seen in this study which may be due to the fact that subjects were given scanned about 1.5 hours after breakfast whereas in the Malik study breakfast was served 3 hours before the scanning, thus subjects might have been less hungry in the ghrelin fMRI study.

6.2.5. Effect of ghrelin on the ventral striatum

The Malik study showed increased striatum activation when viewing food over scenery pictures after infusion of ghrelin compared to saline. This is in agreement with the findings of my ghrelin fMRI study as there was a tendency for increased ventral striatum activation at the Fed Ghrelin visit compared to the Fed Saline visit. As previously discussed (see Introduction), ghrelin has been found to induce feeding through activation of midbrain dopamine neurons which project to the ventral striatum (Jerlhag et al., 2006; Jerlhag et al., 2007). Ghrelin increases dopamine in the ventral striatum when locally administered into the VTA (Naleid et al., 2005).

An interesting finding of the current ghrelin fMRI study was that activation in the ventral striatum when viewing high-calorie foods over objects was increased at the Fasted Dummy and tended to be so at the Fed Ghrelin visit compared to both the Fasted Saline and Fed Saline visit. One possible explanation for this may be that after the initial Fasted Dummy visit there is habituation of the ventral striatum activation which is reversed by ghrelin. Indeed neuroimaging research suggests that the ventral striatum is involved in reward expectancy and novelty processing of rewards (Guitart-Masip et al., 2010). In that study subjects viewed different types of indoor and outdoor pictures (novel or non-novel cues) and following each picture received a novel or expected cue. Activation in the ventral striatum was increased when the cue was novel compared to when not novel (Guitart-Masip et al., 2010). This is in agreement with my ghrelin fMRI study as at the Fasted Dummy visit, the food pictures were novel but on the following Fasted and Fed

Saline visit the food pictures were no longer novel with an associated decrease in activation of the ventral striatum irrespective of nutritional state. The novel suggestion of my study was that ghrelin tended to reactivate the ventral striatum to high-calorie foods despite their lack of novelty as a stimulus.

Interestingly, my ghrelin fMRI study also showed that the reaction time for appeal ratings of all the pictures (but not blurred pictures) were significantly increased at the Fed Ghrelin visit compared to the Fed Saline visit. This maybe be explained by ghrelin which is suggested to increase the motivation to receive new rewards ('wanting') (Berridge, 1996; Guitart-Masip et al., 2010). As discussed later (section 6.4.) previous research hypothesizes that subjects are indeed able to react faster to novel items via enhanced energisation of action through reward and ghrelin might be involved in this enhancement (Bunzeck et al., 2009).

The increased picture appeal rating reaction time could also been linked to increased attention to visual food stimuli by increasing activation in the hippocampus and amygdala through the effect of ghrelin (Vuilleumier and Driver, 2007; Malik et al., 2008). Also, this ghrelin fMRI study demonstrated that ghrelin increases cortisol levels although not significantly different from the Fed Saline visit. Previous studies suggest cortisol to increase vigilant attention (Hopper et al., 2004).

6.2.6. Limitations of the Ghrelin fMRI study

1. This study will have produced supraphysiological pharmacological plasma ghrelin levels which leads to a cautious interpretation of the results. In future studies it would be appropriate to use a ghrelin antagonist or GOAT inhibitor to block the action of ghrelin to investigate its physiological effect on brain reward systems in humans.
2. To date it has not been possible to measure acylated and total ghrelin from the current study to assess whether appropriate ghrelin levels have been achieved in all subjects. This may affect the final analyses.

3. It would be useful to repeat a similar study with two different groups of subjects, such as obese versus lean to examine as obese subjects might have an altered signaling in brain reward systems after ghrelin injection.
4. The fixed *ad libitum* lunch should be substituted by a free meal buffet choice, consisting of high-calorie and low-calorie, savoury and sweet components.
5. The amount of breakfast was not individually calculated for each individual according to their gender, body weight and daily energy needs, which may have increased the variance between subjects.
6. The same pictures were shown at each visit reducing the novelty of the pictures at subsequent visits. Using similar types of food picture which are different in exact nature between visits may have helped avoid this problem especially as regard ventral striatum activation.

6.2.7. Conclusion Fed Ghrelin vs. Fed Saline fMRI study

This is the first study in humans which has shown that ghrelin mimics fasting to bias food appeal towards high-calorie, especially sweet, foods and the associated activation of the anterior OFC when viewing and rating high-calorie foods. The effects on appeal of and activation to low-calorie foods were not significant. There was also a suggestion that ghrelin may also reverse the suppression of activation in the ventral striatum when food pictures are no longer novel. Interestingly these findings were seen despite there being no significant differences in VAS appetite scores or *ad libitum* lunch intake between the Fed Ghrelin and Saline visit. This is either due to the fact that the effects of ghrelin of food reward are independent of hunger (Disse et al., 2010) or that elements of the protocol design (e.g. timing of meal after injection, stress of MRI scanning, showing food pictures) prevented differences in hunger being identified.

Additional factors to nutritional state and hormones may drive hunger and food reward. Individual differences in psychological cues such as reward sensitivity and dietary restraint have been previously described to affect eating behaviour. Identifying the neural

pathways that regulate cognitive, emotional and appetitive responses to different foods is particularly relevant to understanding individual differences in eating behaviour.

6.3. Discussion Behavioural Questionnaires (sub-study, fed-fasted fMRI study)

6.3.1. The main findings of this sub-study are as follows:

(i) Individual differences in dietary restraint (measured by DEBQ-restraint) positively correlated with activation to high-calorie vs. low-calorie foods in both the DLPFC and dorsal ACC, and negatively correlated with activation in both the amygdala and lateral OFC, when fasted overnight.

(ii) Reward sensitivity (measured by BAS-drive) positively correlated with activation to high-calorie vs. low-calorie foods in the ventral striatum.

These findings were independent of gender which itself appeared to have no influence on activation.

6.3.2. The effect of reward sensitivity on brain reward systems

My study demonstrated a positive correlation between reward sensitivity and activation in the ventral striatum to high-calorie over low-calorie foods. This latter result is consistent with a previous study which reported that BAS-drive scores predicted activation to appetizing relative to bland food pictures in the ventral striatum when healthy non-obese subjects had not eaten for 2 hours (Beaver *et al.*, 2006). As previously decribed (introduction), the ventral striatum plays an incentive motivational role in food intake (Berridge, 1996; Kelley, 2004). Obesity, food craving, overeating, binge eating and drug abuse are associated with increased levels of reward drive and sensitivity (Davis et al., 2004; Dawe and Loxton, 2004; Schienle et al., 2009). These data suggest that heightened activity of the ventral striatum to high-calorie foods may be a mechanism for translating reward drive into over-consumption of rewarding foods and hence weight gain. There was no correlation seen in our study between BAS-fun seeking nor BAS-reward responsiveness

in any ROI. This may be because these questions measure the tendency to seek out novel rewarding experiences and positive hedonic responses to the actual receipt of rewards (Beaver *et al.*, 2006). By contrast BAS-drive assesses both hedonic and motivational behavioural responses to reward cues in the immediate environment such as in our fMRI paradigm of rewarding food stimuli (Carver and White, 1994).

6.3.3. The effect of dietary restraint on brain reward systems

The results of my study showed that individual differences in dietary restraint positively correlated with activation to high-calorie over low-calorie foods in both the DLPFC and dorsal ACC when fasted overnight.

As previously decribed (introduction) the dorsal ACC is the cognitive subdivision involved in modulation of attention or executive function, influencing response selection, motivation, conflict monitoring and cost/benefit or reward/risk assessment, and is influenced by anxiety and fearful situations (Bush et al., 2000; Carter and van Veen, 2007). This is consistent with the novel finding in this study of greater activation of the dorsal ACC when fasted in those with higher levels of dietary restraint when viewing high-calorie vs. low-calorie foods. Foods of higher caloric and reward value would be predicted to produce greater conflict and altered reward/risk ratio, and hence engagement of the dorsal ACC in subjects with higher dietary restraint. The consequent mismatch between perceived risk of weight gain and food reward under this highly appetitive motivational state would require greater self-control.

The DLPFC is crucial for cognitive regulation of behaviour, goal-directed decisions and exercising self-control (Heekeren et al., 2004; Rorie and Newsome, 2005; Hare et al., 2009; Kable and Glimcher, 2009). Self-reported dieters with greater self-control over choosing foods to eat on the basis of health than taste show greater activation in the left DLPFC during a food picture choice paradigm using fMRI (Hare *et al.*, 2009). Activation of the DLPFC has previously been reported when addicts view a variety of different drug-related cues that induce craving especially when drug use is expected (Wilson *et al.*, 2004), and subjects view high-calorie compared to low-calorie foods especially when

fasted (Killgore *et al.*, 2003; Goldstone *et al.*, 2009; Schur *et al.*, 2009). The results of this study showing increased activation of the left DLPFC in those with higher levels of dietary restraint when viewing high-calorie vs. low-calorie foods are consistent with these roles of the DLPFC to exert self-control in decision making. The dorsal ACC is reciprocally interconnected with the dorsal PFC. Since top-down control appears to be mostly implemented in the DLPFC, it is suggested that following detection of conflict by the ACC, the lateral areas of the dorsal PFC are engaged in order to reduce conflict (Carter and van Veen, 2007).

