System Interactions in the Regulation of Appetite

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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I would like to dedicate this thesis to my dad, who always said his aim in life was for his children to achieve what he has yet to.

Abstract

Despite the huge range of neurobiological targets within the appetite system, the development of pharmacological anti-obesity agents is making slow progress as a result of limitations on maximal weight loss, adverse side-effects, and/or long-term resistance. However, in principle, the use of drug polytherapy allows for the use of lower doses, possible synergistic/additive weight loss, fewer and less serious side-effects and reduced potential for counter-regulation. Although food intake and/or bodyweight have been and are being researched following co-treatment with a range of agents, there is a distinct gap in the literature regarding the behavioural specificity of the anorectic effects for recently approved and upcoming anti-obesity therapies. The present thesis therefore characterised the acute effects of individual systemic (i.p.) treatment with the general opioid receptor antagonist naltrexone (0.1, 1.0 and 3.0mg/kg), the noradrenaline and dopamine reuptake inhibitor bupropion (10, 20 and 40mg/kg), the serotonin 5-$HT_{1B/2C}$ receptor agonist *m*CPP (0.1, 1.0 and 3.0mg/kg), and the GLP-1R mimetic exendin-4 (0.025, 0.25, and 2.5µg/kg), on food intake, feeding and non-feeding behaviour, the behavioural satiety sequence (BSS), and weight gain. In addition, the acute anorectic response to co-treatment with sub-maximal doses of each non-opioid compound plus an opioid antagonist (naloxone or naltrexone) was assessed. The results suggested that, while the anorectic effects of naloxone and naltrexone were behaviourally-selective, those of rimonabant, bupropion, *m*CPP and exendin-4 may have largely resulted from competing behaviour. The co-treatment studies highlighted concurrent anorexia and an undesirable behaviour for rimonabant, bupropion and, potentially, *m*CPP. However, the anorectic action of *m*CPP and exendin-4 may have largely resulted from malaise. The results further showed that, while only the combination of bupropion and naltrexone produced an additive effect on food intake, co-treatment with an opioid antagonist reduced/eliminated unwanted effects normally associated with higher doses of rimonabant, bupropion and, potentially, *m*CPP. The search for efficacious and safe anti-obesity agents should therefore focus, to an even greater extent than at present, on the therapeutic potential of targeting multiple systems (polytherapy). Overall, current findings have emphasised the value of detailed behavioural analysis of drug effects on appetite. As such, novel treatment combinations may well produce a successful anti-obesity agent, if clinical trials are prefaced by adequate preclinical testing.

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Publications

Publications Arising from the Current Thesis

Wright, F.L., Rodgers, R.J. (in press) Behavioural profile of exendin-4/naltrexone dose combinations in male rats during tests of palatable food consumption. Psychopharmacology. DOI: 10.1007/s00213-014-3507-4.

Wright, F.L., Rodgers, R.J. (2014) On the behavioural specificity of hypophagia induced in male rats by mCPP, naltrexone, and their combination. Psychopharmacology, 231, 787-800.

Wright, F.L., Rodgers, R.J. (2013) Acute behavioural effects of bupropion and naltrexone, alone and in combination, in non-deprived male rats presented with palatable mash. Psychopharmacology, 228 (2), 291-307.

Wright, F.L., Rodgers, R.J. (2013) Low dose naloxone attenuates the pruritic but not anorectic response to rimonabant in male rats. Psychopharmacology, 226, 415-431.

Other Publications

Rodgers, R.J., Wright, F.L., Snow, N.F., Taylor, L.J. (2013) Orexin-1 receptor antagonism fails to reduce anxiety-like behaviour in either plus-maze-naïve or plus-maze-experienced mice. Behavioural Brain Research, 243, 213-219.

Rodgers, R.J., Howard, K., Stewart, S., Waring, P., Wright, F.L. (2010) Anxioselective profile of glycineB receptor partial agonist, D-cycloserine, in plus-maze-naïve but not plus-maze-experienced mice. European Journal of Pharmacology, 646: 31-37.

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Rodgers, R.J. and Wright, F.L. (2013). Evidence against response competition as a factor in acute rimonabant anorexia. Obesity Facts; The European Journal of Obesity, 6 (Suppl Issue 1): T2:P.128.

Rodgers, R.J. and Wright, F.L. (2012). Disruptive effects of bupropion on feeding behaviour and the behavioural satiety sequence in rats. Journal of Psychopharmacology, 26 (Suppl Issue 8): A67, TG03.

Rodgers, R.J., Waring, P., Wright, F.L. (2010) Intrinsic anxiolytic activity of D-cycloserine, a partial agonist at the strychnine-insensitive glycineB recognition site on the NMDA receptor complex. Journal of Psychopharmacology, 24 (Suppl 3): A76, TG16.

List of Abbreviations

2-AG 2-arachidonoylglycerol
5-HT 5-hydroxytryptamine (serotonin)
5,7-DHT 5,7-dihydroxytryptamine
5-HIAA 5-hydroxyindole acetic acid
5-HTP 5-hyroxytryptophan
α-MSH alpha-melanocyte-stimulating hormone
ACTH Adrenocorticotropin hormone
AgRP Agouti-related peptide
ANOVA Analysis of varience
APO AIV Apolipoprotein A-IV
AMY Amygdala
AP Area postrema
ATP Adenosine triphosphate
ARC Arcuate nucleus
β Beta
BBB Blood-brain-barrier
BBR Bombesin receptor
BMI Body Mass Index
BSS Behavioural satiety sequence
c-FOS an amino-acid protein that's expression signifies cellular activation
CART Cocaine- and amphetamine-regulated transcript
CB1 Cannabinoid-1 receptor
CCK Cholecystokinin
cm Centimetre
CNS Central nervous system
CPP Conditioned place preference
CRF Corticotropin-releasing factor
CSF Cebebrospinal fluid
CTA Conditioned taste aversion
ºC Degrees centigrade
δ Delta
df Degrees of Freedom
d-FEN dex-fenfluramine
db/db Genotype for leptin receptor deficiency
DIO Diet induced obesity
DMN Dorsomedial nucleus of the hypothalamus
DMSO Dimethyl sulfoxide
et al And others (from Latin *et alii*)
e.g. For example
EB Erythro-hydrobupropion
EMEA European medicines evaluation agency
FDA Food and drug administration agency
fMRI Functional magnetic resonance imaging
g gram
GABA γ-aminobutyric acid
GHIH Growth-hormone inhibiting hormone
GIP_a Gastric inhibitory polypeptide
GIP glucose-dependent insulinotropic polypeptide
GIT Gastrointestinal tract
GLP Glucagon-like peptide
GRP Gastrin releasing peptide
> Greater than
≥ Greater than or equal to
h hour
HPT Hypothalamic–pituitary–thyroid axis
i.e. That is
i.p. intraperitoneal
ICV Intracerebroventricular
IGF-1R Insulin growth factor receptor
IRR Insulin related receptor
IRS Insulin receptor substrates
κ Kappa

kg Kilogram
KJ Kilo joule
KO Knock-out
< Less than
≤ Less than or equal to
lab Laboratory
lb pound
LCD Low calorie diet
LH Lateral nucleus of the hypothalamus
LiCl Lithium chloride
µ Mu
MAO Monoamine oxidase
MC Melanocortin receptor
MCH Melanin-concentrating hormone
***m*CPP** meta-Chlorophenylpiperazine
mg Milligram
min Minute
ml Millilitre
mRNA Messenger ribonucleic acid
NA Noradrenaline
NAcc Nucleus accumbens
NMB Neuromedin-B
NMS Neuromedin-S
NMU Neuromedin-U
NS Non-significant
NTS Nucleus of tractus solitaries
NT Neurotensin
NPW Neuropeptide W
NPY Neuropeptide Y
ob/ob Genotype for leptin deficiency
OB-R Leptin receptor
OXM Oxyntomodulin
OX-R Orexin receptor
% Percent
± Plus or minus
pCPA Parachlorophenlyanine
PIL Personal license
POMC Pro-opiomelanocortin
PFN Prefornical hypothalamus
PP Pancreatic polypeptide
PPL Project license
PRCP Prolylcarboxypeptidase
PVN Paraventricular nucleus of the hypothalamus
PYY Peptide YY
s Seconds
SCN Suprachiasmatic nucleus
SEM Standard error of the mean
SOCS Suppressor of cytokine signalling
SON Supraoptic nucleus
SS Somatostatin
SPSS Statistical package for the social sciences
SSRI Selective serotonin reuptake inhibitor
THC Δ^9-tetrahydrocannabinol
VMH Ventromedial nucleus of the hypothalamus
VLCD Very low calorie diet
VTA Ventral tegmental area

Abbreviations for Treatment Conditions:

Experiment 1:

VV	Vehicle (methyl cellulose) + Vehicle (Saline)
VNL	Vehicle (methyl cellulose) + Naloxone (0.01mg/kg)
VNH	Vehicle (methyl cellulose) + Naloxone (0.1mg/kg)
RV	Rimonabant (1.5mg/kg) + Vehicle (Saline)
RNL	Rimonabant (1.5mg/kg) + Naloxone (0.01mg/kg)
RNH	Rimonabant (1.5mg/kg) + Naloxone (0.1mg/kg)

Experiments 2 and 3:

VV	Vehicle (methyl cellulose) + Vehicle (Saline)
VN	Vehicle (methyl cellulose) + Naloxone (0.05mg/kg)
RV	Rimonabant (1.5mg/kg) + Vehicle (Saline)
RN	Rimonabant (1.5mg/kg) + Naloxone (0.05mg/kg)

Experiment 4:

V	Vehicle (Saline)
B10	Bupropion 10mg/kg
B20	Bupropion 20mg/kg
B40	Bupropion 40mg/kg

Experiment 5:

V	Vehicle (Saline)
Ntx0.1	Naltrexone 0.1mg/kg
Ntx1.0	Naltrexone 1.0mg/kg
Ntx3.0	Naltrexone 3.0mg/kg

Experiment 6:

VV	Vehicle (Saline) + Vehicle (Saline)
VNL	Vehicle (Saline) + Naltrexone (0.1mg/kg)
VNH	Vehicle (Saline) + Naltrexone (1.0mg/kg)
BV	Bupropion (20mg/kg) + Vehicle (Saline)
BNL	Bupropion (20mg/kg) + Naltrexone (0.1mg/kg)
BNH	Bupropion (20mg/kg) + Naltrexone (1.0mg/kg)

Experiment 7

V	Vehicle (Saline)
*m*CPP0.1	*m*CPP 0.1mg/kg
*m*CPP1.0	*m*CPP 1.0mg/kg
*m*CPP3.0	*m*CPP 3.0mg/kg

Experiment 9

V	Vehicle (Distilled water)
Exn0.025	Exendin-4 0.025µg/kg
Exn0.25	Exendin-4 0.25µg/kg
Exn2.5	Exendin-4 2.5µg/kg

Experiment 8

VV	Vehicle (Saline) + Vehicle (Saline)
VNL	Vehicle (Saline) + Naltrexone (0.1mg/kg)
VNH	Vehicle (Saline) + Naltrexone (1.0mg/kg)
mV	*m*CPP (0.1mg/kg) + Vehicle (Saline)
mNL	*m*CPP (0.1mg/kg) + Naltrexone (0.1mg/kg)
mNH	*m*CPP (0.1mg/kg) + Naltrexone (1.0mg/kg)

Experiment 10

VV	Vehicle (Distilled water) + Vehicle (Saline)
VN	Vehicle (Distilled water) + Naltrexone (0.1mg/kg)
ELV	Exendin-4 (0.025µg/kg) + Vehicle (Saline)
ELN	Exendin-4 (0.025µg/kg) + Naltrexone (0.1mg/kg)
EHV	Exendin-4 (0.25µg/kg) + Vehicle (Saline)
EHN	Exendin-4 (0.25µg/kg) + Naltrexone (0.1mg/kg)

Chapter 1 Neurobiology of Appetite

Feeding is vital for survival, and its regulation depends upon numerous physiological, behavioural, social, psychological and environmental influences (Figure 1-1). Thus, while the present thesis concerns the behavioural pharmacology of appetite, it is important to emphasise that neurobiology is just one aspect of appetite regulation.

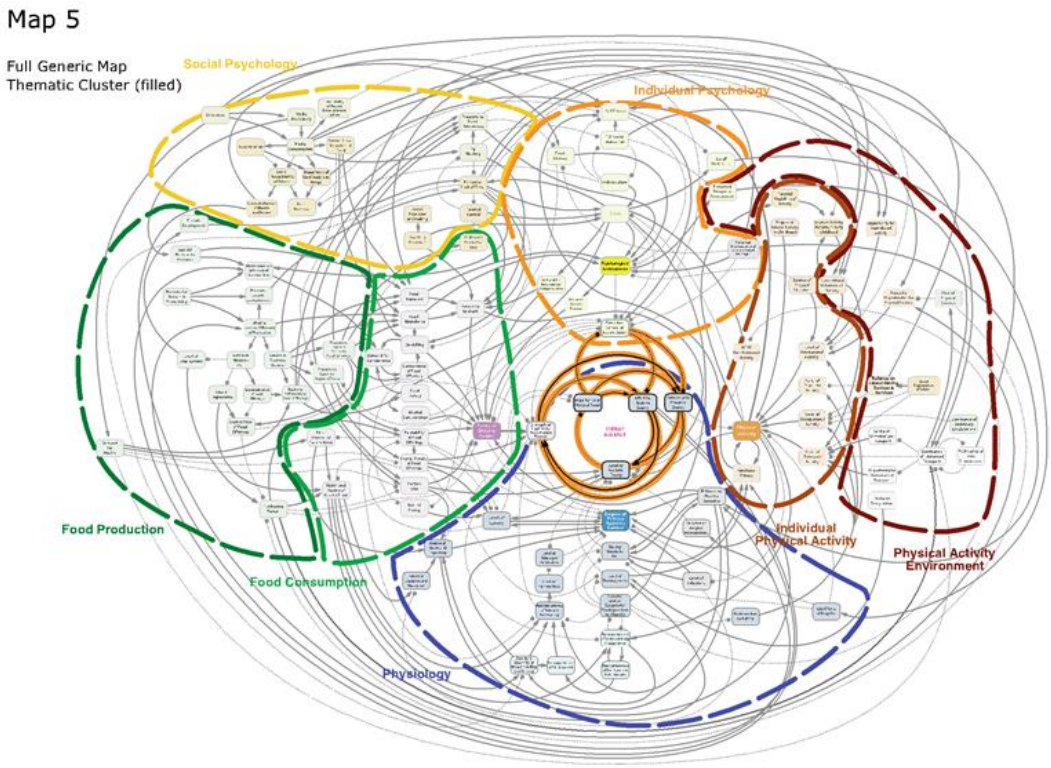

Figure 1-1: The Obesity System Atlas

The obesity system is pragmatically defined here as the sum of all the relevant factors and their interdependencies that determine the condition of obesity for an individual or a group of people. The obesity system, as defined, has been visualised as a causal loop model. (Vandenbroeck., 2007). Yellow, Social Psychology; Orange, Individual Psychology; Red, Physical Activity Environment; Brown, Individual Physical Activity; Blue, Physiology; Light Green, Food Consumption; Dark Green, Food Production

The neurobiological regulation of appetite involves a complex interaction between central and peripheral signals interacting in numerous positive and negative feedback loops to create an intricate mechanism determining calorific intake. The aim of Chapter 1 is to provide an overview of the current understanding of the mechanisms that regulate appetite. This will be done from a historical perspective i.e how our knowledge about key systems has developed over time.

1.1 Historical Overview of Theories and Models of Appetite Regulation

The original “one nutrient hypothesis” was based upon the belief, 2000 years ago, that food provided us with a universal nutrient. Digestion was the mechanism for the extraction of this nutrient and, as a result, growth and development occurred. Prout (1845; cited in Geiger, 1933) then proposed not one but three primary nutrients; saccharine (carbohydrate), oleosa (triglyceride), and albuminosa (protein). Around the same time, Mulder (1839; cited in Geiger, 1933) coined the term “protein”; a nitrogen-containing substance essential for life. Leibig (1843; cited in Geiger, 1933) then suggested two roles of protein; the formation of blood and use in metabolism. He additionally separated food into two distinct groups; ‘‘proteinaceous” (or foods containing nitrogen) and those not. These early concepts introduced nutrient-regulated theories of appetite i.e. that feeding is initiated by a nutrient deficit and terminated by nutrient replenishment.

In the 20th Century, theories of appetite regulation were still highly simplistic but focused instead on the gastrointestinal tract (GIT). In 1912, Cannon and Washburn proposed that gastric contractions were the main influencing factor in food intake (Cannon & Washburn, 1912). Based upon studies whereby balloons were inserted into the stomach to induce extension, an association was identified between contractions and subjective feelings of hunger. More recently, Koopmans et al., (1981) transplanted an extra stomach and intestines into rats and established that food injected into the transplanted stomach decreased eating in proportion to its volume and caloric content. The transplanted stomachs had no nerves, but did have a blood supply, thus allowing the conclusion that some blood-borne chemical was released from the stomach in response to the caloric value and volume of food.

1.1.1 Set-point theories

The next significant advance was the development of set-point theories, based upon the idea that we eat in the presence of an energy deficit to return our energy resources to an optimal level. Set-point systems have three key components: a set-point mechanism (defines the set-point), a detector mechanism (detects deviations) and an effector mechanism (acts to eliminate deviations). Based upon the assumptions of negative feedback systems, whereby feedback from changes in one direction elicits compensatory effects in the opposite direction, these theories saw behavioural changes in feeding as an inevitable outcome of changes in physiological signalling. Grounded in this theory, a range of feedback models was

subsequently developed throughout the mid-1940s to the mid-1950s, each based on a different biochemical signal.

Thermostatic theory (Brobeck, 1946) proposed that food intake could be increased or decreased in direct relation to the thermoregulatory demands on the organism. The basic argument was that heat loss generates heat production, causing the thermosensitive hypothalamus to influence feeding behaviour (Hamilton & Ciaccia, 1971).

The adipostatic model (a.k.a. lipostatic model), introduced by Kennedy (1953), proposed that the amount of energy stored in adipose tissue represents the balance between calorific intake and energy expenditure. He further suggested that, in order to maintain an adipose set-point, homeostatic mechanisms monitor changes in adipose tissue to elicit increases/decreases in intake (coined “indirect calorimetry”). This model implies that the central nervous system (CNS) is sensitive to concentrations of circulating metabolites, a mechanism for which was later suggested by Hervey (1969). It was proposed that a fat-soluble hormone acted as a tracer substance to monitor fat tissue mass via the dilution principle. This proposal was supported empirically and subsequently led to the identification of the protein leptin (Zhang et al., 1994; see Section 1.2.2).

The glucostatic model (Mayer, 1955) argued that the CNS monitors blood glucose levels to control nutrient intake and maintains a glucose concentration set-point. Meal initiation was thought to be determined by “metabolic hypoglycaemia”, i.e. when peripheral concentration differences in blood glucose became too small, glucose no longer entered metabolizing cells (Van Itallie, 1990). Mayer (1955) also argued that set-point theories were complementary rather than mutually exclusive, in that glucostatic theory could account for short-term aspects of feeding (such as meal initiation and termination) while lipostatic theory could account for the long-term regulation of feeding. Bray (1996) later suggested a glucodynamic model, whereby food intake is determined not by the actual level of circulating glucose but rather the rate of glucose utilization, i.e. the changes in glucose concentrations are the triggers that initiate or terminate feeding, known as the “pattern of dynamic change in glucose” (Bray, 2000).

The aminostatic model (Mellinkoff et al., 1956) proposed that amino-acid metabolism predicts hunger, a suggestion supported by the finding of a reciprocal relationship between amino-acid levels and appetite. Increased amino-acid concentrations reduce subjective appetite while increased appetite is accompanied by reduced amino-acid concentrations (Mellinkoff et al., 1956).

Although these set-point theories were supported by experimental validation of blood glucose/adipose/amino-acid concentrations, such manipulations were generally effective only at high magnitudes (Keesey, 1986). In fact, it was found that beliefs about the calorific content of a liquid preload have more influence on the size of a subsequent meal than does actual calorific content (Shide & Rolls, 1995). Furthermore, these set-point theories were inconsistent with eating-related evolutionary pressures, as animals would need to avoid a deficit rather than respond to it (Magdalena Farias et al., 2011). Such theories also failed to recognise major influences such as taste, learning, social and environmental factors.

1.1.2 The dual centre model

In parallel to the set-point theories of appetite control by peripheral signals, brain lesion research strongly suggested that eating behaviour is centrally regulated by two different regions of the hypothalamus: Ventromedial hypothalamus (VMH) and Lateral hypothalamus (LH; Stellar, 1954; see Figure 1-2).

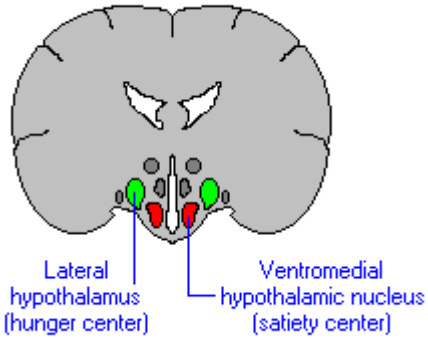

Figure 1-2: Hypothalamus

A coronal section of rat brain showing the positions of the lateral and ventromedial nuclei of the hypothalamus (Bear, 2006).

Classical lesion studies carried out in the 1940s found that large bilateral electrolytic lesions of the VMH in rats produced symptoms of excessive overeating (Hetherington & Ranson, 1940). The VMH Syndrome, characterised by hyperphagia, obesity and finikiness (Graff & Stellar, 1962; Hetherington & Ranson, 1940), has two phases; dynamic and static. The dynamic phase is characterised by several weeks of excessive eating and weight gain, whereas the static phase is characterised by a gradual reduction in consumption to a level sufficient to maintain obesity. The initial experiments demonstrated that VMH lesions typically increase feeding bouts, suggesting an impairment in the termination of feeding, and hence this nucleus became known as the “satiety mechanism” (Brooks et al., 1946). Later

however, Miller et al. (1950) found evidence of a paradoxical dissociation with VMH lesions whereby rats appeared less “hungry” i.e. although the consumption of food increased, the animals were less willing to “work for it” (Teitelbaum, 1957) or to consume unpalatable foods (Miller et al., 1950). Using un-lesioned rats with matched obesity Weingarten et al. (1983) suggested that the latter findings were a consequence of obesity rather of the VMH lesion. Other theories suggested that bilateral VMH lesions increased blood insulin levels which in turn increased lipogenesis (production of body fat) and decrease lipolysis (breakdown of body fat; Powley & Plocher, 1980). The rat is therefore considered to be converting its energy into stores and so must consume more food in order to ensure enough calories for immediate energy requirements (Hustvedt & Lovo, 1972).

In contrast to the developing VMH story, bilateral electrolytic lesions of the LH produced extreme aphagia, a failure to eat resulting in death (Anand & Brobeck, 1951). In LH syndrome, aphagia is also accompanied by adipsia, a failure to drink (Teitelbaum & Stellar, 1954). The converse findings can be elicited through electrical stimulation of these brain areas. That is to say that, VMH stimulation decreases food intake, whereas LH stimulation increases food intake (Wyrwicka & Dobrzecka, 1960).

These early findings led to the development of the dual centre model (Stellar, 1954), whereby it was proposed that termination of eating is controlled by the VMH or “satiety centre”, whereas the LH or “feeding centre” controlled active feeding behaviours. Later, Teitelbaum and Epstein (1962) found that when LH lesioned rats were kept alive via tube-feeding, they were able to recover food and water intake. However, feeding motivation remained impaired, thereby implicating the LH in feeding motivation. The dual centre model was linked to adipostatic theory by Hervey (1969) who, using evidence that lesions to the VMH prevented a “satiety factor” from eliciting behavioural changes, argued that a hormone must relay information about fat tissue mass via the VMH.

Although helpful in the development of our understanding of appetite regulation, there are many limitations to lesion studies. For example, the LH syndrome also produced a wide range of severe motor disturbances and a lack of responsiveness to sensory input; thus deficits in feeding and drinking were just two symptoms of a more general disorder. Furthermore, the effective locus of the LH lesion required to produce aphagia and adipsia overlapped with the nigrostriatal dopamine pathway (Ungerstedt, 1970). Additionally, when the paraventricular nuclei (PVN) of the hypothalamus are damaged (e.g. by bilateral lesions of the noradrenergic bundle or

the PVN), this produces hyperphagia and obesity similar to that produced by VMH lesions. Thus, lesions studies are often unable to target one system independently and findings derived from this methodology alone may be unreliable (Kapatos & Gold, 1973; Leibowitz et al., 1981).

It is now believed that the primary role of the hypothalamus is in the regulation of energy metabolism, and not just eating *per se*.

1.1.3 The Hypothalamus Revisited

Since the original dual centre model of appetite regulation, and as a result of technological developments, many more hypothalamic nuclei (as well as extra-hypothalamic structures) have been implicated in the control of hunger and satiety.

The identification of numerous orexigenic and anorectic mechanisms and their morphological relationships support the role of distinct interconnected circuitry operating within the hypothalamus (see reviews: Broberger, 2005; Broberger & Hokfelt, 2001; Grossman, 1975; Hillebrand et al., 2002; Kalra et al., 1999; Mercer & Speakman, 2001; Schwartz et al., 2000; Williams et al., 2001)

The primary regions of hypothalamus currently identified include, but are not limited to: the arcuate nucleus, the paraventricular nucleus, the dorsomedial hypothalamic area, and the lateral hypothalamic area (see Figure 1-3). Other key structures in the regulation of food intake include the amygdala and the brain stem (Arora & Anubhuti, 2006).

The arcuate nucleus (ARC) lies adjacent to the third ventricle and above the median eminence. The ARC-median eminence area has a modified blood-brain-barrier (BBB) to allow entry of peripheral peptides and proteins (Friedman & Halaas, 1998). Multiple projections from the ARC to other hypothalamic and extra-hypothalamic nuclei support the key role of this nucleus as a hub in the integration of hormonal signals that regulate feeding (Cone et al., 2001).

The paraventricular nucleus (PVN), adjacent to the superior part of third ventricle in the anterior hypothalamus, plays a large role in integrating nutritional signals with the hypothalamic-pituitary-thyroid (HPT) axis (Zoeller et al., 2007). In addition to the localisation of orexigenic producing neurons, it is thought that the PVN has a high concentration of receptor sites for all the main orexigenic signals.

The ventromedial nucleus of the hypothalamus (VMH) is thought to act primarily as a satiety centre, as discussed earlier. Research is progressively supporting its role as a receptive field, whereby feeding is mediated via the convergence of multiple

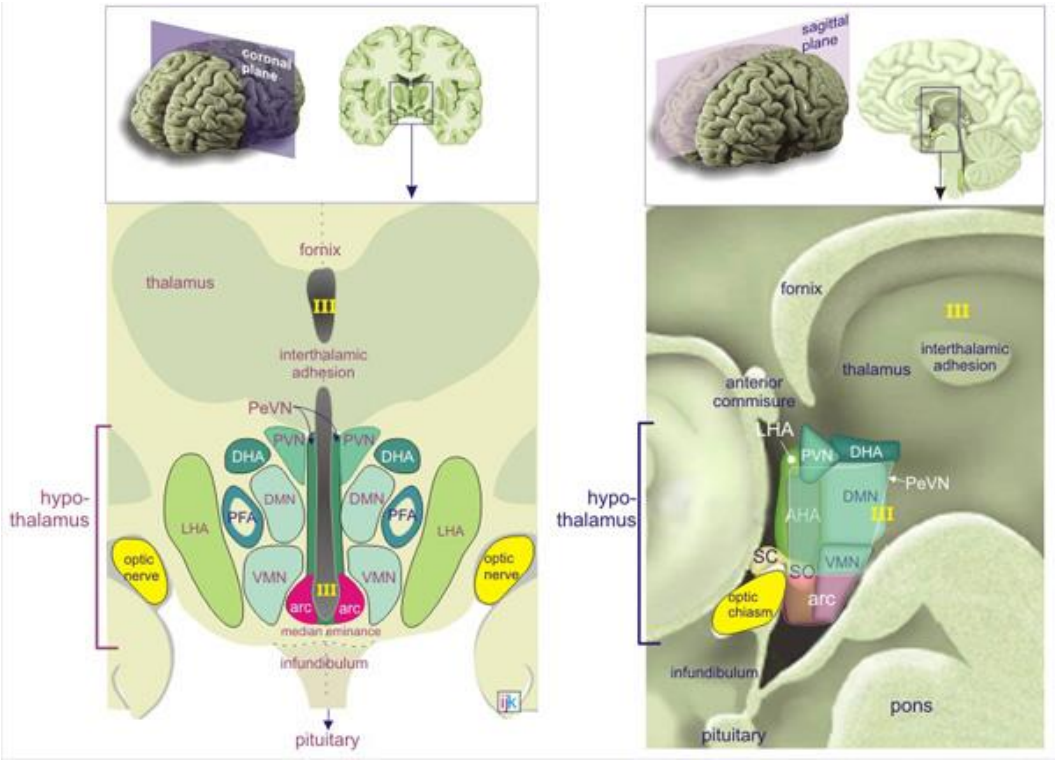

Figure 1-3: Human Hypothalamus (detailed)

Abbreviations: Arc, arcuate nucleus, DHA, dorsal hypothalamic area, DMN, dorsomedial nucleus, LHA, lateral hypothalamic area, PeVN, periventricular nucleus), PFA, parafornicular nucleus, PVN, paraventricular nucleus, VMN, ventromedial nucleus, SO, supraoptic nucleus, SC, surprachiasmatic nucleus. Arc and PeVN are drawn transparently in the sagittal section in order to show the underlying nuclei. (Netterimages.com, 2010)

synaptic inputs, as characterised by extensive links to other hypothalamus sites (Morton et al., 2006).