This study further showed that individual differences in dietary restraint negatively correlated with activation in both the amygdala and lateral OFC to high-calorie over low-calorie foods when fasted overnight.

As previously described, the lateral OFC is involved in encoding reward value and taste processing (Kringelbach and Rolls, 2004), while the amygdala is involved in emotional processing and responses (Pessoa and Ungerleider, 2004). Some amygdala functions are carried out in concert with the OFC, such as updating the values of expected outcomes stored in the OFC (Holland and Gallagher, 2004).

Previous neuroimaging studies have shown greater activity in the lateral OFC and amygdala when viewing appetizing compared to bland or disgusting foods, and high-calorie compared to low-calorie foods, particularly when fasted (Beaver et al., 2008; Schur et al., 2009). In this study activity in the lateral OFC and amygdala was lower in those with high dietary restraint which suggests that the executive control system (DLPFC-dorsal ACC) may have modulated the encoding of reward value and emotional responses in these regions to high-calorie compared to low-calorie foods. These findings are supported by recent studies that voluntary inhibition of hunger during food stimulation (visual, taste and smell) leads to decreased activation in the amygdala and OFC using ^{18}FDG PET (Wang *et al.*, 2009).

These results are in agreement with earlier studies in previously obese subjects who have tried to diet. Successful dieters who had higher levels of dietary restraint

demonstrated greater dorsal PFC activation, albeit on the right, when tasting a liquid meal than non-dieters using ^{15}O-water positron emission tomography (PET) (DelParigi *et al.*, 2007). In the combined groups dietary restraint was positively correlated with dorsal PFC activation and negatively correlated with OFC activation (DelParigi *et al.*, 2007). Successful weight-loss maintainers also showed greater activation using fMRI in bilateral frontal regions to both high-calorie and low-calorie foods compared to normal weight and obese control women (McCaffery *et al.*, 2009).

The findings from this analysis expand the conclusion from earlier studies to include a role for the dorsal ACC and amygdala in addition to the left DLPFC in the cognitive and affective control of food intake related to dietary restraint in non-obese subjects, and emphasize the interaction with different food categories (high-calorie vs. low-calorie), which was either not previously investigated or found in previously obese subjects (DelParigi *et al.*, 2007; McCaffery *et al.*, 2009). Furthermore the results from this study in non-obese subjects without eating disorders are supported by reports of reduced left DLPFC activation to tasting food in obese compared to non-obese subjects (Le *et al.*, 2006), and to food vs. non-food pictures in patients with bulimia nervosa (Uher *et al.*, 2004), which may contribute to reduced-self control leading to overeating. Increased dorsal ACC activation has also been reported in patients recovered from compared to ongoing anorexia nervosa, and patients with bulimia nervosa compared to both non-obese controls and patients with binge eating disorder, where conflict between increased desire to eat and fear of weight gain would be hypothesized to be greater in the former group in each comparison (Uher *et al.*, 2003; Schienle *et al.*, 2009).

The findings of my study are however in contrast to a recently published study comparing brain activation when passively viewing highly vs. moderately palatable foods in fasted normal weight non-dieting women with low or high measures of dietary restraint. They found lower activation in the left DLPFC in subjects with high restraint, and no reported differences in amygdala, OFC or dorsal ACC (Coletta et al., 2009). Potential explanations for these differences between this study and our study may be related to differences in the scan timing, fasting duration and hence degree of hunger (afternoon

scan after 8h fast; morning scan after 16h fast), method of statistical comparison (t-test between highest and lowest quartiles of restraint; correlation with restraint score), restraint questionnaires used (Herman and Polivy's Restraint Scale; DEBQ-restraint), task paradigm (passive viewing; rating appeal of pictures), and choice of food picture categories (highly vs. moderately palatable foods with latter containing some non low-calorie foods e.g. bread, potato; high-calorie vs. low-calorie foods).

Interestingly, Coletta et al. did observe that after consumption of a 500 kCal pre-load subjects with higher restraint had greater activation in OFC, left DLPFC and insula to passively viewing highly vs. moderately palatable foods than those with lower restraint scales (Coletta et al., 2009). As the OFC is involved in reward processing and the DLPFC in cognitive processing, these findings raise the potential confounding phenomenon of counter-regulatory eating and disinhibition. Herman and Polivy (1984) have suggested that restrained eaters are concerned about their body weight leading them to control their food intake cognitively. At the same time, their 'all or none' approach to food makes them more likely to overeat forbidden foods under emotional distress ('disinhibition'). Their history of disinhibition-induced overeating also causes their "satiety boundary" to be shifted upward so that once they become disinhibited, food intake continues beyond the point at which further food intake would normally be inhibited (Herman and Polivy, 1984; Heatherton et al., 1992). When primed by recent food intake they may in fact find food more rewarding and hence overeat ('counter-regulatory eating'). These interpretations are supported by the findings of several studies that dietary restraint did not predict calorie intake in acute feeding studies or over weeks and months of observational studies (Stice et al., 2004; Stice et al., 2007; Stice et al., 2010).

Although the results of my study in the fasted state may reflect the consequences of high dietary restraint in the context of disinhibition due to exposure to highly palatable food cues, they might not reflect the situation in the presence of disinhibition due to emotional distress, social context, counter-regulatory eating responses and priming to ingestion of food. It is also unclear whether our findings in non-obese subjects might be different from those seen in currently or previously obese objects.

Indeed longitudinal studies have suggested that high dietary restraint (especially flexible restraint which able to appropriately adapt to context) may be able to counteract disinhibtion, and hence the interaction of these factors may be the best predictor of weight change (Hays and Roberts, 2008; Savage et al., 2009; Teixeira et al., 2010; Bryant et al., 2010). Comparison of the correlation of measures of dietary restraint (both flexible and rigid) and disinhibition between fasted and fed (using a fixed pre-load) states with activation to different food pictures using a similar experimental fMRI paradigm to this study will therefore be of interest to further investigate these interactions. Comparison of never, currently and previously obese subjects will also be of interest.

6.3.4. The effect of external eating on brain reward systems

Surprisingly the results of my study did not show any correlation between activation to high-calorie over low calorie foods in any ROI with DEBQ-external scores, a measure of sensitivity to the hedonic attributes of food, e.g. desire to eat or buy food that looks or smells delicious. This is in contrast to a previous study of obese subjects (with and without type 2 diabetes mellitus) in whom DEBQ-external scores were positively correlated with activation in the medial OFC when viewing food over non-food pictures. The differences between these findings and the current results may be related to differences in the study cohort (obese, non-obese), duration of fasting (3h, 16h), food picture contrast (mixture of high-calorie and low-calorie foods vs. non-food, high-calorie vs. low-calorie foods), range of individual DEBQ-external scores in each cohort, and task involved (passive viewing, rating appeal) (Chechlacz et al., 2009). Future studies using a similar paradigm to ours would benefit from including a cohort of subjects with a wide range of individual scores for both DEBQ-external and DEBQ-restraint.

However, the findings of my study were in agreement with another fMRI study of non-obese subjects fasted for at least 2 hours in which no correlation of DEBQ-external scores was found with BOLD activation *per se* to viewing (but not rating) appetizing over bland foods in the amygdala, striatum or ACC (Passamonti et al., 2009). Researchers of this study did find correlations of external food sensitivity with changes in connectivity

between regions when viewing appetizing over bland foods. Connectivity changes between ventral striatum (source) and amygdala were positively correlated and between ventral striatum and dorsal ACC were negatively correlated with DEBQ-external score; while connectivity changes between amygdala (source) and both ventral and dorsal ACC were negatively correlated with DEBQ-external score (Passamonti et al., 2009). This evidence for variation in network coupling among neural structures implicated in feeding as opposed to activation itself is worthy of further exploration through such a functional connectively analysis in further analysis of our cohort.

6.3.5. Study limitations

1. Relatively small number of subjects compared to the number of examined correlations of several different psychological traits with activation in eight *a priori* ROIs. There is therefore a significant risk of a Type 1 error in the findings of this study since data has not been corrected for multiple correlations. The conclusions therefore remain preliminary until they are re-examined in further study populations with larger subject numbers.
2. Relatively small number of subjects with high dietary restraint scores which is over 30 according to the cut-off level in the DEBQ. Future studies using a similar paradigm would benefit from including a cohort of subjects with a wide range of individual scores for both DEBQ-external and DEBQ-restraint.
3. In further analysis of this study it is also important to use the functional connectively analysis approach as there is evidence for variation in network coupling among neural structures implicated in feeding as opposed to activation itself.