The lateral hypothalamus (LH), as discussed earlier, acts primarily as a feeding centre. It has glucose sensitive neurons, allowing it to mediate the hyperphagia associated with hypoglycemia (Bernardis & Bellinger, 1996). Furthermore, it is the source of multiple orexigenic peptides (see Section 1.3.2; α-MSH and Section 1.4.3; Orexins).

The dorsomedial hypothalamus (DMN) is well connected to other medial (VMH) and lateral (LH and PVN) hypothalamic nuclei. It acts to mediate both neural and humoral signals from these pathways that are associated with feeding and bodyweight (Elmquist, Ahima, et al., 1998; Elmquist, Maratos-Flier, et al., 1998).

The nucleus of tractus solitarius (NTS) is a brainstem nucleus in the upper medulla that provides reciprocal connections between the hypothalamus and the brainstem (Bray, 2000). The NTS receives viscerosensory information, i.e. it receives sensory innervation from internal organs, such as the GIT, that is subsequently integrated

with homeostatic signals from central nuclei (e.g. the pons, diencephalon and forebrain; Grill & Hayes, 2009). Of primary importance to the role of the NTS in appetite regulation is its highly perforated endothelial cells that provide a permeant blood-brain barrier (Gross et al., 1990), and thus the potential for integration with the systemic circulation, allowing a range of peptides to contribute to the regulation of appetite. Furthermore, the NTS has a high density of anorexigenic binding sites (see Section 1.4.1; NPY).

The amygdala (AMY) is located within the medial temporal lobes. Traditionally considered to be an “emotional” part of the brain (Kluver & Bucy, 1939; Weiskrantz, 1956), more recent research into orexigenic and anorectic signal receptor expression has implicated the AMY in appetite regulation (Parker & Bloom, 2012). Lesions of the AMY lead to obesity and the preference of a high carbohydrate diet (King et al., 2003; King et al., 1994). It is also now been shown that a number of neuropeptides administered directly into the AMY, can regulate feeding behaviour (Heilig et al., 1993; Zhang, Li, et al., 2011; see Section 1.4.1; NPY). In addition, the AMY is thought to play a role in the learning and experiencing of food through reward mechanisms (i.e. conditioned taste aversion; CTA).

1.2 Adiposity Signals

It is known that numerous signals convey information about food intake and body fat status from the periphery to brain areas that control energy homeostasis. The first discovered signals were found to circulate in proportion to body fat mass and are referred to as "adiposity signals".

1.2.1 Insulin

Insulin is a 51-amino-acid polypeptide hormone, initially extracted in 1921 by Banting and Best (1922). After the discovery by Minkowski and von Mering in 1889 that pancreatic removal in dogs induces diabetes, the islets of Langerhans within the pancreas were isolated and insulin extracted (Banting & Best, 1922; Luft, 1989). The administration of this extract to a diabetic human drastically improved the patient’s condition (Macleod, 1924).

Insulin is secreted from pancreatic β cells (islets of Langerhans) in response to increased blood glucose levels following a meal (Polonsky et al., 1988). It acts in three major ways to (1) promote the use of glucose as the primary source of energy, (2) initiate the conversion of blood-borne fuels to forms that can be stored

and, (3) promote the storage of glycogen within the liver, fat in the form of adipose tissue, and amino-acids as proteins. (UFSC, 2010).

Insulin is not just involved in glucose homeostasis, linking back to Kennedy's (1953) adipostatic model of appetite, insulin was thought to be the missing link between adipose tissue and the brain. It was discovered that elevations in circulating insulin are directly proportional to fat mass (Bagdade et al., 1967). As such, insulin levels are elevated with a positive energy balance and decreased with a negative energy balance (Woods & Porte, 1974).

The insulin receptor is part of a large receptor family of tyrosine kinases, including insulin growth factor receptor (IGF-1R), insulin related receptor (IRR) and several insulin receptor substrates (IRSs). The highest concentrations of insulin receptors are found in the olfactory bulbs and hypothalamus (e.g. ARC, DMH, PVN, suprachiasmatic and periventricular nuclei; Arora & Anubhuti, 2006). Pro-opiomelanocortin neurons (POMC; see Section 1.3.1) express IRSs, via which insulin directly inhibits their firing. Although produced peripherally, once in the brain it acts as an anorectic signal, enhancing catabolic pathways and inhibiting the anabolic NPY and melanocortin systems (Arora & Anubhuti, 2006; Valassi et al., 2008). CNS and cerebrospinal fluid (CSF) administration of insulin therefore results in a reduction of food intake in rodents (Baskin et al., 1987; Benoit et al., 2004; Brown et al., 2006) and humans (Hallschmid et al., 2004). Furthermore, IRS-R1 and IRS-R2 knock-out (KO) mice exhibit hyperphagia and increased fat stores in addition to insulin resistance and reduced glucose tolerance, whereas IRS-R3 and IRS-R4 KO mice exhibit relatively normal phenotypes (for review see: Saltiel & Kahn, 2001). It is as yet unclear as to the specific roles of the insulin receptor subtypes in the regulation of appetite.

Insulin is found to interact with other signals to regulate energy balance; for example, the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Although there is limited evidence for a direct effect of insulin on GABA receptors, insulin-induced hyperphagia can be blocked by GABA receptor antagonists (Kamatchi et al., 1984), thereby suggesting that GABA receptors play some role in the effects of insulin on the regulation of food intake (for review see: Pang & Han, 2012). Insulin also appears to act upon adipocytes to increase leptin secretion, potentially via increased gluconeogenesis and suppressed glycogenolysis to decrease net hepatic glucose production (Ramnanan et al., 2010).

Interestingly, elevated levels of insulin, in both the basal state and in response to glucose, are associated with obesity (Bagdade et al., 1967), indicating a link

between obesity and insulin resistance. It is therefore unsurprising that, in 1996, the Food and Drug Administration (FDA) approved a modified version of human insulin (Humalog®) for the treatment of type-1 and type-2 diabetes (Nobelprize.org, 2013).

1.2.2 Leptin

In 1950, the Jackson Laboratory discovered the genotypes *ob/ob* (leptin deficiency) and *db/db* (leptin receptor deficiency), both phenotypically characterised by hyperphagia, decreased energy expenditure and early onset obesity in mice (Hummel et al., 1966; Ingalls et al., 1950). Five years later, Jeffery Friedman's laboratory successfully cloned the *ob* gene in mice and its homolog in humans, terming the purified gene product "leptin" (Zhang et al., 1994). At the time, this discovery was thought to be the physiological feedback system that regulates energy balance and was soon followed by an explosion of research.

It is now known that leptin is a 16-kDa protein hormone encoded by the *ob* gene. Secreted from adipocytes in proportion to body fat mass, it was originally thought to be produced only in white adipose tissue. However, it is has since been found to also be synthesised in brown adipose tissue, the stomach, placenta, mammary gland, ovarian follicles, and in fetal organs such as the heart, bone and cartilage (Arora & Anubhuti, 2006; Margetic et al., 2002). Despite peripheral production, leptin is able to cross the BBB and act upon receptors (leptin receptors; OB-R) that are highly expressed in hypothalamic regions such as the ARC, VMH, DMH and PVN (Mercer et al., 1996). Leptin receptors are of two distinct types, long (OB-RL) and short (OB-RS), with the difference lying primarily in the intracellular domains. It is the long form that is found primarily within the hypothalamus and which is responsible for regulating energy balance (see review: Tartaglia, 1997).

In laboratory animals, leptin administration decreases food intake and increases energy expenditure (Ahima & Flier, 2000b; Friedman & Halaas, 1998), centrally-produced anorectic effects thought to be mediated by activation of pro-opiomelanocortin (POMC; Section 1.3.1) neurons in the ARC. Activation of these neurons inhibits feeding by both increasing the release of α-melanocyte-stimulating hormone (α-MSH; Section 1.3.2) and down-regulating neuropeptide-Y (NPY; Section 1.4.1), agouti-related peptide (AgRP; Section 1.4.2) and melanin concentrating hormone (MCH; Section 1.5.4.2; Ahima & Flier, 2000a; Ahima & Flier, 2000b; Ahima et al., 2000; Arora & Anubhuti, 2006; Harrold et al., 2012). Conversely, as a dual regulator, NPY/AGRP circuits are enhanced and catabolic circuits blocked during periods of low circulating leptin, resulting in increased meal size and reduced energy expenditure (Schwartz, 2000; Valassi et al., 2008).

Somewhat incongruously, circulating leptin is increased considerably in obesity. Although rising levels of leptin should signal the storage of excess energy, and thus bring about a decrease in appetite and increase in energy expenditure, chronic hyperleptinemia leads to leptin desensitization/resistance (Kennedy et al., 1997). The specific mechanisms for leptin resistance are still under investigation, but may reflect dysregulation of synthesis, secretion or transport, or the existence of abnormal receptors or post-receptor signalling (Ahima et al., 2000; Munzberg et al., 2005; Myers et al., 2008). OB-RL signal attenuation is also thought to play a crucial role in leptin resistance, as supported by the identification of SOCS-3 (suppressor of cytokine signalling-3) as a potential mediator of leptin signalling. Elevation of SOCS-3 in direct response to leptin treatment, as well as its affinity for OB-RL, is thought to be associated with its role in inhibiting leptin signalling (Bjorbaek et al., 1998) and may therefore contribute to leptin resistance (Myers et al., 2008).

Leptin dysregulation is also seen at the other extreme. Although leptin levels decrease rapidly following periods of fasting or prolonged exercise (Matejek et al., 1999; Neary et al., 2004; Zheng et al., 1996), and while patients with anorexia nervosa or those with restricted food intake have significantly reduced circulating leptin, they experience un-expectantly high leptin transport rates (Arora & Anubhuti, 2006; Frederich et al., 1995; Mantzoros et al., 1997; Margetic et al., 2002).

The phenomenon of leptin resistance ultimately thwarted optimism that leptin could be the “anti-obesity” hormone. Despite initial studies demonstrating that *ob/ob* and *db/db* phenotypes could be normalised by leptin administration (Campfield et al., 1995; cited in Harrold et al., 2012), and findings that recombinant leptin could normalise the eating behaviour of humans with similar leptin inhibiting mutations (Farooqi et al., 1999; Licinio et al., 2004; Montague et al., 1997), clinical trials on obese populations yielded disappointing results (Heymsfield et al., 1999; Hukshorn et al., 2003).

There has also been a suggested synergy between leptin and peripheral signals such as cholecystokinin (CCK; Section 1.5.1.1). Low dose leptin administration has *per se* been found to enhance CCK-induced c-Fos expression in the PVN, thereby suggesting that centrally administered leptin acts via the PVN to enhance satiety signals such as CCK (Halford et al., 2003). As an alternative therapeutic route, sub-threshold administration of leptin and CCK has been found to dose-dependently reduce food intake (Emond et al., 1999).

This rapid progress in the understanding of leptin’s role in energy intake and metabolism was a watershed event for yet further major developments in our

understanding of the CNS, its signals, and their significance in the regulation of appetite. Table 1-1 summarises the wide range of centrally-released chemical signals thought to either stimulate or inhibit appetite.

Table 1-1: Centrally Released Appetite Modulators

The table displays both anorexigenic and orexigenic signals, that are primarily thought to be released centrally.

Appetite Simulants

Peptide	Biochemistry	Site of synthesis	Receptors	Receptor localization
Neuropeptide Y	36-amino-acid neuropeptide	ARC	Y_1 and Y_5	Hypothalamus, Hippocampus, AMY
Agouti-related protein	132-amino-acid neuropeptide	ARC	MC3R and MC4R	ARC, PVN, AMY
Melanin concentrating hormone	19-amino-acid neuropeptide	LH	MCH-1R and MCH-2R	Cerebral cortex, AMY, hypothalamus, thalamus
Orexins	28- and 33-amino-acid peptide	dorsal and lateral hypothalamus	OX-1R and OX-2R	VMH, PVN, hippocampus and raphe nucleus
Galanin	29-amino-acid peptide	Hypothalamus	GAR-1R, GAL-2R, and GAL-3R	PVN, LH, VMH, and AMY
Galanin-like peptide	Identical first 13-amino-acids	ARC		
Endogenous Opioids	B-endorphin	Wide distribution throughout the brain	μ - and δ -	All present within hypothalamic nuclei, differential distribution for each subtype
	dynorphin		κ -	
	enkephalins		δ -	
Endocannabinoids	lipids	Wide distribution throughout the brain	CB1 and CB_2	Differential distribution for individual subtypes

Appetite Suppressants

Peptide	Biochemistry	Sit of synthesis	Receptors	Receptor localization
Pro-opiomelanocortin	241-amino-acid peptide	The pituitary, ARC, PVN, NTS, and VTA	MC1R – MC5R	ARC, VMH, PVN and AMY
Cocaine and amphetamine-regulated transcript	CART(42–89) and CART(49–89)	PVN, ARC, PBN	Not yet identified	Not yet identified
α-Melanocyte stimulating hormone	13-amino-acid polypeptide	ARC	MC3R and MC4R	ARC, PVN, AMY, and spinal cord
Neuropeptide W	NPW30 and NPW23	PVN, VMH, ARC, LH and NTS	NPBWR1 and NPBWR2	PVN, SON, DMH, VMH and ARC
Neurotensin	13-amino-acid peptide	PVN, VMH, SON	NTS-1, NTS-2 and NTS-3	DH and VTA
Oxytocin	Neuro-hypophysial hormone	PVN and SON	OTR	Prefrontal cortex, AMY, ventral striatum
Serotonin	Indoleamine transmitter	Dorsal raphe nucleus	$5\text{-}HT_{1-7}$	Widespread distribution

1.3 The Melanocortin System

Historically, the POMC- and NPY- neurons within the ARC were identified as the two neurochemical sites of leptin receptor expression in the CNS and the site of c-Fos activation by both peripheral and central administration of leptin (Cowley et al., 2001; Elias et al., 1999). The central melanocortin system is now widely known as the system of neurons that expresses NPY/AgRP or POMC and their associated receptor subtypes (see Figure 1-4 and reviews: Balthasar et al., 2005; Benoit et al., 2001; Butler & Cone, 2002; Cone, 1999; Cummings & Schwarts, 2000; Ellacott & Cone, 2004; Gantz & Fong, 2003; Vergoni et al., 2000; Wisse & Schwartz, 2001)

1.3.1 POMC

Pro-opiomelanocortin is a precursor 241-amino-acid peptide. Expressed in the periphery, the pituitary, and the CNS (primarily the ARC, PVN, NTS, and the ventral tegmental area; VTA; Morton et al., 2006; Schwartz et al., 2000), POMC neurons co-express cocaine amphetamine-related transcript (CART) and GABA (Millington, 2007). POMC is a potent anorectic agent, with agonists inhibiting and antagonists stimulating feeding behaviour / food intake (Boston et al., 1997; Fan, 1997; Hansen et al., 2005; Murphy et al., 1998; Sainsbury, Cooney, et al., 2002).