6.3.6. Conclusion correlations with behavioural questionnaires (sub-study, fed-fasted fMRI study)

These executive-control (DLPFC and dorsal ACC) and affective-reward systems (amygdala and lateral OFC) are therefore implicated in mediating how evaluation and processing of

foods of differing caloric and reward values are influenced by cognitive factors controlling eating behaviour that promote or avoid weight gain. This provides a neurobiological network that may underlie the relationship between individual personality traits and excessive or reduced intake of high-calorie foods. This raises the possibility that modulation of the executive-affective top-down system by behavioural programs or other interventions may help maintenance of a healthy weight by encouraging dietary restraint towards high-calorie foods (Shaw et al., 2005; Uher et al., 2005; Camus et al., 2009). Verification of the importance of this network in the promotion or avoidance of weight gain will however need inclusion of subjects with differing weight histories, analysis of interactions between dietary restraint and other psychological traits that influence eating behaviour, such as disinhibition, and the effects of recent food intake in larger longitudinal studies.

6.4. Discussion of reproducibility of eating behaviour studies

Measuring eating behaviour can be very challenging as appetite studies are not performed under real-life conditions. It is therefore suggested to include practice dummy visit to acclimatise the patients to the study environment (Blundell et al., 2010).

In this sub-study of the ghrelin fMRI study, patients always had one initial Fasted Dummy visit before any other visit to help with habituation to the protocol. The results from the Fasted Dummy visit were compared to the Fasted Saline visit for the following measurements: VAS appetite scores, VAS stress scores, blood stress hormones, lunch intake, food picture appeal rating and fMRI activation to food and object pictures.

6.4.1. Main findings of this sub-study:

The Fasted Dummy and Fasted Saline visits were identical apart from the order in which they occurred. The Fasted Dummy was always first while the Fasted Saline visit was either the 2nd, 3rd or 4th visit.

(i) Differences in mean results between fasted visits:

a. VAS appetite scores did not differ between the fasted visits but there was a trend for fullness to be greater and pleasantness to be reduced on the Fasted Dummy visit.
b. Lunch intake was significantly reduced and there was a tendency for lunch pleasantness to be reduced at the Fasted Dummy visit.
c. Anxiety and nausea ratings were significantly higher at the Fasted Dummy visit.
d. High-calorie foods (but not low-calorie foods or objects) were less appealing on the Fasted Dummy visit. High-calorie foods were more appealing than low-calorie foods at the Fasted Saline visit but not at the Fasted Dummy visit.
e. There was a significant decrease in the reaction time for appeal rating of any of the picture categories at the Fasted Saline visits compared to the Fasted Dummy visit.

f. Activation in the ventral striatum when viewing high-calorie foods (but not low-calorie foods) was significantly greater at the Fasted Dummy compared to the Fasted Saline visit (while there was no difference for amygdala, insula or anterior OFC).

(ii) Correlation between results from the fasted visits:

a. VAS appetite scores and lunch intake were positively correlated between the fasted visits.

b. VAS stress ratings and plasma prolactin, but not serum cortisol, were positively correlated between fasted visits.

c. Appeal ratings for all pictures types positively correlated between fasted visits.

d. There was a tendency for a positive correlation of anterior OFC activation to high-calorie or low-calorie foods, and a significant positive correlation to objects pictures between the fasted visits. However there was no correlation for activation in any of the other ROIs (amygdala, insula or anterior OFC) to any food picture between fasted visits.

6.4.2. Differences in results between fasted visits

Stress and novelty can alter the results of appetite studies. Previous research suggests that anxiety and stress can alter feeding behaviour (Adam and Epel, 2007; Chandarana et al., 2009). This is in agreement with the results from this study because anxiety and sickness ratings were significantly elevated on the Fasted Dummy visit. Also, *ad libitum* lunch intake was significantly decreased on the Fasted Dummy visit. Studies in humans and rats show that stress alters the intake of high-calorie, high-palatable food (Adam and Epel, 2007). Nevertheless, there were no significant differences in VAS appetite ratings between the visits but there was a trend for VAS fullness to be greater and pleasantness to be reduced on the Fasted Dummy visit. Furthermore the appeal data demonstrated that at the Fasted Dummy visit appeal bias towards high-calorie foods was absent compared to

the Fasted Saline visit. This supports the assumption that appetite and therefore the intake of highly palatable energy dense food is generally decreased on the first study visit and that a dummy visit is important to acclimatise the patients to the study environment before making any assumptions about a stimuli (food/ hormone) to influence appetite (Chandarana et al., 2009). This is further supported by previous research which has shown that the study visit order can affect anxiety, plasma cortisol, and plasma PYY3–36, with increased anxiety and cortisol concentrations on the first study day (Chandarana et al., 2009). Plasma cortisol area under the curve (AUC) correlated positively with plasma PYY3–36 AUC. PYY3-36 is a satiety hormone released from the gut and elevated after food intake. Cortisol may influence the reward value of food via neuroendocrine/peptide mediators such as leptin, insulin and NPY. However my study did not show a significant difference in serum cortisol levels between fasted visits. In this study correlations between appetite ratings and appetite hormones have not yet been performed. Nevertheless, the findings of this study confirm that accurate assessments of appetite, feeding behavior, and gut hormone concentrations benefit from subject acclimatization to the study protocol (Chandarana et al., 2009).

Interestingly, there was a significant difference in the percentage intake of fat and carbohydrates between the Fasted Saline and Fasted Dummy visit the supper prior to the study day with in increased intake of carbohydrates on the Fasted Dummy visit and increased intake of fat at the Fasted Saline visit. Although there is no evidence that these differences in macronutrient composition could affect appetite the next study day however in the future studies a fixed meal for each subjects should be recommended.

Activation in brain reward systems did not differ between the fasted visits except for the ventral striatum. Activation was significantly reduced when viewing high-calorie foods over objects at the Fasted Saline visit suggesting habituation of ventral striatum activation. This function of the ventral striatum was discussed in more detail in the ghrelin discussion (Section 6.2.5.). The results of this analysis agree with the hypothesis that the ventral striatum is involved in reward expectancy and novelty processing (Guitart-Masip et al., 2010) because the activation was reduced on subsequent visits. This data is supported by

the picture reaction time for appeal rating of any of the picture categories which was significant increased at the Fasted Dummy compared to the Fasted Saline visit. It could be possible that the 'wanting' of the reward was increased at the first visit because the reward was novel (Guitart-Masip et al., 2010). Indeed, previous research hypothesizes that behaviorally, subjects are able to react faster to novel items via enhanced energisation of action through reward (Bunzeck et al., 2009).

This demonstrates the importance of counter-balancing the order of any intervention in fMRI studies when looking at ventral striatum activation. This may not always be easy to do, for example in examing the effects of bariatric surgery, or chronic dietary or drug interventions, in which case a control group with no or placebo intervention should be included.

6.4.3. Correlations of results between fasted visits

Appetite studies are suggested to be biased as they are not performed under real-life circumstances (Blundell et al., 2010). Previous studies however show that VAS appetite and stress ratings, *ad libitum* lunch intake, and appeal ratings positively correlated between the fasted visits thus demonstrating the reliability and reproducibility of the appetite data from this study.

Unfortunately the test-retest reliability of the fMRI activation to food and non-food pictures was not as robust with the exception of the anterior OFC activation. One reason for the anterior OFC to be more reproducible than the other ROIs may be explained by the function of the OFC in encoding reward value. The task in the MRI scanner was to specifically rate the appeal of the food pictures at each of the visits. These results are similar to a previous research study where activation in the OFC was decreased after investigators specifically asked the subjects to actively inhibit their desire for food and suppress their feelings of hunger while they were presented with foods (food was placed on their tongues so they could taste it) rather than passively tasting (Wang et al., 2009).

The other ROIs in my study showed greater variations between visits and subjects. This occurred despite controlling for confounding variables between visits including the

time of the menstrual cycle, time since supper and the type of food at supper as well as the time of the day. There was however a significant difference in the macronutrient composition at supper prior to the study between the fasted visits. Fat intake was increased before the Fasted Saline visit and carbohydrate intake was increased before the Fasted Dummy intake although subjects where instructed to eat the same supper before each study visit. This difference in food intake could influence measurements at each visit particularly brain reward activation, however to date no study investigated the effect of macronutrtient composition at supper on brain reward activation.