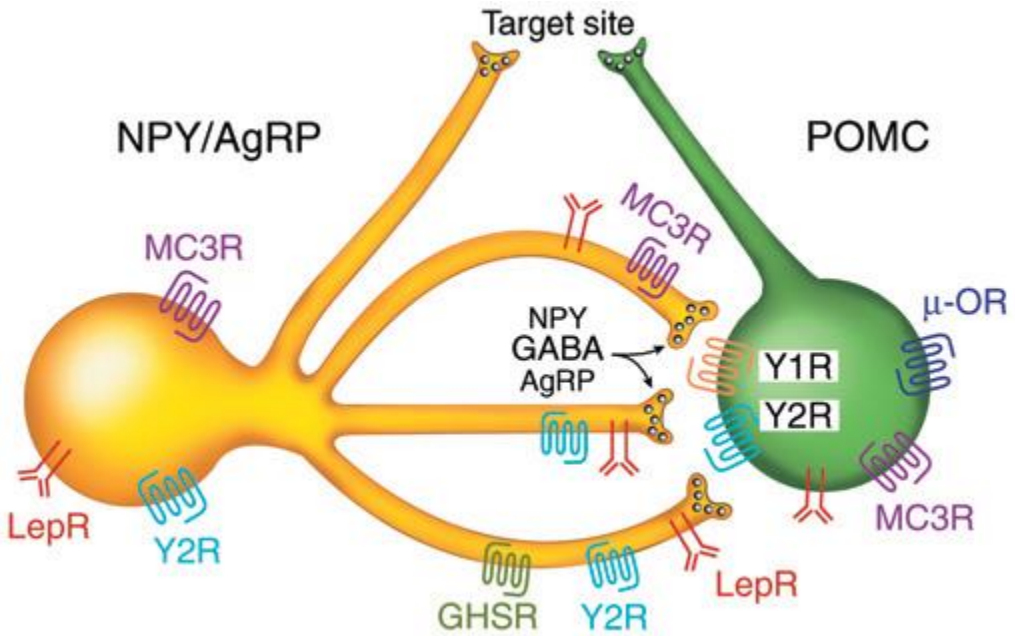

Figure 1-4: Melanocortin System

Schematic of the melanocortin system within the ARC of the hypothalamus (Cone, 2005). Lep-R, leptin receptor; μ-OR, μ-opioid receptor; Y1-R, type 1 NPY receptor; Y2-R, type 2 NPY receptor; MC3-R, melanocortin type 3 receptor.

POMC is the precursor to adrenocorticotropin hormone (ACTH), β-endorphin (see Chapter 4) and α-, β-, γ- melanoctye-stimulating hormones (α-, β-, and γ-MSH), with the latter mediating their effects through five G-protein coupled receptors; MC1R – MC5R (Coll & Tung, 2009). Peripheral melanocortin peptides in skin and hair regulate the production of yellow-red/brown-black pigments through MC1R, whereas ACTH regulates adrenal glucocorticoid production through MC2R, and MC5R expressed in the periphery (Cone, 2005). The remaining receptors, MC3R and MC4R, are expressed primarily within the ARC and VMH and are considered key regulators in appetite control (see review: Butler & Cone, 2002).

MC4R is more widely distributed than MC3R, and is considered particularly important in regulating food intake. Selective MC4R agonists have been shown to significantly reduce food intake and bodyweight (McMinn, Sindelar, et al., 2000), conversely, antagonists dose-dependently increase feeding and induce bodyweight gain (Murphy et al., 1998). Whole-body deletion of MC4R in mice produces hyperphagic obesity and hyperinsulinaemia. Interestingly, the hyperinsulinaemia is out of proportion to the degree of obesity, which may suggest an interaction with insulin/leptin. The hyperphagic obese profile is also mirrored by POMC neuron ablation (Gropp et al., 2005). As might be expected, selective MC4R knock-out mice are obese and hyperphagic. However the administration of MC3R agonists reduces food intake substantially less in these mice than in wild-type controls, thereby further supporting MC4R as the primary receptor in food intake regulation (Chen, Williams, et al., 2004).

Comparatively, selective MC3R antagonists/agonists do not alter food intake (Abbott et al., 2000), and MC3R knock-out mice exhibit only a mild overweight phenotype; exhibiting a small increase in bodyweight and fat mass but lacking hyperphagia (Butler & Cone, 2002; Chen et al., 2000). However, this does not exclude the role of MC3R in energy regulation. For example, it is thought that MC3R may act as an inhibitory auto-receptor on ARC POMC neurons, creating a positive feedback circuit whereby agonists (such as α-MSH) act to stimulate their own release (Cowley et al., 2001). Additionally, human mutations of both receptor subtypes (MC3R and MC4R) produce an obese phenotype (Mencarelli et al., 2004). Indeed, the inability to synthesise melanocortin ligands from POMC is linked to early onset obesity syndrome (Fan, 1997).

1.3.2 α-Melanocyte Stimulating Hormone

α-melanocyte stimulating hormone (α-MSH) is a 13-amino-acid polypeptide. Its precursor ACTH, a 39-amino-acid polypeptide tropic hormone, is cleaved from

POMC expressed in the pituitary gland (Mountjoy & Wong, 1997). Expressed also in the anterior hypothalamus (DMH, PVN and ARC), it is a tonic inhibitory signal in appetite regulation, reducing food intake and bodyweight (Giraudo et al., 1998) via action at MC3R and MC4R (Marks & Cone, 2001).

In addition to the literature on the effects on intake of MC3 and MC4 receptor ligands, specific central administration of α-MSH has been found to elicit an anorectic action (Matsuda et al., 2006; McMinn, Wilkinson, et al., 2000). Conversely α-MSH antagonists, such as SHU-9119, increase food intake and bodyweight (Raposinho et al., 2000). Further support for the significance of α-MSH in appetite regulation comes from studies on prolylcarboxypeptidase (PRCP), an enzyme that cleaves α-MSH and inactivates it. Mice lacking PRCP demonstrate higher α-MSH levels, have lower bodyweights and exhibit hypophagia (Wallingford et al., 2009).

1.3.3 Cocaine and Amphetamine-Regulated Transcript

The cocaine and amphetamine-regulated transcript (CART) gene encodes a peptide, of 116-amino-acid residues, expressed peripherally in the pituitary, pancreatic islets and stomach, and centrally, in the ARC, PVN and NTS (Koylu et al., 1998; Zhang, Han, et al., 2012). Within the hypothalamus, CART is thought to interact with a variety of other signals. For example, within the PVN, CART is co-expressed with oxytocin; in the LH, it is thought to be co-localised with MCH; in the VTA, it is co-expressed with dopamine; and in the ARC, it is co-localised with POMC (Larsen & Hunter, 2006; Parker & Bloom, 2012; Vrang, 2006; see review: Zhang, Han, et al., 2012)

Although there are no currently identified CART specific receptors, CART antiserum has been found to increase food intake. Similarly, endogenous CART is anorectic when administered centrally, suggesting that it plays some role in the regulation of feeding (Aja et al., 2001; Kristensen et al., 1998; Lambert et al., 1998; Larsen et al., 2000; Vettor et al., 2002). Surprisingly, despite its co-localization with POMC in the ARC, its action does not appear to be mediated via the melanocortin system (Edwards et al., 2000). In fact, discrepancies between the effects of hypothalamic and ICV (intracerebroventricular) injections suggest that its anorectic effects are not in fact mediated by hypothalamus. It has been suggested that CART could have opposing effects in the hypothalamus and brainstem (Aja et al., 2001). This is supported by the observation that CART null mutants exhibit increased food intake and obesity, whereas the heterozygotes demonstrate reduced food intake (Asnicar et al., 2001; Kokkotou et al., 2005; Wierup et al., 2005). CART could therefore be a component of both anorectic and orexiogenic circuits (Parker & Bloom, 2012).

1.4 The ARC NPY/AgRP System

1.4.1 NPY

NPY, a 36-amino-acid residue with tyrosine at either end (hence the Y), was isolated from porcine hypothalamus in 1982 (Adrian et al., 1983; Allen et al., 1983). It is a member of the PP-fold family of proteins consisting of NPY, polypeptide Y (PYY; Section 1.5.1.7) and pancreatic polypeptide (PP; Section 1.5.1.8), named after a common tertiary structural motif known as the “PP fold”. It is synthesised in the brain stem and hypothalamus (ARC and DMN), with the latter projecting to the PVN (Currie & Coscina, 1996; Kalra et al., 1999; Ramos et al., 2005). Central or peripheral administration of NPY potently induces hyperphagia and obesity, by promoting meal initiation, increasing meal size and the duration of feeding (Kamiji & Inui, 2007; Stanley et al., 1986; Stanley & Leibowitz, 1984; Tiesjema et al., 2007). Conversely, NPY antagonists suppress food intake (Clark et al., 1984; Corp et al., 2001; Levine & Morley, 1984; Myers et al., 1995; Stanley & Leibowitz, 1984) and deprivation-induced feeding (Ishihara et al., 1998).

There are six currently identified receptor sub-types for NPY (Y_1 – Y_6). However, it is generally considered that only receptors Y_1 and Y_5 are important in mediating the effects of NPY on appetite (see review: Blomqvist & Herzog, 1997; Kalra et al., 1991). Central administration of a selective Y_1 receptor agonist, such as N-acetyl [Leu28, Leu31] NPY (24-36), stimulates food intake (Silva et al., 2002), whereas Y_1 receptor antagonists inhibit food intake and dose-dependently induce weight loss (Kask et al., 1998; MacNeil, 2007; Poindexter et al., 2002). Together with Y_1 receptor KO mice, which display a hypophagic phenotype in addition to an impaired fasting-induced re-feeding (Kanatani, 2000), these findings would support a significant role for Y_1 receptors in the control of food intake and obesity.

It was initially thought that Y_1 receptors alone mediated the feeding-related effects of NPY. However, selective Y_1 receptor agonists have been shown to elicit only 50% of maximum feeding response (Oshea et al., 1997). Unfortunately, the precise role of these receptors in appetite regulation cannot be understood until a highly selective Y_1 receptor antagonist reduces food intake independent of other systems; no such agent has yet been found (Chamorro et al., 2002). Currently-developed Y_1 antagonists (although similar results are found with Y_5 antagonists) suppress food intake in NPY-deficient mice and Y_5 KO mice, suggesting they act beyond the Y_5 receptor and are not specific (Bannon et al., 2000).

Y_5 receptor agonists are similarly orexigenic when administered centrally, and Y_5 receptor antagonists, such as GlaxoSmithKline's Y_5 receptor antagonist GW438014A, are found to reduce food intake, bodyweight and fat mass in rodents (Daniels et al., 2002). On the other hand, some studies have found that selective Y_5 receptor agonists have no influence on food intake, but do contribute to the maintenance of the NPY feeding response (Flynn et al., 1999). Y_5 receptor knock-out studies have also yielded conflicting results (Marsh et al., 1998).

NPY is thought to interact with a wide variety of other pathways. For example, it has been shown to be stimulated by the gut peptide ghrelin (see Section 1.5.3.1) and inhibited by amylin (see Section 1.5.1.6), insulin, leptin and serotonin (Harrold et al., 2012; Valassi et al., 2008; Wang & Leibowitz, 1997). Ghrelin increases c-Fos activation in ARC, and increases NPY mRNA (Wang et al., 2002). Administration of amylin, a pancreatic hormone, can block the hyperphagic effects of NPY (Morris & Nguyen, 2001). Activation of serotonin receptors (5-HT_{1B} and/or 5-HT_{2A}) is found to suppress levels of NPY in the PVN and to induce hyperphagia, conversely, antagonising 5-HT receptors increases NPY function (Halford et al., 2003).

As discussed earlier, NPY is thought to be the key component responsible for obesity in leptin-deficient mice (*ob/ob*). The co-expression of leptin receptor mRNA and NPY mRNA in ARC implies that the two peptides may act together. This was confirmed when *ob/ob* mice were also bred to be NPY-deficient, reducing obesity by 50% compared to *ob/ob* mice alone (Sainsbury, Schwarzer, et al., 2002). Further study found that insulin receptor agonism reduces levels of NPY mRNA, thereby inhibiting NPY synthesis and secretion (Arora & Anubhuti, 2006; Kalra et al., 1991; Schwartz et al., 1992). As NPY-induced hyperphagia can be blocked by leptin administration (Smith et al., 1998), NPY inhibition may be the mechanism of action for leptin-induced hypophagia.

1.4.2 Agouti-Related Peptide

The agouti gene was identified in 1992 (Bultman et al., 1992), when it was discovered to be the basis of Ay obesity, a monogenetic obesity model. The hypothalamic protein homolog, agouti-related peptide (AgRP), subsequently discovered in 1997 (Shutter et al., 1997), is a 132-amino-acid peptide, co-expressed with NPY in the ARC and PVN (Broberger et al., 1998). The central administration of AgRP has a strong orexigenic action (Ebihara et al., 1999; Ollmann et al., 1998), so much so that acute AgRP administration can increase food intake for up to a week (Hagan et al., 2000). In contrast, overexpression produces hyperphagia and obesity (Arvaniti et al., 2001; Shutter et al., 1997). AgRP

acts to increase food intake via competitive antagonism of central MC3R and MC4R, thus blocking the anorectic action of the agonist α-MSH (Ollmann et al., 1997). Interestingly, AgRP knock-out mice exhibit no unusual phenotype (Qian et al., 2002), suggesting that AgRP may have an indirect a role in energy expenditure. Studies have demonstrated that reduced AgRP levels elicit increases in energy expenditure and weight loss, without food intake changes (Makimura et al., 2002).

There is conflicting evidence for the role of AgRP in obesity. Studies have shown that models of diet-induced-obesity (DIO; mice fed on 22week schedule of high fat diet) have reduced hypothalamic AgRP mRNA expression and increased MC4R expression (Huang et al., 2003). Similarly, suppressed AgRP mRNA expression is seen in obese mice (Tritos, Elmquist, et al., 1998), and yet obese humans express elevated levels of AgRP (Barsh et al., 2000).

Further, evidence suggests that leptin acts to reduce food intake via the suppression of AgRP (Korner et al., 2001). The expression of AgRP within peripheral tissues (including the adrenal gland) further emphasises the potential role for AgRP within leptin-regulated feedback mechanisms (Stutz et al., 2005). This, in addition to leptin resistance seen in obese humans, may explain the elevated AgRP levels in obese humans (Barsh et al., 2000).