Problems with fMRI reproducibilty between visits have been found in other studies. Meta-analysis of fMRI signals when performing a classification learning task known to activate frontostriatal circuitry have generally found similar results across visits, though there is often a widespread varialibility (Aron et al., 2006). A number of previous imaging studies have examined the reproducibility of fMRI signals in experiments with visual stimuli (Rombouts et al., 1997; Machielsen et al., 2000), fear and disgust (Stark et al., 2004), auditory stimuli (Kiehl and Liddle, 2003), working memory (Wei et al., 2004), and sensorimotor control (Loubinoux et al., 2001). The test-retest interval for most of these studies was only short (e.g. a few weeks). This was similar in this study, where fMRI sessions were on average 20 days apart. In the study of Stark et al. the interval was only 1 week and they used a repeated picture presentation as in this fMRI study (Stark et al., 2004). The result was that previously observed brain activations (thalamus, visual cortex, amygdala, hippocampus, and PFC) dissappeared when viewing disgust-inducing pictures, with the exception of the temporo-occipital activation. In contrast, a study by Aron et al. showed that behavioural performances and fronto-striatal activation were highly concordant at a group level at both time points. This research examined eight, healthy adults on two sessions, 1 year apart, while performing a classification learning task (Aron et al., 2006). The relatively low magnitude of activation in brain reward systems during food reward based tasks such as our study compared to the activation seen when performing cognitive tasks may also increase variability because of low signal / noise ratio.

Researchers take a critical view on the reproducibility of fMRI data (Aron et al., 2006). They criticize the statistical approach to analyse fMRI data specifically to assess reliability. Previous test-retest studies used an approach where they compared group activation maps or single-subject activation maps at different time-points. This is not ideal for test-retest reliability (Aron et al., 2006) as thresholding of images can exaggerate very small differences between maps; the signal levels could be highly reliable, yet small differences in the signal or noise could result in substantial differences in the threshold maps due to nonlinearity of the thresholding operation. Aron and colleagues extracted signal change for each subject at each time point and computed intra-class correlation coefficients (ICCs) to assess reliability (Aron et al., 2006). ICC represents the ratio between-subject variance to total variance and is the appropriate metric for assessing within-subject reliability, rather than Pearson's correlation coefficient, because the observations are not independent (Shrout and Fleiss, 1979). However this Fasted Dummy/ Fasted Saline fMRI study did not compare group or single activation maps but calculated the average activation in particular identical voxels between each visit. In future studies it would be useful to use this statistical approach to test fMRI reproducibility.

Another important characteristic of an fMRI task is that it is shown to exhibit minimal practice effects. The nature of the task in Arons study was identical for the 2 sessions, but the material to be learned changed in each session (Shrout and Fleiss, 1979). In this Fasted Dummy/ Fasted Saline fMRI study, the same pictures were used in a pseudo-randomised block design. In future fMRI studies it might be helpful to choose similar pictures but with different colours and shapes of the food pictures for each of the study visits. However, the disadvantage to apply this to further studies is that changes in brain reward system are very small in appetite studies and differences in activations when viewing foods may vary when different food pictures are presented at different study visits. Therefore, it would be interesting to assess the reproducibility of the audio-motor-visual task of the ghrelin fMRI study or the visual checkerboard of the first fed-fasted fMRI study which activates other networks rather than the brain reward system. Unfortunately due to MRI scanner problems there was not enough time to analyse this data.

6.4.4. Conclusion reproducibility of eating behaviour studies

Since appetite studies cannot be performed in a fully controlled environment, stress could potentially influence data collection, especially fMRI data where only small differences in BOLD activation are normally found between visits and subjects. The data of this sub-analysis showed that measures of appetite and stress vary between two fasted visits and that subjects show blunted bias to high-calorie foods and appetite on the Fasted Dummy visit. Activation in brain reward systems decreased in the ventral striatum when viewing high-calorie foods at the subsequent Fasted Saline compared to the initial Fasted Dummy visit. Therefore subject acclimatization to the study protocol should be considered when planning feeding behaviour studies.

This is the first study to retest fMRI changes between two identical visits using a food reward task. There is a considerable variance in activations between visits. Only one brain area, the anterior OFC activation showed a trend towards a positive correlation between the two fasted visits. Maybe the OFC is the only brain region which activates consistently in food reward tasks during fMRI studies. Data from large populations must be obtained to further investigate intra- and intersubject variance, and more insight into possible confounding factors (gender, age, psychological factors) is needed to reduce or control this variance.

6.5. Overall discussion, conclusion and impact on public health

In this research project, I was able to use the technique of fMRI to investigate the complex regulation of appetite and food intake. Previous research has focused on the homeostatic system and internal hormonal signals interacting with this system. However, in the last decade, researchers have increasingly looked at hedonic corticolimbic areas in brain reward systems and their impact on eating behaviour and the development of obesity (Figure 1.5.). This system is influenced by the (i) environment, such as viewing different types of food (external cues), (ii) nutritional state, such as being fasted or fed, (iii) by internal cues, such as appetite hormones (ghrelin, PYY, GLP-1, leptin), and (iv) individual differences in eating behaviour and food preferences (psychological cues) (Figure 1.1.).

6.5.1. Summary of the most relevant findings of all the fMRI studies in this PhD thesis:

(i) Hunger and pleasantness are increased and fullness decreased when fasted compared to when fed (Study 1 - Chapter 3 and Study 2 – Chapter 5, Section 5.2.); and there is tendency for hunger to be greater after injection of ghrelin (Study 2 – Chapter 5, Section 5.3.).

(ii) *Ad libitum* lunch intake is increased when fasted compared to when fed (Study 2 – Chapter 5, Section 5.2.). However, *ad libitum* lunch intake was not increased by ghrelin (Study 2 – Chapter 5, Section 5.3.).

(iii) Food picture appeal is biased towards high-calorie foods when fasted and after injection of ghrelin, especially for the high-calorie sweet but not svaoury foods (Study 1 - Chapter 3 and Study 2 – Chapter 5, Section 5.2. and 5.3.).

(iv) Activation in the ventral striatum, amygdala, insula, medial and lateral OFC respond to visual food stimuli, especially high-calorie compared to low-calorie foods when fasting but not when fed in the first study (Goldstone et al., 2009) (Study 1 - Chapter 3).

(v) Activation in the anterior OFC when viewing high-calorie foods is increased when fasted compared to when fed (Study 2 – Chapter 5, Section 5.2.) and after injection of ghrelin compared to injection of saline in fed subjects, mimicking the effects of fasting (Study 2 – Chapter 5, Section 5.3.).

(vi) Activation in the ventral striatum is increased at the Fasted Dummy visit and tended to be increased at the Fed Ghrelin visit when viewing high-calorie foods, but not at the Fasted Saline and Fed Saline visit or when viewing low-calorie foods (Study 2 – Chapter 4 and Chapter 5, Section 5.3.).

(vii) Individual differences in appeal correlated with individual differences in hunger and pleasantness-to-eat, and also activation in both the medial OFC and lateral OFC for the high-calorie vs. low-calorie contrast between fasted and fed states (Study 1 - Chapter 3).

(viii) Activation in each ROI for each contrast did not correlate with hunger rating in the scanner at the Fasted Saline visit. By contrast, at the Fed Saline visit, average hunger rating in the scanner positively correlated with activation in the ventral striatum, anterior OFC and insula when viewing high-calorie foods vs. objects and when viewing low-calorie foods vs. objects (Study 2 – Chapter 5, Section 5.2.).

(ix) When fasted, activation in the insula when viewing high-calorie (but not low-calorie) foods positively correlated with lunch intake. When fed, activation in the insula and anterior OFC when viewing high-calorie foods correlated with lunch intake (with a trend for low-calorie foods). However, VAS hunger ratings (just before lunch) did not correlate with food intake (Study 2 – Chapter 5, Section 5.2.).

(x) Individual differences in dietary restraint positively correlated with activation to high-calorie vs. low-calorie foods in both the DLPFC and dorsal ACC, and negatively correlated with activation in both the amygdala and lateral OFC, when fasted overnight (Study 1 - Chapter 3).

(xi) Reward sensitivity positively correlated with activation to high-calorie vs. low-calorie foods in the ventral striatum (Study 1 - Chapter 3).

(xii) Lunch intake was significantly increased and there was a tendency for lunch pleasantness to be increased at the Fasted Saline visit compared to the Fasted Dummy visit (Study 2 – Chapter 4).

(xiii) Anxiety and sickness ratings were scored significantly higher at the Fasted Dummy visit compared to the Fasted Saline visit (Study 2 – Chapter 4).

(xiv) High-calorie foods were less appealing on the Fasted Dummy visit visit compared to the Fasted Saline visit (Study 2 – Chapter 4).

(xv) VAS appetite and stress scores, lunch intake, and appeal ratings positively correlated between fasted visits (Study 2 – Chapter 4).