1.4.3 Orexins

The discovery of the orexin system was reported almost simultaneously by two separate research groups. de Lecea et al., (1998) identified the mRNA for a pro-hormone pre-pro-hypocretin and its products hypocretin-1 and hypocretin-2. Independently, Sakurai et al. (1998) identified two peptide ligands; orexin-A and orexin-B and its pro-hormone, pre-pro-orexin (see review: Rodgers et al., 2002).*

The orexins are 33- and 28- amino-acid peptides (Orexin-A and Orexin-B, respectively) localised to the DMH, LH and PVN (Sakurai et al., 1998) and which act at via two G-protein coupled receptors; Orexin-1 (OX-1R) and Orexin-2 (OX-2R). Orexin-A has an equal affinity for both receptor types whereas orexin-B has a much greater preference for the OX-2R (Sakurai et al., 1998). Both receptors are widely expressed within the hypothalamus; OX-1R chiefly in the VMH, whereas OX-2R is found principally in the PVN (Trivedi et al., 1998). OXR is also found

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* Due to the more common use of the term “orexin” in the appetite literature, the term “orexin” will be used throughout this discussion.

elsewhere in CNS; DMH, VTA, nucleus accumbens (NAcc) Shell, and NTS (Rodgers et al., 2002). Peripherally, OX-1 receptors are found in brown adipose tissue and OX-2 receptors in the adrenal medulla.

Both orexins are orexigenic in that, when injected centrally, they dose-dependently increase food intake and water consumption in rats, mice (Sakurai et al., 1998) and goldfish (Volkoff & Peter, 2001). Conversely, the administration of selective orexin receptor antagonists (Duxon et al., 2001; Porter et al., 2001; Rodgers et al., 2001; Smart et al., 2001) or anti-orexin antibodies produce the reverse effect (Yamada et al., 2000). Furthermore, orexin KO mice (prepro-orexin gene) are hypophagic with genetic ablation of orexin neurons associated with a 30% reduction in bodyweight (Hara et al., 2001), and narcolepsy (Chemelli et al., 1999; Chemelli et al., 2001).

It is possible that orexins increase food intake indirectly via fluctuations in arousal levels. To assess behavioural specificity the behavioural satiety sequence methodology is often employed (BSS; see Chapter 3; Halford et al., 1998; Rodgers et al., 2010). It uses the orderly transition of eating, active grooming to resting to discriminate between drugs that reduce food intake via natural physiological mechanisms or those that do so by interference (Halford et al., 1998; Rodgers et al., 2010). Data using this technique has demonstrating that orexin-A increases food intake by delaying the onset of the BSS. This delay is then associated with elevated levels of locomotion, sniffing, rearing and feeding (Rodgers et al., 2000).

In contrast, orexins may elicit their orexigenic action via direct regulation of central neurotransmitters. For example, orexins act on NPY, POMC and glucose-responsive neurons in the ARC and VMH, in a manner reciprocal to leptin (Muroya et al., 2004; Tsujino & Sakurai, 2009). It is also possible that orexins act in the hindbrain to inhibit post-ingestive feedback from peripheral signals (Baird et al., 2009). This is based on evidence that orexin administered directly into the 4th ventricle increases intake and meal duration (Zheng et al., 2005).

Orexins are also linked to hedonic aspects of feeding. High levels of orexin neurons are found in brain regions associated with motivation and reward aspects of feeding, such as the VTA (Fadel & Deutch, 2002; Korotkova et al., 2003). Furthermore, administration of both OX-A and OX-B increases the firing frequency of dopaminergic neurons in the VTA (Borgland et al., 2009; Borgland et al., 2006), while intra-VTA administration of OX-A increases dopamine activation in the NAccShell (Narita et al., 2006; Vittoz et al., 2008). Additionally, increased place preference induced by a reward is associated with increased activation of orexin neurons (Harris et al., 2005). Specifically, OX1R antagonists block place preference

for morphine reward (Harris et al., 2007), whereas OX2R antagonism is ineffective (Smith, See, et al., 2009).

Interestingly, depleted nutritional state (such as that produced by 48-hour fasting) causes an up-regulation of pre-pro-orexin (Cai et al., 1999; Griffond et al., 1999; Mondal et al., 1999; Yamamoto et al., 2000). Furthermore, orexin neurons expressing both orexin and leptin receptors have been identified in the GIT and are activated during starvation. It is therefore unsurprising that peripheral administration of orexins increase blood insulin levels. Additional peripheral effects include increases gastric acid secretion and gut motility (Bengtsson et al., 2007; Rodgers et al., 2002; Takahashi et al., 1999).

1.5 Other Systems

The central melanocortin and NPY systems are sensitive to episodic and tonic signals expressed within the hypothalamus as well as those that are able to cross the BBB from the periphery. In other words, NPY/AgRP and POMC/CART neurons act as downstream targets for circulating hormone signals to regulate appetite (see Figure 1-5).

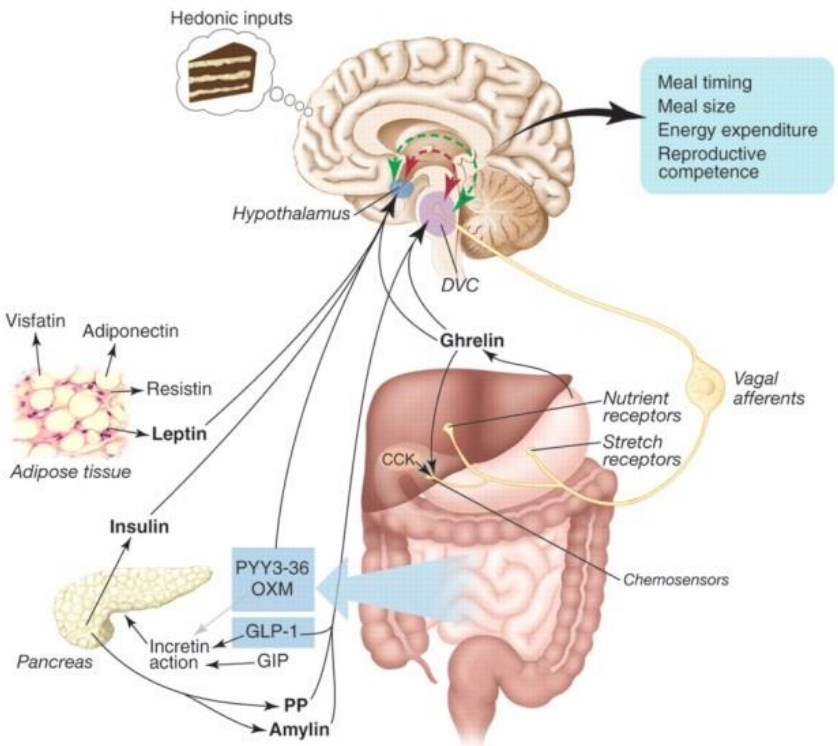


Figure 1-5: Peripheral appetite signals

Schematic of the integration of peripheral signals including, adiposity signals, gut peptides, and pancreatic peptides (Badman & Flier, 2005). DVC, dorsal vagal complex; GIP, gastric inhibitory polypeptide; PP, polypeptide

Table 1-2: Peripherally Released Appetite Peptides and Adiposity Signals

The Table displays both anorexigenic and orexigenic peptides, that are primarily thought to be released peripherally

Orexigenic Peripheral Signals

Peptide	Biochemistry	Discovery	Site of synthesis	Site of Action	Receptors
Ghrelin	28-amino-acid peptide	1999	Gastric mucosa, DMN, VMH, PVN and ARC	Hypothalamus, Hippocampus, VTA, pituitary	GHS-R1

Anorectic Peripheral Signals

Peptide	Biochemistry	Discovery	Site of synthesis	Site of Action	Receptors
Leptin	cytokine	1994	Adipose tissue	ARC, VMH, DMH, and PVN	OB-R
Insulin	51-amino-acid polypeptide	1921	Pancreatic β cells	Hypothalamus, Hippocampus, Olfactory bulb	IR-A, IR-B, IGF
CCK	58-, 39-, 33- and 8-amino-acid peptide	1928	ARC, VMH, VTA and Duodenum	PVN, DMN, SON, NAcc and Pancreas and Vagal nerve	CCK-1, CCK-2
GLP-1	31-amino-acid peptide	1980	L cells, NTS, ARC and PVN	ARC, PVN, VMH, SON	GLP-1R
Peptide YY	36-amino-acid peptide	1980	Lower GI tract (distal gut)	Hypothalamus and Pancreas	NPY Y-2
Bombesin	14-amino-acid peptide	1986	GI Tract, spinal cord and hypothalamus	AMY	BBR-1, BBR-2 and BRS-3
Corticotropin releasing hormone	41-amino-acid neurohormone	1981	PVN	Anterior lobe and pituitary gland	CRF-1 and CRF-2
Somatosatin	14- and 28-amino-acid peptide	1973	Stomach, Hypothalamus and Pancreas	Stomach, Duodenmum, pancrease	SST_{1-5}
Oxyntomodulin	37-amino-acid peptide	1981	Colon, and NTS	ARC, PVN, VMH, SON	GLP-1R
Pancreatic Polypeptide	36-amino-acid peptide	Early 1970s	Pancreatic islets (F-cells)	Arc, PVN, AMY, Thalamus	NPY Y_4 > Y_1, Y_5
Enterostatin	pentapeptide	1988	Pancreas	Unknown	beta subunit of F1-ATPase
Apolipoprotein A-IV	glycoprotein	1977	small intestine	NTS	As yet Unknown
Amylin	37-amino-acid peptide	1987	Pancreatic islets (B-cells)	NTS and hypothalamus	AMY-1, -2 and -3

1.5.1 Anorectic –Peripheral

Appetite and food intake are regulated peripherally by a variety of tissues and organs such as adipose tissue and the GIT. Signals released from the periphery

(see Table 1-2) are then relayed via the general circulation and vagal nerve to the brain, where they interact with receptors in the hypothalamus, NTS and elsewhere.

1.5.1.1 Cholecystokinin

Cholecystokinin (CCK) is a family of hormones, comprising a varying number of amino-acids (58-, 39-, 33- and 8-amino-acid peptides). Originally identified in 1928 (Ivy & Oldberg, 1928), it is secreted from duodenal and ileal cells in response to nutrients (primarily fat and protein). Endogenous CCK rises rapidly post-prandially, remaining elevated for up to 5 hours (Koulischer et al., 1982).

There are two currently identified types of CCK receptor; CCK1, originally named CCK-A ‘alimentary type” and CCK2, originally named CCK-B ‘brain type’ (Little et al., 2005). CCK1 receptors are primarily located in the CNS; particularly regions associated with appetite regulation, such as the NTS, the DMN and the area postrema (AP; Little et al., 2005; Moran, 2009; Moran, 2000). Peripherally, CCK enters the gastrointestinal lumen and binds to CCK1 receptors located on the vagal sensory terminals (Valassi et al., 2008).

Centrally, CCK increases expression of CART and NPY_2 receptors, while inhibiting the expression of MCH, its receptors and cannabinoid receptors (Harrold et al., 2012). Lee et al. (2011) found that the absence of CCK1 receptors is linked to an elevation of ghrelin-induced hyperphagia, potentially mediated by CART.

Peripherally, CCK mediates satiety and meal termination (Antin et al., 1975; Burton-Freeman & Schneeman, 2002; Gibbs et al., 1973; Gutzwiller et al., 2000; Lieverse et al., 1995) through many actions within the GIT including; the slowing of gastric emptying (Debas et al., 1975), modulation of gastrointestinal motility (Brennan et al., 2005), and pancreatic secretion (Brennan et al., 2007; Owyang, 1996).

Central administration of CCK (Ballinger et al., 1995; Figlewicz et al., 1992; Gibbs et al., 1973; Hirosue et al., 1993; Pisunyer et al., 1982) and agonists for CCK1 and CCK2 receptors dose-dependently suppresses energy intake by reducing meal size and duration (Bignon et al., 1999; Kissileff et al., 1981; Stacher et al., 1982). As might be expected, the blockade of both CCK receptor subtypes (via antagonists or CCK antibodies), in addition to genetic deletion of CCK receptors, results in hyperphagia and obesity (Beglinger et al., 2001; Corwin et al., 1991; Dourish et al., 1989; Funakoshi et al., 1995; Gutzwiller et al., 2000).

It should be noted, however, that the majority of peptide dose levels used to induce feeding effects are likely to have produced supraphysiological plasma concentrations (Little et al., 2005). Furthermore, research has found that only

specific CCK1 receptor agonists suppress appetite, while receptor deficiency or blockade results in hyperhagia and obesity in rodent models (Moran & Bi, 2006). In humans, the hypophagic effect is very modest (7% increase in intake; Beglinger et al., 2001). Clinical findings show that obese patients produce higher CCK secretion following a high energy meal than their lean counterparts (French et al., 1993). However, bulimic patients have impaired CCK secretion in response to food intake, which correlates with an impaired sense of post-meal satiety (Geracioti & Liddle, 1988; Hannon-Engel, 2012). In this context, there appear to be differential effects in lean and obese rats (Meereis-Schwanke et al., 1998), in addition to gender and diet composition effects (Maggio et al., 1988; Strohmayer & Smith, 1987).

1.5.1.2 Oxyntomodulin

Oxyntomodulin (OXM) is a naturally occurring 37-amino-acid peptide discovered in 1981 (Bataille et al., 1981; see review: Pocai, 2012). Found in the colon, it is produced by the epithelial cells of the oxyntic (fundic) mucosa. It is released in response to caloric intake, rises 5-10 minutes after a meal, and peaks at 30mins post-ingestion (Anini, 1999).

Central or peripheral administration of OXM dose-dependently inhibits feeding in fasted and non-fasted rats (Dakin et al., 2001; Dakin et al., 2004). Chronic OXM administration studies confirm an inhibition of food intake, with no evidence of tolerance (Dakin et al., 2002). Additionally, chronic OXM-induced reductions of intake and bodyweight can be produced in obese subjects, an effect that maintained for up to 4 weeks (Wynne et al., 2006; Wynne et al., 2005). Furthermore, a OXM-induced reduction in hunger scores and energy intake has been demonstrated in healthy humans (Cohen et al., 2003).

These anorectic actions are very similar to the actions of glucagon-like peptides (GLP; see Chapter 7). There are two glucagon-like peptides; GLP-1 and GLP-2, both impacting at different levels on the regulation of appetite and energy homeostasis. GLP-2 research has primarily focused on its therapeutic use to treat gastrointestinal disorders, whereas research on GLP-1 is more relevant in the present context (Janssen et al., 2013; Kieffer & Habener, 1999). Identified in 1980 (Lund et al., 1980), GLP-1 is an anorectic incretin hormone. GLP-1 has two distinct forms: GLP-$1_{7\text{-}36amide}$ and GLP-$1_{7\text{-}37}$, the former being the main circulating peptide associated with feeding (for a detailed discussion see Chapter 7). All GLP-1 subtypes act on GLP-1 receptors (GLP-1R) which are widely expressed in the pancreatic islets, lung, heart, kidney, intestine and the CNS; hypothalamus and brainstem (Baggio & Drucker, 2007; Drucker, 2006).

No OXM-specific receptors have been currently identified. However, OXM has affinity for GLP-1R two orders of magnitude lower than that of GLP-$1_{7\text{-}36amide}$, yet it produces a bioequivalent anorectic effect (Dakin et al., 2001). Furthermore, the anorectic effects of OXM can be blocked by GLP-1 receptor antagonist, exendin9-39, whereas OXM fails to alter feeding in GLP-1R KO mice (Baggio et al., 2004a). These findings would support an action of OXM at the GLP-1 receptor.

Similarly, OXM has a weak affinity for the glucagon receptor. OXM may act as a glucagon mimetic within the liver and pancreas (Baldissera et al., 1988), whereby it simulates insulin secretion (Drucker, 2007). However, knock-out studies demonstrate that although OXM may act at these receptors, its administration to Gcgr-/- mice (glucagon receptor-deficient mice) can still elicit OXM-induced anorexia (Baggio et al., 2004a).

Some evidence suggests that the anorectic effects of OXM may not be direct. For example, OXM administration has metabolic actions; whereby it increases heart rate (Sowden et al., 2006) and activity levels (Wynne et al., 2006). Furthermore, OXM acts peripherally to inhibit meal-stimulated gastric acid secretion (Jarrousse et al., 1986).

1.5.1.3 Bombesin

Bombesin is a 14-amino-acid peptide, originally isolated from the skin of an amphibian (Bombina bombina; Spindel, 1986). It has two known homologues in mammals (known as bombesin-like peptides); Neuromedin B (NMB) and gastrin-releasing peptide (GRP). Gastrin is a peptide hormone that stimulates the release of hydrochloric acid in the stomach and which aids gastric motility (Arora & Anubhuti, 2006). NMB was originally isolated from pig spinal cord, but has now been shown to be present in human CNS and GIT (Minamino et al., 1983). Bombesin and related peptides have three, currently known, G-protein-coupled receptor subtypes: BBR-1 (NMB specific), BBR-2 (GRP specific), and BRS-3 (as yet uncharacterised; Battey & Wada, 1991; Ohki-Hamazaki et al., 2005), all of which are widely expressed in the CNS and GIT (Moran et al., 1988; Plamondon & Merali, 1994; Wada et al., 1992).

Central administration of bombesin or GRP elicits a suppression of food intake (Gibbs et al., 1979; Muurahainen et al., 1993; Sayegh, 2013). Conversely, bombesin receptor antagonists both enhance food intake and block bombesin-induced satiation (Flynn, 1997); the PVN and NTS are particularly sensitive to these effects (Flynn, 1992). Furthermore, when bombesin is systemically administered, central pre-treatment with antagonists can attenuate the satiation effects (Kirkham

et al., 1994). For these reasons, bombesin-induced feeding effects are believed to be centrally activated. Specifically, as GRP-R KO mice (but not NMB-R KO mice) exhibit increased weight gain (Maekawa et al., 2004; Wada et al., 1997), these actions are probably mediated via the BBR-2 receptor.

Similarly, BRS-R3 KO mice display a mildly obese phenotype and hyperphagia. However they also show elevated circulating leptin levels (Maekawa et al., 2004). Typically, when leptin is administered ICV food intake is inhibited in wild-type mice, however, this effect is attenuated in BRS-3 KO mice (Maekawa et al., 2004), suggesting that bombesin may act via other central satiety mechanisms. Bombesin is also thought to at least partly mediate its feeding effects through CRF (corticotropin-releasing factor) release, as CRF antagonists have been found to attenuate the bombesin-induced satiety effect (Plamondon & Merali, 1994). Furthermore, BRS-3 KO mice display elevated MCH levels, and present an orexigenic response to MCH administration. The up-regulation of MCH could be, at least partially, responsible for the hyperphagia and leptin resistance in BRS-3 KO mice (Maekawa, 2004). This research implies a significant mediating role of MCH in the feeding effects of bombesin in normal animals. Interestingly, although pharmaceutical research is focusing on BRS-3 receptor agonists for the treatment of obesity (Guan et al., 2010; Guan et al., 2011; Jensen et al., 2008; Ramos-Alvarez et al., 2013), bombesin actually has a low affinity for the BRS-3 receptor (Jensen et al., 2008), despite its name and sequence similarity.

1.5.1.4 Somatostatin

Somatostatin (SS), identified and isolated in 1973 from the sheep hypothalamus (Siler et al., 1973), is also known as growth-hormone inhibiting hormone (GHIH). It is a peptide formed by cleavage from pre-somatostatin, and has two active forms; primarily as the tetradecapeptide somatostatin-14 (SS-14) but also as somatostatin-28 (SS-28; Yamada et al., 1992). SS is generally found in the GIT and pancreas, where it is produced by paracrine and endocrine-like D cells to inhibit gastrointestinal endocrine secretion (McIntosh et al., 1978). High levels of SS are also found in the hypothalamus and limbic regions, including the AMY and hippocampus (Johansson et al., 1984). SS acts on five different SS receptors, four of which do not differentiate SS-14 and SS-28 (Patel, 1999).

The effect of SS on feeding is inconsistent. Whereas, SS is found to increase food intake in rats (Aponte et al., 1984; Feifel & Vaccarino, 1994) and chicks (Tachibana et al., 2009), it is reported to reduce intake in goats (Mogi et al., 2003) and baboons (Lotter et al., 1981). Central administration of ‘physiological doses’ in rats increases

feeding at 1hour post-treatment, while ‘pharmacological doses’ decrease food intake, and no effects at all are found with dark-phase administration (Vaccarino et al., 1990). This suggests that the inconsistencies may be due to variation in doses and/or testing protocols. It is also thought that the differential effects of SS between studies are due to the relative affinities of the compounds used.

SS is also believed to exert its action via suppressed release and absorption of peripheral appetite signals (Eisenbraun & Ehrlein, 1996). For example, SS suppresses circulating ghrelin (Norrelund et al., 2002), and insulin (Kleinman et al., 1994). It also inhibits digestive processes such as gastric motility (Scalera & Tarozzi, 1998) and gastric emptying, via the suppression of CCK-induced smooth muscle contraction (Kabemura et al., 1991). However, studies shows that a vagotomy abolishes SS-induced effects on food intake (Levine & Morley, 1982), suggesting an ultimate central mechanism of action (Stengel et al., 2010).

Much of the current research focuses now on S2 receptor agonists such as octreotide (SMS 201–995; Grace et al., 2006), and ODT8-SST (Erchegyi et al., 2008; Stengel et al., 2010; Tachibana et al., 2009), both of which act to stimulate gastric acid secretion and gastric emptying (Yoneda et al., 1991).

1.5.1.5 Fat-Specific Satiation Peptides

Fat-specific satiation peptides are gastrointestinal peptides specifically stimulated by the ingestion of fat, and which regulate the intake and metabolism of lipids (Cummings & Overduin, 2007). Two known fat-specific satiation peptides are; Enterostatin and Apolipoprotein A-IV (APO-AIV).

Enterostatin is a pentapeptide, discovered in 1988 (Erlanson-Albertsson, 2011). It is primarily secreted from pancreatic procolipase during fat digestion (Erlansonalbertsson & Larsson, 1988) but has also been found centrally in the PVN, supraoptic nucleus (SON), ARC, DMH and AMY (Lin et al., 2006; Lin & York, 1997; York & Park, 2006). The receptor for enterostatin is the beta subunit of F1-ATPase (Berger et al., 2002; Park et al., 2004).

Both peripheral (Erlansonalbertsson & Larsson, 1988) and central (Lin et al., 1994; Shargill et al., 1991) administration of enterostatin dose-dependently suppresses food intake in rats. In fact, administration of enterostatin produces a preferential reduction in dietary fat intake (Lin et al., 1997; Lin et al., 2007; Lin & York, 1998; York & Park, 2006). However, this particular effect has not proven to be replicable in humans (Kovacs et al., 2003). Conversely, administration of enterostatin antagonists increases dietary fat intake (Shargill et al., 1991).

Timecourse methodology has established that enterostatin decreases food intake directly by accelerating satiety (Lin et al., 2003). This effect is potentially due to the inhibition of gastric motility and gastric emptying (Erlanson-Albertsson, 2011), through the stimulation of other peptides such as CCK (Lin, 2003) and amylin (Arsenijevic et al., 2005). Enterostatin is also known to interact with serotonin (Lin & York, 2005) and the melanocortin system (Lin et al., 2007).

APO-AIV is a glycoprotein secreted from the small intestine in response to lipid absorption and may be involved in the inhibition of food intake following fat ingestion (Fujimoto et al., 1992; Swaney et al., 1977). Fujimoto et al. (1992) found an inhibition of food intake by intestinal lymph collected from rats actively absorbing fat. After ruling out the possibility that the mere presence of lipid in the chylous lymph is responsible for inhibiting food intake, the authors concluded that the agent responsible was APO-AIV. In APO-AIV-deficient chylous lymph failed to produce any significant effects on feeding behaviour (Yoshimichi et al., 2012). Hypothalamic administration of APO-AIV produces a dose-dependent reduction in food intake that is 50-fold greater than that seen with peripheral administration (Fujimoto et al., 1993); conversely, APO-AIV antibodies increase intake (Derosa & Salvadeo, 2011). It was later found that intracisternal administration of purified APO-AIV dose-dependently inhibits gastric acid secretion and gastric motility (Okumura et al., 1996). As intravenous administration failed to elicit the same gastric effects, a hindbrain/brainstem site of action seems likely (Stan et al., 2003).

1.5.1.6 Amylin

Amylin, a 37-amino-acid known as an islet amyloid polypeptide, was first identified in 1987 (see reviews: Arora & Anubhuti, 2006; Lutz, 2009; Reda et al., 2002). It is co-released with insulin from pancreatic β-cells in response to food consumption (Pittner et al., 1994).

Central and peripheral administration of endogenous and exogenous amylin dose-dependently reduces food intake (Bhavsar, Watkins, et al., 1998b; Chance et al., 1991; Chapman et al., 2005; Kelly & Cline, 2007; Lutz et al., 1994; Mack et al., 2003; Morley & Flood, 1991; Rushing et al., 2000). Conversely, administration of amylin antagonists, such as AC187, increases food intake and meal size (Mollet et al., 2004; Reidelberger et al., 2004; Rushing et al., 2001). As expected, amylin-deficient mice exhibit the typical obese phenotype characterized by hyperphagia and increased adiposity (Devine & Young, 1998). Similarly, Pramlintide, a human amylin analogue, reduces food intake and lowers subjective feelings of hunger, in both lean and obese subjects (Chapman et al., 2007).

Amylin receptors are expressed primarily in the AP (Zuger et al., 2013) In fact, the satiating effect of peripheral amylin seems to be mediated by direct action on AP neurons. Amylin administration induces c-fos activation on AP neurons and AP lesions profoundly attenuate the amylin-induced anorectic response (Becskei et al., 2007; Lutz et al., 1998). Amylin appears to decrease food intake and meal size via a combination of central and peripheral mechanisms (see review: Lutz, 2013). Although all of these mechanisms are not completely understood at the present time, amylin has been shown to: inhibit the digestive secretion of gastric acid and pancreatic enzymes (Young et al., 2005), inhibit nutrient-stimulated glucagon secretion (Fineman et al., 1998), but not hypoglycaemia-stimulated glucagon secretion (Silvestre et al., 2001); slow gastric emptying (Jodka et al., 1996) via amylin receptors in the AP (Edwards et al., 1998); reduce the expression of orexigenic neuropeptides in the LH (Lutz, 2005); up-regulate leptin receptor expression and increase leptin binding in the rat VMH (Lutz, 2010); and increase the release of noradrenaline via AP neuron activation (Potes et al., 2010).

Amylin is also thought to act as an adiposity signal. Although, evidence shows that obese patients have higher mean basal amylin concentration than their lean counterparts (Reda et al., 2002), research on the sensitivity of amylin receptors in obese models remains inconclusive. For example, some DIO models suggest an attenuated sensitivity to peripheral amylin (Boyle et al., 2011), implicating some form of amylin resistance. Furthermore, amylin also elicits effects on bodyweight and food intake via a metabolic action. Amylin increases energy expenditure (Arsenijevic et al., 2005; Osaka et al., 2008; Roth et al., 2006) by means of increases in body temperature (Bouali et al., 1995), actions which may have a secondary effect on food intake. Amylin also decreases locomotor activity (Clementi et al., 1996) and increases anxiety like behaviours in rodents (Kelly & Cline, 2007).

1.5.1.7 Peptide YY

Peptide YY is a 36-amino-acid peptide identified in 1980 (Tatemoto & Mutt, 1980). Secreted from the L cells of the GIT in response to ingested calories, circulating PYY exists in two forms; PYY_{1-36} and PYY_{3-36} (Adrian et al., 1985). The latter is claimed to be peripherally active anorectic signal, and has a relatively selective affinity for Y_2 receptors as expressed on NPY-releasing neurons in the ARC.

Acute and chronic administration of PYY_{3-36} to both fasted and free-feeding rodents and monkeys has been reported to inhibit food intake (Adams et al., 2006; Batterham et al., 2002; Halatchev et al., 2004; Koda et al., 2005; Koegler et al., 2005; Moran et al., 2004; Sileno et al., 2005; Sloth et al., 2007; Stoeckel et al.,

2008). This effect is lost in Y_2 receptor KO mice, and attenuated by selective Y_2 antagonists (Scott et al., 2005; Talsania et al., 2005). Research using BSS methodology has demonstrated a selective action of PYY_{3-36} to accelerate yet preserve the behavioural satiety sequence in rats (Boggiano et al., 2005; Scott et al., 2005; Tschop et al., 2004). As expected, PYY_{3-36} knockout mice exhibit a phenotype characteristic of increased bodyweight and body fat together with increased food consumption (Batterham et al., 2006). Interestingly, obese humans and rodents have low circulating PYY_{3-36}, compared to lean controls (le Roux et al., 2006), and yet retain sensitivity to exogenous administration. Clinical studies have shown that PYY_{3-36} infusions reduce calorific intake in both lean and obese subjects (Batterham, Cohen, et al., 2003; Batterham et al., 2002; Degen et al., 2005).

It has been proposed that PYY_{3-36} elicits its anorectic effect via inhibitory Y_2 auto-receptors on NPY neurons. It dampens NPY release and stimulates reciprocal POMC neurons and orexigenic circuit activation (Ueno et al., 2008; Zhang, Nguyen, et al., 2012). Systemic administration of PYY_{3-36} significantly decreases NPY mRNA expression and increases that of POMC mRNA (Parkinson et al., 2008). Additionally, PYY_{3-36} acts peripherally to delay gastric emptying, and the release of pancreatic and stomach secretions, while increasing the rate of ileum absorption (Steinert et al., 2010).