(xvi) There was a tendency for or a significant positive correlation of anterior OFC activation to high-calorie and low-calorie foods and object pictures between the Fasted Dummy and Fasted Saline visit (Study 2 – Chapter 4).

In the ghrelin fMRI study, I was able to replicate some of the results of the first fed-fasted fMRI study although the two study protocols were different in several points.

This is the first study to show that ghrelin mimics fasting by biasing appeal towards high-calorie foods and activating the OFC toward high-calorie foods. These studies especially proved that the OFC plays an important role in encoding the reward value of high-calorie and low-calorie foods and that the ventral striatum may be implicated in novelty processing when viewing high-calorie foods.

In summary, these results demonstrated that nutritional state and ghrelin play an important role in influencing the hedonic brain reward system and therefore food appeal and pleasantness in an environment where high-palatable, high-calorie foods are constantly available (Cabanac, 1971, Uher et al., 2006, Stoeckel et al., 2007). As both fMRI studies examined healthy volunteers it needs to be further investigated how brain

reward systems differ in lean and obese people and what are the differences in their underlying physiological mechinsms.

6.5.2. How can these findings be linked to obesity?

As previously described, natural rewards such as palatable food and ghrelin can affect dopamine signaling in the brain reward system (Nestler, 2001; Naleid et al., 2005).

Human key focus in research of addiction disorders and obesity is that *hypo*-dopaminergic functioning (Reward Deficiency Syndrome - RDS) in brain reward pathways is a key risk factor for their development (Blum et al., 2000). The premise is that addictive substances such as food, which increase brain dopamine levels, are used as a form of "self-medication" to boost a sluggish dopamine system and increase hedonic capacity and promote excessive behaviour. The same arguments have been recently extended to the risk for obesity; focusing on dopamine D2 receptors. There is reasonable evidence that individuals with the Taq1A+ allele (polymorphism of D2 receptor) have reduced brain dopamine function due to a 30-40% reduction in D2 receptor density in the striatal region (Stice et al., 2008).

The counter argument is that *hyper*-sensitivity to reward contributes to increased risk of obesity because of enhanced motivation to approach pleasurable activities such as eating (Beaver et al., 2006, Davis et al., 2007). Addictive drugs and food could also share a tolerance of their rewarding properties by the downregulation of dopamine receptors in the striatum (Volkow et al., 2002), and a decrease in the function of the extended amygdala, which produces negative affect and anxiety associated with abstinence (Koob, 2006). There is now evidence that highly palatable foods cause the same neuroadaptions which increase strong cravings, and contribute to the symptoms of withdrawl (Grigson, 2002, Pelchat, 2002). Davis and Carter suggest a dual vulnerability to addictions whereby both *hypo*-dopaminergic functioning and *hyper*-sensitivity to reward can confer risk, albeit in different individuals and perhaps with different levels of severity (Davis and Carter, 2009).

Obese patients, especially women, binge eaters and patients with increased reward sensitivity previously showed increased activation in brain reward areas when viewing or tasting high-calorie foods compared to their lean counterparts (Gautier et al., 2000; Gautier et al., 2001; DelParigi et al., 2004; DelParigi et al., 2005; Le et al., 2006; DelParigi et al., 2007; Le et al., 2007; Rothemund et al., 2007; Stice et al., 2008; Stoeckel et al., 2008; Bohon et al., 2009; Martin et al., 2009; Schienle et al., 2009; Stoeckel et al., 2009; Davids et al., 2010; Stice et al., 2010). In bulimia nervosa patients, who had increased ventral striatum volumes, BMI and purgeing severity were correlated with striatal grey matter volume abnormalities (Schafer et al., 2010). A voxel-based morphometric functional MRI study showed that pathologic “sweet tooth” was associated with grey matter volume loss in the right anterior insula, the lateral OFC, the DLPFC and temporal poles; hyperphagia was associated with bilateral grey matter loss in anteromedial portions of the OFC (Whitwell et al., 2007). The new insight into the meachnisms of brain reward systems and their role in eating behaviour may be used to provide rationale for new psychological or pharmacological approaches for treatment of obesity (Adan et al., 2008). For example, targeting specific regions of the brain reward systems with new strategies of deep brain stimulation for managing eating disorders (Halpern et al., 2008; Camus et al., 2009). Another pharmacological treatment could involve ghrelin antagonists to block the action of ghrelin and its receptor in order to suppress its action on brain reward systems.

The findings of the fMRI studies in this thesis are potentially of relevance to understand some of the factors which play a role in the development of obesity and how rewarding properties of food become represented in terms of subjective hedonic values and can contribute in driving excessive food intake.

6.6. Future work

6.6.1. Ghrelin fMRI study

- Increase the number of subjects to at least 20. In fMRI, especially food reward tasks, it is only possible to measure small changes in brain reward systems, so the higher the number of subjects, the more likely the result is robust.

- Include obese subjects and compare them to lean controls in the fasted and fed state to analyse differences in brain reward systems.

- Assess resting state functional connectivity to examine the effect of ghrelin in brain reward systems when no stimulus is applied. Resting connectivity allows the assessment of coordinated activity between brain regions in the absece of an external input (Damoiseaux et al., 2006). These include brain regions involved in executive functioning or 'executive control network' (ECN, involving anterior cingulate and dorsolateral pre-frontal cortex), and random episodic silent thought, the so-called 'default-mode network' (DMN, involving the precuneus, posterior cingulate and pre-frontal cortex). Motivational salience networks involving the anterior cingulate and frontoinsular cortex are also described (Seeley et al., 2007). A recent study demonstrate that nicotine administration in abstinent smokers to suppress craving modulates dynamic interactions between large-scale cognitive brain networks in the resting state, for example inverse coupling between the DMN and ECN (Cole et al., 2010). It is unknown how the activity in these resting state networks is altered during states of increased hunger (where attention may switch away from random silent thought to thinking about food) and/or whether additional resting state networks are activated (e.g. within brain centres involved in reward, craving, pleasure and emotion). This data will be analysed to see how activity and connectivity in resting state networks changes when healthy non-obese subjects are fasted or given the hormone ghrelin.

- Analyse audio-motor-visual scans to look for non-specific changes in activation in brain reward systems.

- Measure gut hormones, including acylated and total ghrelin, PYY, GLP-1, PP and leptin to correlate them with activation in brain reward systems and feeding behaviour.

- Include different test lunch; ghrelin is suggested to preferentially increase the appeal and perhaps intake of sweet over savoury food such as used in our study (pasta and curry). It would be also interesting to use different types of foods at lunch, such as high-calorie sweet, high-calorie savoury, low-calorie sweet and low-calorie savoury food, thus subjects need to make a choice such as when rating the appeal of the food pictures in the scanner.

- Investigate the effect of ghrelin on dopamine release in humans. Previous PET imaging studies showed smoking-induced dopamine realease in the ventral striatum using bolus-plus-continuous-infusion of (11)C-raclopride (D2 recpetor antagonist) (Brody et al., 2010) or amphetamine-induced dopamine release in the ventral striatum using [(11)C]-(+)-4-propyl-9-hydroxynaphthoxazine ((+)-PHNO) (D2/D3 recpetor agonist) (Willeit et al., 2008).

Chapter 7

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Chapter 8

APPENDICES

Appendix 1. Patient information sheet

Imperial College London

MRC Clinical Sciences Centre

Ref number 07/Q0406/19 Version 03 11/10/2007

Robert Steiner MRI Unit, Imaging Sciences Department
MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College
Hammersmith Hospital Campus
Du Cane Road, London W12 0NN

THIS INFORMATION SHEET IS VALID FOR USE UNTIL: **30th September** 2009

INFORMATION SHEET FOR RESEARCH PARTICIPANTS

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

STUDY TITLE: HOW DOES GHRELIN INCREASE APPETITE IN THE BRAIN: A FUNCTIONAL MAGNETIC RESONANCE IMAGING STUDY?

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

We will be happy to let you have a copy of the leaflet entitled 'Medical Research and You' published by Consumers for Ethics in Research (CERES). This leaflet gives more information about medical research and looks at some questions you may want to ask.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation. Thank you for reading this.

WHAT IS THE PURPOSE OF THE STUDY?

Ghrelin is a naturally-occurring substance found in the blood of healthy people. It is made in the stomach, released when fasting and levels in the blood fall after a meal. Ghrelin is known to increase appetite in healthy individuals. This study aims to see how ghrelin acts in the brain to increase appetite. This is an important step towards finding out how ghrelin naturally regulates appetite. This will help with the development of medicines to treat obesity and loss of appetite.

WHY HAVE I BEEN CHOSEN?

Healthy individuals are being recruited to take part in this study.