Despite the above reports, there are major inconsistencies within the literature, and many findings have not been successfully replicated (Tschop et al., 2004). Dramatically, Boggiano (2005) claims that 90% of the literature has failed to demonstrate an anorectic effect using PYY_{3-36}.

1.5.1.8 Pancreatic Polypeptide

Pancreatic Polypeptide (PP) is a 36-amino-acid peptide discovered in the early 1970's. Secreted by pancreatic islets in response to food ingestion (Adrian et al., 1976), it mediates its appetite effects centrally via NPY Y_4 and Y_5 receptors (Larhammar, 1996).

Peripheral administration or the transgenic overproduction of PP decreases food intake and bodyweight (Asakawa et al., 2003; McLaughlin & Baile, 1981); conversely, peripheral administration of anti-PP antiserum increases food intake (Ueno et al., 1999). Systemic infusions can also decrease calorific intake in healthy human subjects by 22% (Batterham, LE Roux, et al., 2003).

However, there are discrepancies within the literature. For example, Clark et al. (1984) found that PP injected directly into the brain can in fact increase food intake.

Similarly, Okumura et al. (1994) found that the central administration of PP increases food intake, via a dose-dependent acceleration of gastric emptying. More recent research demonstrates that PP acts peripherally to decrease the rate of gastric emptying (Katsuura et al., 2002). It is also pertinent to note that PP increases oxygen consumption, suggesting that its effect on bodyweight could be a result of increased energy expenditure rather than a direct effect on appetite. Due to these inconsistencies, the mechanism by which PP elicits an anorectic response is currently uncertain.

Plasma PP levels are reduced in obese patients (Glaser et al., 1988; Reinehr et al., 2006), while anorexia nervosa sufferers experience an increased response to PP (Batterham, LE Roux, et al., 2003; Fujimoto et al., 1997; Uhe et al., 1992). Additionally, Prader-Willi syndrome, characterised by dramatic hyperphagia and obesity, is associated with a deficiency in basal and meal-stimulated levels (Berntson et al., 1993).

1.5.2 Anorectic – Central

1.5.2.1 Neuropeptide W

Neuropeptide W (NPW) has two forms: NPW30 (comprising 30 amino-acids) and NPW23 (comprising 23 amino-acids; see review: Takenoya et al., 2010). Identified in 2002 from porcine hypothalamus (Shimomura et al., 2002), NPW is expressed primarily within the substantia nigra and spinal cord of humans, and the PVN, VMH, ARC and LH of rats (Dun et al., 2003). NPW, is also found peripherally in the gastric antral G cells (Mondal et al., 2006), pancreatic islets (Hochol et al., 2007), and stomach mucosa (Caminos et al., 2008).

NPW interacts with two G protein-coupled receptors, Neuropeptide B/W receptor 1 (NPBWR1; also known as GPR7) and Neuropeptide B/W receptor 2 (NPBWR2; also known as GPR8). It should be noted that the latter is not present in rodents (Tanaka et al., 2003). NPBWR1 are found in the hippocampus, AMY and hypothalamus, including the PVN, SON, DMH, VMH and ARC (Fujii et al., 2002).

Generally, central administration of neuropeptide W increases food intake in rats (Levine et al., 2005; Shimomura et al., 2002). However, continuous infusion of NPW has been found to suppress feeding and bodyweight gain, while anti-NPW (neutralising) antibodies stimulate feeding (Mondal et al., 2003).

NPW is thought to elicit its effect on intake via the MC4R signalling pathways (Date, 2010). Using RT-PCR and electro-physiological methodologies, findings show that NPW increases POMC mRNA expression and decreases AgRP mRNA, but doesn’t

appear to have an effect on that of NPY or CART (Date et al., 2010). Furthermore, MC4R antagonism has been found to inhibit the anorectic effects of NPW (Date et al., 2010).

Neuronal interactions have also been observed between NPW and both orexin- and MCH- producing neurons (Takenoya et al., 2008). For example, NPW ICV administration increases c-fos activation in orexin-producing neurons (Levine et al., 2005). There is also evidence that NPW interacts with leptin function, as NPW levels are significantly up-regulated in leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice (Date et al., 2010). Other views suggest that NPW acts to inhibit growth hormone release via SS neuron activation (Price et al., 2008).

1.5.2.2 Neurotensin and Neuromedin

Neurotensin (NT) is a 13-amino-acid neuropeptide, first isolated from extracts of bovine hypothalamus (Carraway & Leeman, 1973). Although distributed throughout the CNS, highest levels are found in the AMY, NAcc and many hypothalamic sites associated with feeding and bodyweight regulation (Manberg et al., 1982). In the gut, NT is produced in endocrine N cells and regulates gastrointestinal motility as well as pancreatic and biliary secretion (Mazella et al., 2012). NT acts through two G-protein-coupled receptors; NT-1 and NT-2, in addition to the single transmembrane receptor NT-3 (also known as sotilin-1; Vincent et al., 1999).

Central administration of NT decreases food intake (Cooke et al., 2009; Levine, Kneip, et al., 1983; Luttinger et al., 1982), an action thought to be mediated by the NT-1 receptor. Research shows that genetic deletion of NT-2R does not cause a specific phenotype in mice (Maeno et al., 2004), whereas NT-1R KO mice exhibit increased weight gain and hyperphagia in addition to blocked NT-induced anorexia (Remaury et al., 2002).

ICV administration of neurotensin has been found to block MCH-induced, but not NPY-induced, hyperphagia. This suggests a complex functional interaction between central anorectic and orexigenic systems (Tritos, Vicent, et al., 1998). NT is also involved in the mediation of leptin’s action on feeding. NT levels are reduced in ob/ob mice (Wilding et al., 1993), and NT neurons in the hypothalamus express leptin receptors (Mazella et al., 2012). Furthermore, leptin-induced satiety is reversed both by NT immunoneutralization and NT receptor antagonism (Sahu et al., 2001). Likewise, NT is thought to play a role in the regulation of CRF. NT has been found to stimulate CRF release (Rowe et al., 1995), while NT antagonism decreases CRF mRNA levels in the PVN (Rowe et al., 1997). In this respect, NT has been also been linked with prolactin and dopamine (Stolakis et al., 2010).

Xenin, a neurotensin-related peptide, has also been found to reduce food intake in rodents and chicks via stimulation of hypothalamic areas (Cline et al., 2007; Cooke et al., 2009; Leckstrom et al., 2009). Xenin is a 25-amino-acid peptide discovered in 1992 (Feurle et al., 1992). Co-expressed in intestinal K-cells with GIP (Anlauf et al., 2000), xenin is thought to play a role in the regulation of glucose homeostasis and may potentiate the action of GIP on glucose-mediated insulin release via NT-1R (Mazella et al., 2012). However, there is also evidence for a non-neurotensin receptor-mediated effect of xenin (Heuser et al., 2002).

Neuromedin-U (NMU) and Neuromedin-S (NMS) are 25- and 36- amino-acid peptides, respectively (Peier et al., 2011). NMU is expressed both peripherally and centrally, with highest levels found in the hypothalamus (VMH and ARC), the striatum (Hosoya et al., 2000) and the GIT (Nakashima et al., 2010). In contrast, NMS is found primarily in the suprachiasmatic nucleus (SCN).

Both peptides are endogenous ligands for two orphan G protein-coupled receptors, FM-3/GPR66 and FM-4/TGR-1; also known as NMU receptor type 1 (NMU1R) and type 2 (NMU2R; Ida et al., 2005). NMU1R is located widely in peripheral tissues, such as the intestine and pancreas, whereas NMU2R is thought to be limited to the brain and specifically the PVN (Ida et al., 2005).

Acting similarly to NT, NMU and NMS suppress food intake and reduce bodyweight when centrally administered (Howard et al., 2000; Ida et al., 2005; Nakazato et al., 2000; Peier et al., 2011), although it should be noted, that activation of NMU2R also increases locomotor activity and core body temperature (Peier et al., 2009; Zeng et al., 2006). As would be expected, transgenic overexpression of NMU results in hypophagic, lean phenotypes (Kowalski et al., 2005), whereas NMU deficient mice are obese and have reduced energy expenditure (Hanada et al., 2004). In further support for a role of NMU in appetite regulation, human NMU variants are also associated with obesity (Hainerova et al., 2006). Despite these interesting findings, however, the exact mechanism/s responsible for regulation of feeding and energy metabolism by NMU and NMS remains unclear (Nakahara et al., 2010).

1.5.2.3 Oxytocin

Oxytocin is a neurohypophysial hormone that was primarily thought to play a role in reproduction and attachment but, in 1989, was also found to be an anorectic agent (Arletti et al., 1989). Expressed primarily within the PVN and SON, oxytocin has only one, currently identified, receptor (OTR). Interesting, OTRs are also found peripherally in adipocytes (Ho & Blevins, 2013).

Typically, when food is consumed, plasma levels of oxytocin rise (Verbalis et al., 1986; Zhang, Bai, et al., 2011), while oxytocin mRNA is reduced during periods of fasting (Kublaoui et al., 2008). Food consumption also increases c-Fos activation in hypothalamic oxytocin neurons (Johnstone et al., 2006). When administered either centrally (Arletti et al., 1989) or peripherally (Morton et al., 2012) oxytocin is anorectic, while, conversely, OTR antagonism stimulates food intake (Blevins et al., 2004; Zhang, Bai, et al., 2011). Interestingly, oxytocin KO mice display an increased preferential intake of carbohydrate (Olszewski et al., 2010).

It is thought that oxytocin mediates its feeding effects by decreasing meal size. In line with this, oxytocin antagonists dose-dependently increase meal size (Blouet et al., 2009). Furthermore, oxytocin neurons link extensively to the NTS, where it is thought they modulate responsivity to peripheral satiety signals such as CCK. For example, as impaired oxytocin expression blunts the anorexgienic effects of CCK administration, oxytocin may contribute to CCK-induced satiety (Baskin et al., 2010; Blevins et al., 2003; Blevins et al., 2004; Ho & Blevins, 2013; Verbalis et al., 1986).

Recent research has found that oxytocin-induced changes in body mass are independent of food intake (Deblon et al., 2011). As oxytocin also elicits effects on energy expenditure, through an increase in heart rate, body temperature, and oxygen consumption (Ho & Blevins, 2013), as well as effects on gastric motility (Qin et al., 2009; Wu et al., 2003), it may not have a direct impact upon appetite regulation *per se*.

1.5.2.4 Corticotropin-releasing factor

Corticotropin-releasing factor (CRF) is a 41-amino-acid glycoprotein hormone, secreted by the PVN (Vale et al., 1981). Its primary function is to stimulate the synthesis and release of pituitary ACTH which, in turn, stimulates corticosterone secretion from the adrenal cortex (Owens & Nemeroff, 1991).

CRF exerts its actions via two receptor subtypes: CRF-1 and CRF-2 (Behan et al., 1996; Chalmers et al., 1996), although it is thought that CRF-2 is more important in mediating the effects of CRF and CRF-related peptides on appetite (Martinez et al., 1998). CRF-2R antagonists (Cullen et al., 2001), but not CRF-1R antagonists or CRF-1R genetic deletions, block the anorectic effects of CRF administration (Bradbury et al., 2000; Sekino et al., 2004). In contrast, the two endogenous ligands for CRF-2, Stresscopin and Urocortin, both reduce food intake and delay gastric emptying (Arora & Anubhuti, 2006; Inoue et al., 2003; Zorrilla et al., 2004).

Central administration of exogenous CRF, specifically into the PVN, decreases feeding, while chronic administration leads to sustained anorexia and weight loss (Levine, Rogers, et al., 1983; Morley & Levine, 1982; Wang, Stengel, et al., 2011). As might be expected, CRF overexpressing mice exhibit reduced food intake in response to fasting (Stengel et al., 2009), whereas, mice that overexpress CRF binding protein, which sequesters CRF, exhibit increased food intake. CRF may act via tonic restriction of orexigenic signals within the hypothalamus. This is supported by evidence that CRP receptor blockade enhances NPY-stimulated feeding (Hulsey et al., 1995). It should be noted that, CRF also exerts stimulatory effects on arousal and locomotor activity and elicits “anxiogenic-like” effects in rats (Britton et al., 1982; Monnikes et al., 1992; Morley & Levine, 1982; Zorrilla & Koob, 2004; Zorrilla et al., 2004), suggesting its effects on food intake may not be direct.

1.5.3 Orexigenic – Peripheral

1.5.3.1 Ghrelin

Ghrelin, a 28-amino-acid acylated peptide, discovered in 1999, is found primarily in the “A-X like” cells of the oxyntic glands in the gastric mucosa of the stomach (Kojima et al., 1999). Also found in the ARC, ghrelin is a specific GHS-R ligand (growth-hormone secretagogues receptor; located primarily in the hypothalamic pituitary unit, especially on NPY neurons) that stimulates the release of growth hormone (GH; Wren et al., 2000). GHS-R receptors are found in the VMH, ARC and PVN, as well as the hippocampus and VTA (Guan et al., 1997).

Known as the circulating appetite stimulant, ghrelin increases two-fold just before a meal and falls rapidly post-prandially. This rise and fall within an hour of eating suggests that ghrelin plays an important role in meal initiation (Cummings et al., 2001). Ghrelin administration, both central (ICV) and peripheral (i.p.) results in an immediate and sustained increase in food intake similar to that seen with NPY ICV administration (Wren et al., 2001); indeed, ghrelin administration can increase the calorific value of one meal by 28% (Wren et al., 2001). Conversely, ghrelin neutralising antibodies are anorectic (Nakazato et al., 2001). Ghrelin is also thought to play a role in long-term appetite regulation, as supported by the hyperphagic response to chronic ghrelin administration (Tschop et al., 2000).

Although ghrelin regulates GH release from the pituitary via GHS-R (Kojima et al., 1999), it is believed that ghrelin-stimulated appetite and weight gain are independent of ghrelin-mediated GH release. Evidence demonstrates that ghrelin-induced adiposity is not attenuated in GH-deficient dwarf rats compared to intact rats (Tschop et al., 2000). Instead, ghrelin is thought to achieve its orexigenic action

via stimulation of hypothalamic neurons (Nakazato et al., 2001) and inhibition of gastric vagal afferents (Date et al., 2002). Ghrelin stimulates ARC NPY neurons and orexin neurons in the LH (Kohno et al., 2003). Although it has been found that the presence of NPY is not obligatory for ghrelin's role in stimulating fat accretion and reducing lean body mass (Tschop et al., 2000), ghrelin-induced hyperphagia does appear to be mediated by an enhancement of NPY/AgRP and inhibition of POMC (Park et al., 2005). Central ghrelin administration has been found to increase c-Fos expression in NPY and AgRP neurons; antibodies and antagonists that inhibit NPY and AgRP have been found to modulate ghrelin-induced feeding (Lawrence, Snape, et al., 2002; Wang et al., 2002); and administration of ghrelin fails to produce hyperphagia in NPY/AgRP KO mice (Chen, Trumbauer, et al., 2004).