You should **not** take part in this study if you:
1) have any illnesses which we feel make you unsuitable
2) if you take any medication which we feel make you unsuitable
3) if you are pregnant or breast feeding
4) if you have given blood in the last three months

It is important that you **should not become pregnant** during the course of the study or for one month following the study, as the effects of ghrelin in pregnancy are not known. It is

therefore important that you have adequate and reliable contraception during this period.

DO I HAVE TO TAKE PART?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

Screening visit

If you agree to take part in this study, you will first be examined by one of the research doctors and have a number of blood tests after an overnight fast (no more than 50ml blood equivalent to around 3 tablespoons), a pregnancy test (if female) and a heart recording (ECG) to ensure you are fit and healthy. You will be asked to complete a few short questionnaires about your eating habits, personality and mood, and keep a record of all food and drink consumed for three days. You will also be asked to taste a number of different meals which will be used later on in the study.

You will also have your height and weight taken and your body fat content measured using a 'bio-electrical impedance' machine. This is a painless safe method which involves measuring the electrical current from your body and takes only about 5 minutes. As long as these medical checks are satisfactory and you are still happy to participate, you will then be asked to attend for the first of up to 4 study visits.

With your permission, we will also take a sample of DNA from blood or saliva to look for changes in your genes that may be involved in the how the body controls appetite, body weight and responds to ghrelin. This will enable us to see what effect such changes may have on your response to the injections.

Number of visits

You will be asked to attend the Clinical Investigation Ward at Hammersmith Hospital as an outpatient on up to another 4 occasions, following your screening interview, each separated by at least 3 days. These will be completed on dates convenient to you and the investigators, but should usually be completed within a maximum of 3 months.

Study design

On each study visit you will be asked to have nothing to eat and only water to drink from 8 pm on the evening before the study. You will be asked to attend in the morning and each visit will last around 5 hours. You will be asked to abstain from alcohol and strenuous exercise for 24 hours before the visit. We will also ask you to keep a record of all food and drink consumed for three days before the visit, the day of the visit and for one day afterwards. You will also have a pregnancy test on each visit (if female), and have your height, weight and body fat content measured. You will also complete questionnaires about your mood on each study visit.

On each study day you will have a small plastic cannula tube inserted into a vein in one arm. A vein is the type of blood vessel commonly used for taking blood samples. You may feel some discomfort whilst the cannula is being inserted. After the cannula tube has been inserted this will be used to take blood samples. We will also ask you to score how you are feeling (e.g. rating your hunger) by placing a mark on a line called a visual analogue scale.

You will then be either given a moderate size breakfast to eat over 20 minutes, or continue

fasting. On each of the visits, you will then receive either a saline (salt water) or ghrelin injection under the skin on the thigh or tummy. You may fell some slight discomfort at the time of the injection.

The saline is a placebo or dummy treatment that is commonly used in studies of this nature. It contains no active ingredient and is not expected to alter your appetite. The saline treatment will serve as a baseline measurement to which all the active treatments are compared. This is a randomised, double-blind trial. This means that neither you, nor your research doctors, will know what treatment you are being given on some visits (although, if your doctor needs to find out, he/she can do so). Throughout the study, we will monitor your heart rate and blood pressure.

Further blood samples will be taken and you will be asked to complete further visual analogue scales over the visit. The total amount of blood taken on each study visit will not be more than 150ml (about 10 tablespoonfuls). The total amount of blood taken over all your visits will not be more than 500 ml (a little less than a pint and the same amount taken when making a single donation of blood for blood transfusion). During these visits you will be seated or lying on a couch, and can read or watch television.

Thirty minutes after the injection, you will have a magnetic resonance imaging (MRI) brain scan by lying in an MRI scanner for up to 1 hour. This will take place in the Robert Steiner Magnetic Resonance Imaging Unit, nearby the Clinical Investigation Ward. This will enable us to look at the structure and activity of your brain.

During the functional brain scans we look at the activity of the brain at rest, while you look at a variety of pictures on a screen, and perform simple tasks like viewing a flashing light, pressing a button, reading, listening, speaking, recalling, thinking about words or numbers. You will be asked to make responses to the pictures while in the scanner using a keypad. You will have the opportunity to practice lying in the scanner while looking at various pictures on the screen. This will enable us to ensure that you can follow the instructions and lie still while in the scanner.

At the end of the brain scan you will be taken back to the Clinical Investigation Ward. Fifteen minutes after the end of the MRI scan you will be presented with a buffet meal and you will be asked to eat as much as you want of the meal until you feel comfortably full.

We will continue to monitor you for another hour after the buffet meal, after which you are free to go (4 hours after the cannula was inserted). At the end of the final visit you will also be asked to score how much you usually like to eat the foodstuffs shown in the food pictures using a visual analogue scale.

WHAT DO I HAVE TO DO?

The only restrictions on your lifestyle are that you will be asked to have nothing to eat and only water to drink from 8pm on the night before the meal days. You will need to keep a record of all food and drinks consumed in the three days before every study and for the twenty-four hours afterwards. For twenty-four hours before each study meal you will be asked to refrain from taking strenuous exercise and drinking alcohol.

Female volunteers should have adequate contraception for the period of the study and for one month afterwards as the effects of ghrelin in pregnancy are not known. Pregnancy tests will be carried out to confirm that women of child bearing age are not pregnant on the morning before each study injection.

WHAT IS THE DRUG THAT IS BEING TESTED?

Ghrelin is a naturally occurring hormone found in the circulation of healthy people. Ghrelin has been used in several other studies in our department and worldwide, in men and women, without harmful effects. The dose of ghrelin that you will be given are small and will increase the levels of ghrelin in your body to a levels naturally found after a prolonged fast.

WHAT ARE THE SIDE EFFECTS OF TAKING PART?

From our previous studies we do not expect any significant side effects, but the unexpected can occur. During the study, at least one experienced doctor will monitor you closely. If you suffer from any ill effects during the study you should report them to the doctors monitoring you immediately. If you suffer from any ill effects afterwards you should report them to one of the research doctors at the contact number below or when you next see them. You may ask for the study to stop at any time without giving a reason. If any unexpected side effects occur, the study will be stopped.

MRI is a powerful, diagnostic body scanning technique, which is used in hospitals worldwide to create images of the inside of the body. MRI has been used safely for several decades and has no known side-effects. Each scan is directed to the specific requirements of your referring doctor or to research study in which you are taking part.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?

Ghrelin has been administered in several studies by our laboratory and therefore we do not anticipate any problems with the injection. Insertion of the cannula (drip) into your arm on each of the study days may cause minor discomfort or superficial bruising, as may the injection of saline or ghrelin under the skin.

Magnetic resonance imaging (MRI) is a procedure that allows doctors to look inside the body by using a scanner that sends out a strong magnetic field and radio waves. MRI does not use X-rays. This procedure is used routinely for medical care and is very safe for most people, but you will be monitored during the entire MRI scan in case any problems occur. The risks of having an MRI scan are:

- The MRI scanner contains a very strong magnet. Therefore, you will not be able to have the MRI if you have any type of metal implanted in your body, for example, any pacing device (such as a heart pacer), any metal in your eyes, or certain types of heart valves or brain aneurysm clips. Someone will ask you questions about this before you have the MRI.

- There is not much room inside the MRI scanner. You may be uncomfortable if you do not like to be in close spaces (“claustrophobia”). During the procedure, you will be able to talk to and hear the MRI staff through a speaker and earphone system, and, in the event of an emergency, you can tell them to stop the scan. You will be closely monitored and repeatedly checked on to make sure you are as comfortable as possible. While your head is in the scanner, we will support it, so you can’t move it. If this upsets you, you will be able to signal and speak to the investigator and stop the scan through the use of a radio system and a signaling button. You will have the opportunity during the first MRI scan to ensure that you can tolerate having the scan before the next two scans are done.

- The MRI produces a “hammering noise”. You will wear earplugs and headphones to prevent discomfort or damage to hearing. The headphones will also allow you to be able to hear us talk to you.

- You will be fully awake during the MRI scan and will not be sedated at any time. We will make every effort to ensure your comfort during this experiment.

It should be noted that the MRI brain scan cannot be viewed as a comprehensive health screening procedure. However, vary rarely, unexpected information can be detected which may warrant further investigation. In this event, you will be informed and a report will be sent to your GP, who will arrange further tests and coordinate your further care. In the rare event that we find a significant abnormality on your structural brain scan on the first visit this may exclude you from continuing with the rest of the study.

It is possible that if the treatment is given to a pregnant woman it will harm the unborn child. Pregnant women must therefore not take part in this study; neither should women who plan to become pregnant during the study. Women who are at risk of pregnancy will be asked to have a pregnancy test before taking part, to exclude the possibility of pregnancy. Women who could become pregnant must use an effective contraceptive during the course of this study. Any woman who finds that she has become pregnant while taking part in the study should immediately tell her research doctor.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

The information that we get from this study will help us to better understand normal appetite regulation and may help us to better treat future patients who suffer from being overweight or reduced appetite.

If any of the screening questionnaires or blood tests reveal any medical problems (e.g. depression, diabetes, high cholesterol, thyroid, kidney or liver problems), your GP will be informed so that they can coordinate your further care, arrange any further tests, and refer you on to Hospital Doctors if necessary.

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

Once the study has finished, the results of the study can be made available to you and/or your GP should you wish. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided below.

WHAT IF SOMETHING GOES WRONG?

In the event of any adverse events occurring as a consequence of your participation in this study, you will be compensated through the Imperial College School of Medicine's 'No-fault' Compensation scheme.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

It is a requirement that your GP is informed, with your consent, of your participation in this study, at the start of the study.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results are likely to be published in the year following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP should you wish.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

This study is being organised and funded by the Department of Imaging Sciences, MRC Clinical Sciences Centre and the Department of Metabolic Medicine, Imperial College London.

PAYMENT

You will receive a fixed payment to cover expenses including travel costs. This sum of £20 for the screening visit and £50 for each injection visit will be paid when you have completed your visits.

WHO HAS REVIEWED THE STUDY?

This study has been reviewed by the Hammersmith Hospitals Research Ethics Committee.

CONTACT FOR FURTHER INFORMATION

If you experience any problems during the study, you may withdraw at any stage. The doctor involved in the study, Dr Goldstone, will be available by telephone during working hours (020 8383 1029 or via the paging system 0845 0921000 bleep 5530). The hospital switchboard (020 8383 1000) has home and mobile phone numbers for all the doctors involved in the study and can contact them at any time outside normal working hours.

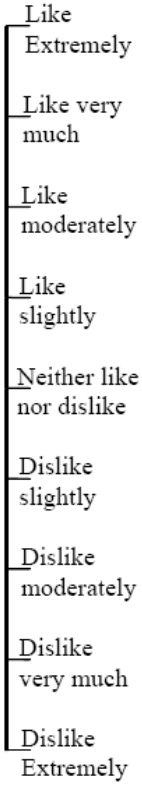
If you agree to take part in the trial, you will also be given the mobile phone numbers of the doctors.

Appendix 2. Food preference scale

Food Preference

Name:	**Date:**
ID:	

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



Comments:

Appendix 3. SCOFF

SCOFF QUESTIONNAIRE

Initials:

ID:

Date:

Do you make yourself sick because you feel uncomfortably full?

YES / NO

Do you worry you have lost control over how much you eat?

YES / NO

Have you recently lost more than one stone in a 3 month period?

YES / NO

Do you believe yourself to be fat when others say you are too thin?

YES / NO

Would you say that food dominates your life?

YES / NO

Appendix 13. SCOFF Questionnaire 07-Q0406-19

Appendix 4. BIS/ BAS Questionnaire

BIS / BAS Scales

Each item of this questionnaire is a statement that a person may either agree with or disagree with. For each item, indicate how much you agree or disagree with what the item says. Please respond to all the items; do not leave any blank. Choose only one response to each statement and mark your answer with a tick. Please be as accurate and honest as you can be. Respond to each item as if it were the only item. That is, don't worry about being "consistent" in your responses.

Nº	Question	Very true for me	Somewhat true for me	Somewhat false for me	Very false for me
1.	A person's family is the most important thing in life.				
2.	Even if something bad is about to happen to me, I rarely experience fear or nervousness.				
3.	I go out of my way to get things I want.				
4.	When I'm doing well at something I love to keep at it.				
5.	I'm always willing to try something new if I think it will be fun.				
6.	How I dress is important to me.				
7.	When I get something I want, I feel excited and energized.				
8.	Criticism or scolding hurts me quite a bit.				
9.	When I want something I usually go all-out to get it.				
10.	I will often do things for no other reason than that they might be fun.				
11.	It's hard for me to find the time to do things such as get a haircut.				
12.	If I see a chance to get something I want I move on it right away.				
13.	I feel pretty worried or upset when I think or know somebody is angry at me.				
14.	When I see an opportunity for something I like I get excited right away.				
15.	I often act on the spur of the moment.				
16.	If I think something unpleasant is going to happen I usually get pretty "worked up."				
17.	I often wonder why people act the way they do.				
18.	When good things happen to me, it affects me strongly.				
19.	I feel worried when I think I have done poorly at something important.				
20.	I crave excitement and new sensations.				
21.	When I go after something I use a "no holds barred" approach.				
22.	I have very few fears compared to my friends.				
23.	It would excite me to win a contest.				
24.	I worry about making mistakes.				

Appendix 5. DEBQ

DUTCH EATING QUESTIONNAIRE

Volunteer Initials: _____ **Date:** _____

Volunteer No. _____

Please place an (✓) in the box which applies best to each of the numbered statements. All of the results will be *strictly* confidential. Most of the questions directly relate to food or eating, although other types of questions have been included. Please answer each question carefully. Thank you.

1. If you have put on weight, do you eat less than you usually do?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

2. Do you try to eat less at mealtimes than you would like to eat?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

3. How often do you refuse food or drink offered because you are concerned about your weight?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

4. Do you watch exactly what you eat?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

5. Do you deliberately eat foods that are slimming?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

6. When you have eaten too much, do you eat less than usual the following days?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

7. Do you deliberately eat less in order not to become heavier?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

8. How often do you try not to eat between meals because you are watching your weight?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

9. How often in the evening do you try not to eat because you are watching your weight?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

10. Do you take into account your weight with what you eat?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

11. Do you have the desire to eat when you are irritated?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

12. Do you have a desire to eat when you have nothing to do?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

13. Do you have a desire to eat when you are depressed or discouraged?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

14. Do you have a desire to eat when you are feeling lonely?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

15. Do you have a desire to eat when somebody lets you down?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

16. Do you have a desire to eat when you are cross?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

17. Do you have a desire to eat when you are approaching something unpleasant to happen?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

18. Do you get the desire to eat when you are anxious, worried or tense?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

19. Do you have a desire to eat when things are going against you or when things have gone wrong?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

20. Do you have a desire to eat when you are frightened?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

21. Do you have a desire to eat when you are disappointed?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

22. Do you have a desire to eat when you are bore or restless?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

23. Do you have a desire to eat when you are emotionally upset?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often ❑ Not Relevant

24. If food tastes good to you, do you eat more than usual?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

25. If food smells and looks good do you eat more than usual?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

26. If you see or smell something delicious, do you have the desire to eat it?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

27. If you have something delicious to eat, do you eat it straight away?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

28. If you walk past the baker do you have the desire to buy something delicious?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

29. If you walk past a snackbar or a café, do you have the desire to buy something delicious?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

30. If you see others eating, do you also have the desire to eat?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

31. Can you resist eating delicious foods?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

32. Do you eat more than usual, when you see others eating?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

33. When preparing a meal are you inclined to eat something?

❑ Never ❑ Seldom ❑ Sometimes ❑ Often ❑ Very Often

Appendix 6. PANAS Questionnaire

PANAS Scale

Initials: ID: Date:

This scale consists of a number of words that describe different feelings and emotions.

Read each item and then mark [x] the appropriate answer in the space next to the word.

Indicate to what extent you have felt this way **on average during the past week**.

Use the following scale to record your answer:

No.	Feeling	very slightly or not at all	a little	moderately	quite a bit	extremely
1	interested					
2	distressed					
3	excited					
4	upset					
5	strong					
6	guilty					
7	scared					
8	hostile					
9	enthusiastic					
10	proud					
11	irritable					
12	alert					
13	ashamed					
14	inspired					
15	nervous					
16	determined					
17	attentive					
18	jittery					
19	active					
20	afraid					

Appendix 7. Dietary record (example page)

DIETARY RECORD SHEET - EXAMPLE

Record **ALL** food and drink consumed during the day including snacks, nibbles, sauces and dressings.

Record method of cooking, type and quantity of food
eg: 6 tbsp boiled wholemeal spaghetti
2 egg sized roast potatoes.

DAY: Example DATE: 1st June 1994

MEAL/ SNACK	QUANTITY EATEN	DETAILS OF FOOD & DRINK	Leave Blank
Early Morning:	1 cup 1 tbsp	Tea with Skimmed milk	
Breakfast:	3 heaped tbsp ¼ pint 1 medium slice 1 tsp 2 mugs	Branflakes (Kellogg's) Skimmed milk for cereal & drinks Wholemeal bread (large loaf) Flora extra light margarine Coffee	
During Morning:	1 mug 1 tbsp 1 medium	Coffee with skimmed milk Apple (eaten with skin)	
Midday:	4 slices 4 level tsp 2 thin slices 1 large 1 large 1 can (330ml)	Sandwiches: wholemeal bread (Allinsons) large loaf, sliced Flora extra light margarine Ham (no fat) Tomato Banana Diet Tango	

DAY: Example DATE: 1st June 2006

MEAL/ SNACK	QUANTITY EATEN	DETAILS OF FOOD & DRINK	Leave Blank
During Afternoon:	1 glass 25g pkt	Low Calorie squash made with concentrated squash KP roasted salted peanuts	
Evening Meal:	4 heaped tbsp 1 apple sized 3 tbsp 1 x 150g tub 1 glass 1 cup 1 tbsp	Chicken & mushroom casserole (home-made with skimmed milk in the sauce) Jacket potato Broccoli, boiled Shape raspberry yoghurt Half mineral water/half natural orange juice Tea with skimmed milk	
During Evening:			
Bedtime Snack:	1 mug 1 tsp ½ mug 2	Ovaltine made with: Ovaltine ordinary silver top milk, the rest water Rich Tea biscuits (Sainsburys).	

Appendix 8. MRI Metall Check List

Check list for contra-indications

Study title

Ghrelin and functional MRI of appetite 07/Q0406/19

To be checked by the participant

Patient Name: ……

Date of birth: ……

Please tick the following

	YES	NO	DETAILS
Cardiac pacemaker or defibrillator			
Mechanical heart valve			
Coronary stent			
History of foreign body in eye			
Occupation as metal worker, grinder, welder			
Metallic implant, joint replacements, orthopaedic plates or screws			
Shrapnel			
Aneurysm clip/haemostatic clip			
Ear Implants			
Artificial Eye			
Coloured contact lenses			
Pregnancy			
Intra-uterine contraceptive device (Coil)			
Implantable pumps/neurostimulators			
Allergies			
Are you wearing a watch or hair clip?			
Are you wearing jewellery or body piercing?			
Have you removed any clothing containing metal,			

If the answer to answer to any of the above questions with "YES" please give details.
Certain devices may still be permitted if they are MR-compatible.

It is extremely hazardous to bring any metal object near the scanner.

This is to confirm that the above checklist has been completed.

Signed:…… (patient) Date: ……

Signed:…… (MR staff) Date: ……

Appendix 9. Mood / Depression questionnaire

V 0477

Mood/depression questionnaire

CRTN: _____ CRF number: _____

patient inits: _____

Mood/Depression Assessment Questionnaire

1. Since your last visit have you felt depressed, sad or blue much of the time?

   yes ☐
   no ☐

2. Since your last visit have you often felt helpless about the future?

   yes ☐
   no ☐

3. Since your last visit have you had little interest or pleasure in doing things?

   yes ☐
   no ☐

4. Since your last visit have you had trouble sleeping many nights?

   yes ☐
   no ☐

Are two (2) or more of the above questions marked YES while undergoing treatment in this protocol?

yes ☐ → *complete a Beck Depression Inventory. If score is 28 or less, patient may continue in the study. If score is ≥ 29, patient will need to complete all final assessments and be dropped from the study. The investigator may recommend that the patient be referred for a professional psychiatric assessment.*

no ☐

Appendix 10. Visual Analaogue Scale

Time -15

HOW HUNGRY DO YOU FEEL RIGHT NOW?

NOT AT ALL EXTREMELY

HOW SICK DO YOU FEEL RIGHT NOW?

NOT AT ALL EXTREMELY

HOW PLEASANT WOULD IT BE TO EAT RIGHT NOW?

NOT AT ALL EXTREMELY

HOW ANXIOUS DO YOU FEEL RIGHT NOW?

NOT AT ALL EXTREMELY

Time -15

HOW MUCH DO YOU THINK YOU COULD EAT RIGHT NOW?

NOTHING A LARGE AMOUNT

HOW FULL DO YOU FEEL RIGHT NOW?

NOT AT ALL EXTREMELY

HOW STRESSED DO YOU FEEL RIGHT NOW?

NOT AT ALL EXTREMELY

HOW SLEEPY DO YOU FEEL RIGHT NOW?

NOT AT ALL EXTREMELY

Appendix 11. Blood sample preparation

1. AEBSF (protease inhibitor to prevent breakdown of active ghrelin).

Sigma A856-500MG £252.50 per 500mg

Added in advance to 10ml orange plastic lithium heparin tubes to which add whole blood.

Bottle contains 500mg powder.
Add 2ml distilled water to AEBSF bottle = 250mg/ml = 0.25 mg/ul
Final [AEBSF] = 1mg / ml whole blood.
10ml blood tubes.
Need to add 40ul AEBSF solution per 10ml blood tube = 10mg.
Store tubes at -80C.
Defrost day before into fridge.

2. Trasylol = Aprotinin (protease inhibitor to prevent breakdown of certain gut hormones).

Order Nordic Pharma UK 01189298233
Cost £20.53 per 50ml bottle (+£20 admin charge if < £300 order)
Need to complete named patient form and covering letter stating that for research.

Add 200ul to each 10ml orange blood tube day before study and store in fridge.

3. HCl (to prevent breakdown of active ghrelin).

Added to plasma (not whole blood).
Add to aliquots to which will add plasma for active ghrelin (yellow A tubes) on day before study and store in fridge.

Stock [HCl] = 11M.
Dilute 784ul of stock with 10ml distilled water to give 10.784ml 0.8M HCl.

Add 10ul of 0.8M HCl to 150ul plasma to give final [HCl] = 0.05M

Appendix 12. fMRI scanning sheet

MRI SCANNING SHEET										
Date of scan:										
Initials:		ID #:			Hospital #:					
Fasted / Fed			Injection #:	D		S		K		L
Visit no:	1		2	3		4				
Food picture runs:	AB		CD	EF		GH				
MR personnel: Tony Goldstone/Giuliana Durighel/Emer Hughes/Christina Prechtl/Sam Scholtz /Other:							Check buttons work in WordPad ☐			
3T MRI #:					IFIS hood / Data logging PC / (IFIS PC Folder#:)					
Attach: Respiratory belt (under arms) Pulse oximeter (L ring, red on pulp) Check audio cable										
Time into Philips 3T scanner:					Time out scanner:					
Scans:	Test*	Rest*	Food x 2	Field	AMV*	T1 or	FLAIR + T2	or	DTI	or T1
Time (mins):	2	9	10 x 2	4	6	6	6 + 3		10	6
No. of vols:		192	192 x 2		114	(* = hunger rating included)				
Prep phase		Full	Auto	Auto	Auto (ensure CLEAR ON)				Full	
Time of scan: /										
T1/T2/T2W_FLAIR Anatomical scan: Parallel to AC-PC Line										
fMRI scans: gradient epi BOLD, TR 3000, TE 30, FOV 280x220x143, 2x2x3.25 mm, FA 90, 44 slices ascending contiguous, SENSE 2. Slice pos: 30° to AC-PC Line, CLEAR ON										
Initial RL tilt		degrees	Add Initial *r[0] =			+ 0.0926 to -		(parallel AC-PC)		
-30° RL tilt		degrees	Adjusted *r[0] =			-		(30° to AC-PC)		
Rest: 192 vols. Eyes shut, awake. Initial 6 vols discarded. **At end: Awake or Asleep**										
Field map: spin echo TR 800, TE 20, flip 90, 3.25 x 2 x 2 mm, δTE 0 & 2.5										
Food: 2 x 192 vols. Stimulus duration 2.5 sec; ISI 3.0 sec. ITI 0.5 sec; 3 conditions x 5 blocks x 6 pictures x 2 runs (high calorie, low calorie, household object) + 16 inter-condition blurred blocks per run. Block length 18 sec. Initial 6 vols to be discarded.										
AMV: 114 vols. Auditory (story), visual (4Hz colour checkboard), motor (tap R index finger 1Hz)										
DTI: 32 dir, b factor 1000, TR 13951, TE 59, 73 slices, SENSE 2.5, 224x224, 1.75x1.75x2mm, AC-PC line, no z-gradient correction, full prep, **PB-volume shim**										
Hunger VAS button: Test Rest AMV			**Problems:**							
☐ Download edat files x5 to memory stick					☐ Save edat files to Appetite folder					
☐ Save LabChart Pro data to laptop					☐ Download LabChart Pro data to Appetite					
☐ Upload MR scans to MRIdb					☐ Save MR scans to disc DVD #:					
☐ Upload T1 T2 MR scans to PACS					☐ Export fieldmap raw data to Export/Appetite					
☐ Photocopy MRI front sheet x2 (case folder and master file) - after 4th visit only										
☐ GP letter sent - 1st visit only					☐ Photocopy x2 (case folder, hospital notes)					
☐ MRI T1 T2 FLAIR anatomical report					☐ Pcopy x3 (case folder, master file, hosp notes)					
☐ MRI anatomical GP letter - after 2nd visit					☐ Pcopy x2 (case folder, hosp notes)					