Interestingly, the effect of ghrelin on feeding behaviour seems to be the exact opposite to that of leptin, suggesting competitive interaction in appetite regulation. As discussed, ghrelin causes an increase in food intake and bodyweight gain by stimulating the production of NPY and AgRP in the ARC, whereas leptin acts to decrease food intake and bodyweight gain by stimulating the production of POMC in the ARC. Ghrelin and leptin are complementary players in this regulatory system, whereby ghrelin may act centrally to directly counter-regulate leptin and insulin signalling in the hypothalamus (Horvath et al., 2001; Zigman & Elmquist, 2003).

Despite the apparent role ghrelin in hyperphagia and weight gain, obese patients are found to have reduced circulating ghrelin levels compared to age-matched lean controls (Ariyasu et al., 2001; Tschop et al., 2001). This corresponds with findings that ghrelin levels increase with chronic over-eating (Shiiya et al., 2002). Although ghrelin is down-regulated in human obesity, ghrelin levels are elevated in those with a negative energy balance, for example during high levels of exercise or anorexia nervosa (Neary et al., 2004). This is similar to insulin resistance, whereby the negative feedback loop that stimulates ghrelin release is absent (Finlayson et al., 2007).

Recent findings suggest that ghrelin may play a role in the rewarding and motivational aspects of feeding. GHS-Receptors are co-located on neurons known to play a role in hedonics; for example, GABAergic and dopaminergic neurons in the VTA (Parker & Bloom, 2012). Administration of ghrelin to this area increases the firing frequency of dopaminergic VTA neurons and preferences for highly palatable food (King et al., 2011; Parker & Bloom, 2012). GHS-R antagonism and genetic KO studies have also demonstrated ghrelin's involvement in other hedonic behaviours,

such as operant responding for sucrose and food-induced conditioned place preference (Blum et al., 2009; Egecioglu et al., 2010; Jerlhag et al., 2010; Jerlhag et al., 2009; Parker & Bloom, 2012; Skibicka et al., 2011). Furthermore, human fMRI studies show that ghrelin administration produces increases in neural activity in areas associated with hedonic aspects of eating, including the amygdala, orbitofrontal cortex, anterior insula, and striatum (Malik et al., 2008).

1.5.4 Orexigenic - Central

1.5.4.1 Galanin and Galanin-like peptide (GALP)

Galanin is a 29-amino-acid neuroendocrine peptide, originally identified from porcine intestinal extracts (Tatemoto et al., 1983). Found in the brain and GIT (Bonnefond et al., 1990), it is co-produced in NPY-releasing neurons in the brainstem and hypothalamus. It also co-exists with GABA, NA and 5-HT (Melander et al., 1986). Its receptors (GalR-1, GalR-2 and GalR-3) are widely distributed, including the PVN, LH, VMH, AMY and NTS (Waters & Krause, 2000).

Although central administration of galanin into the PVN and NTS has been found to increase feeding behaviours (Koegler & Ritter, 1998; Schick et al., 1993), genetic models of over-expression or KO have failed to produce a phenotype characterised by altered food intake or bodyweight (Hohmann et al., 2003). However, galanin may have a preferential effect on macronutrient intake. For example, PVN administration of galanin increases fat intake, while galanin antagonists injected into the PVN reduce fat intake (Leibowitz & Kim, 1992; Nagase et al., 2002).

Galanin-like peptide (GALP), a 60-amino-acid peptide, is structurally similar to galanin and is identical for first 13 amino-acids (Ohtaki et al., 1999). GALP expression, unlike that of galanin, is primarily limited to the basomedial ARC and DMH (Jureus et al., 2000; Larm & Gundlach, 2000). GALP is able to interact with the same receptors as galanin, although it has a higher affinity for GalR-3 (Lang et al., 2005). However, as GalR-2/3 agonists fail to elicit any change in feeding behaviours (Man & Lawrence, 2008), these receptors are not those that mediate GALP's action on food intake. At present, the identity of the latter remains unknown (Parker & Bloom, 2012).

Initial studies demonstrated ICV injection of GALP stimulates food intake with a much greater potency than galanin itself (Matsumoto et al., 2002). However, further investigation revealed that, while central administration of GALP stimulated feeding over a 2h period, it reduced bodyweight and food intake over 24-h (Lawrence, Baudoin, et al., 2002). Some theories propose that GALP may initially act upon

galanin receptors, but that subsequent anorectic effects are a result of stimulation of an unidentified GALP receptor (Krasnow et al., 2003). This pattern might also suggest that GALP's action is biphasic in nature, a proposal supported by germline knock-out mice which exhibit no specific phenotype (Dungan Lemko et al., 2008).

1.5.4.2 Melanin-concentrating hormone

Melanin-concentrating hormone (MCH) is a cyclic 19-amino-acid neuropeptide. Initially discovered in chum salmon pituitaries as a regulator of skin colour change (Nahon, 1994), it is cleaved from the precursor prepro-MCH (ppMCH) found primarily in the LH (Bittencourt et al., 1992). MCH acts at two types of G-protein coupled receptor; MCH-1R and MCH-2R. MCH has higher affinity for MCH-2R, which widely distributed in the hippocampus, AMY and cerebral cortex of humans although, interestingly, it is not present in rodents. MCH-1R is expressed predominantly in pituitary tissue and GH cell adenomas.

MCH is orexigenic when administered centrally, and studies show that fasting increases MCH expression (Georgescu et al., 2005; Qu et al., 1996). Conversely, MCH antagonist administration has anorectic effects (Della-Zuana et al., 2012; Verty et al., 2013). However, it should be noted that MCH antagonism also results in increased locomotor activity and energy expenditure (Borowsky et al., 2002), suggesting that effects on feeding behaviour may be secondary.

As would be expected, genetic studies have shown that mice over-expressing MCH display hyperphagia and increased bodyweight (Ludwig et al., 2001), while global MCH knockout mice exhibit hypophagic phenotypes and are 28% lighter than the wild-type controls (Shimada, 1998). Similar findings are seen with specific MCH neuron ablation (14% reduction in bodyweight; Alon & Friedman, 2006). Interestingly, rats lacking MCH display reduced operant responding for high fat foods (Mul et al., 2011). Conversely, MCH administration has been found to increase the hedonic value of sweet foods (Lopez et al., 2011), suggesting that this peptide may also play a role in the hedonic aspects of feeding.

1.5.5 Summary

An understanding of the neurobiology of appetite in normal circumstances is critical in characterising clinical disturbances of appetite regulation, such as obesity. Effective manipulation of the systems that regulate appetite are critical for the development of novel therapeutics.

Chapter 2 Obesity: Prevalence and Treatment

Advances in our understanding of the basic neurobiology of appetite (see Chapter 1) are important not only for their own sake but also because of their potential to inform therapeutic innovation for disorders such as obesity.

2.1 The obesity problem

Obesity is the excessive accumulation of body fat a condition currently diagnosed using the body mass index (BMI). BMI is a measure of body fat based on height and weight, that defines people as overweight if their BMI is greater than 25 kg/m^2, and obese when it is greater than 30kg/m^2 (WHO., 2013). It is pertinent to note that BMI alone cannot always accurately portray a patient's risk. BMI can be misleadingly dependent on levels of muscle tissue, or oedema. However, the more accurate methods to measure body fat, such as air displacement, bioelectrical impedance, dual-energy x-ray absorptiometry, and skin fold calipers, also have limitations and are not currently used in clinical practice (Burkhauser & Cawley, 2008).

Obesity has reached global epidemic (i.e pandemic) proportions. As of 2008, more than 1.4 billion adults were overweight, of whom 200 million men and nearly 300 million women were obese (WHO., 2013). The incidence of obesity in children is also on the rise; in 2011, more than 40 million children under the age of five were overweight (WHO., 2013). Obesity is a growing concern in children and adolescents, due to the serious long-term health and psychological consequences (Adair, 2008; Franks et al., 2010; Reilly & Kelly, 2011). Children exhibiting weight gain and those who are exposed to unhealthy eating habits have a greater risk of obesity in later life. The “developmental origin hypothesis” (Volkow & O'Brien, 2007) suggests calorie content and nutrient exposure, as early as pregnancy, can alter how the brain and body develop in anticipation of future environment. Therefore, even foetal exposure to high fat foods can lead to obesity through the selection of similar nutrients in later life (Anzman et al., 2010).

Obesity is not just a cosmetic problem. The health ramifications for overweight and obese people are serious and potentially life-threatening. Obese patients experience day-to-day problems with back pain and osteoarthritis (Lean et al., 1998), in addition to sleep apnea and breathing problems, associated with the excess fat reducing lung volume (Kopelman, 2007). Obesity is a major risk factor in the development of: chronic metabolic disorders, such as type-2 diabetes and

hypertension; some cancers, such as endometrial, breast, and colon; musculoskeletal disorders, especially osteoarthritis; and cardiovascular diseases, primarily heart disease and stroke (Kopelman, 2007; WHO., 2013). It has been estimated that obesity can reduce life expectancy by up to 20 years (Fontaine et al., 2003).

Furthermore, the financial costs of obesity are huge. In England, it has been estimated that the annual cost of obesity and its consequences is £3.3 – 3.7 billion, increasing to £6.6 – 7.4 billion when including overweight patients. That is roughly equivalent to 2.5% of the total net NHS expenditure in 2001/2002 (Commons, 2004). Effective interventions that can reduce bodyweight and decrease the prevalence of obesity-related diseases in the long-term are therefore essential.

2.2 Treatment Options

Current options for the treatment of obesity include lifestyle interventions (dieting, exercise and behavioural therapies), surgical procedures (mal-absorptive and restrictive), and pharmacotherapy (see reviews: Brown et al., 2009; Wyatt, 2013).

2.2.1 Lifestyle Interventions

Energy balance is the key to obesity treatment. A positive energy balance occurs when the calories consumed exceed those used through metabolism and exercise; this leads to weight gain and obesity. Interventions that encourage a negative energy balance, whereby calories are restricted or energy expenditure is increased, have the potential to reduce obesity and associated risk factors (see reviews: Douketis et al., 2005; Wadden et al., 2012).

2.2.1.1 Diet

The “thrifty genotype” hypothesis (Neel, 1999) states that evolution has shaped the way that our bodies access and store food. It proposes that our ancestors lived with limited resources, and that we therefore evolved to maximise our intake at every possible opportunity in order to avoid a nutritional deficit and death. This helps explain increased food consumption in the modern world, where there is a surplus of palatable, high fat and energy dense foods (Holt, 2005).

Diet modification therapies aim to restore energy balance and decrease bodyweight via food restriction and the reduction of caloric intake (Astrup et al., 2002). Diet therapies typically reduce caloric intake by 600 kcal/day (LCD; low calorie diet), or up to 800kcal/day (VLCD; very low calorie diet; Astrup et al., 2000). A caloric deficit of 500-1000 kcal/day could enable a loss of 0.5-1.0 kg/week to achieve a 10%

reduction in bodyweight over 6 months of therapy. An average loss of 10kg is usually observed over 6 months (Health, 2000). Dietary interventions vary in the method by which they achieve calorie restriction. For example, some reduce portion size (Rolls et al., 2002), while others emphasise calorie counting or manipulate the specific macronutrient intake, such as low dietary fat (Bray & Popkin, 1998), low carbohydrate (Saris et al., 2000), low sugar (Ebbeling et al., 2006), low glycemic and high protein (Martens et al., 2013) and mediterranean (Hedner et al., 2013) diets.

Despite the range of dietary interventions, the best predictor for weight loss is not the specific programme but adherence to the diet (Dansinger et al., 2005; Webber et al., 2010). As such, dietary interventions generally only exhibit short-term success. Patients reach maximum weight loss at 6 months and then regain the weight for non-significant outcomes at 24-36 months, possibly due to compensatory eating following restricted intake (Franz et al., 2007; Littman et al., 2005).

2.2.1.2 Exercise

A decline in manual labour and the increase in sedentary lifestyle has also contributed to the prevalence of obesity (Holt, 2005). Physical activity interventions increase levels of exercise to roughly 30-60mins of moderate-to-intense exercise (500kcal per session) 5-7 days a week (Donnelly et al., 2009). This reduces sedentary time and promotes energy expenditure and weight loss, in addition to direct and indirect benefits to other parameters including reducing the risk of chronic diseases and adverse health conditions, such as type 2 diabetes, cardiovascular diseases, osteoporosis, and some cancers (Fang et al., 2003; Gorely et al., 2011; Kriska et al., 2003; Sesso et al., 2000; Wannamethee & Shaper, 2001). As increases in physical activity alone produce minimal weight reductions (Franz et al., 2007; Jakicic & Otto, 2005), exercise therapies are thought to be more beneficial in the maintenance of weight loss (Catenacci & Wyatt, 2007; Jakicic et al., 2008; Turk et al., 2009; Wing & Hill, 2001). Therefore, lifestyle therapies should be used in conjunction with exercise to achieve the maximal effects (Foster-Schubert et al., 2012; Hill & Astrup, 2003). Although, more recent literature has suggested that the disappointing outcomes from weight loss interventions can be largely explained by the huge inter-individual variability in predictors and correlates of outcomes (Stubbs et al., 2011), therefore the perceived failure of these interventions may be improved by the use of a more individualized approach.

2.2.2 Behaviour Therapy

Socio-economic status, occupation and individual experiences all influence psychological food-related beliefs, preferences and behaviour (Kumanyika, 2008). Furthermore, theories such as the “Berridge model of food reward” (Berridge, 1996) propose that overconsumption is a result of “hedonic hunger” as opposed to “metabolic hunger” (Lowe & Butryn, 2007). This is considered by some to be similar to ‘need’ states seen in drug users (Cota et al., 2006). Thus, driven by the incentive salience of food sources (wanting), and the appealing taste of food (liking), the motivation to eat is enhanced (Finlayson et al., 2007; Volkow & Wise, 2005).

Behaviour therapy is based on these theories and focuses on the differentiation between food hunger and appetite, as well as psychological concepts of target attainment, self-monitoring through the use of food and exercise diaries, problem-solving and stimulus control. Behaviour therapy can teach obese patients about social and environmental cues, and provide skills to modify bad habits to avoid undesirable eating (snacking), such as stress management, contingency management, and cognitive restructuring (Lang & Froelicher, 2006). With counselling, obese patients can achieve modest but clinically significant (3 to 5 kg), sustained (1 to 2 years) weight loss (Appel et al., 2011; Keranen et al., 2009), in addition to improvements in stress eating (Daubenmier et al., 2011) and life satisfaction (Niemeier et al., 2012).

2.2.3 Surgical Interventions

Research has shown that lifestyle therapies (dieting, exercise and psychological therapies) are not always effective in the long-term maintenance of weight loss (Klein et al., 2004). An alternative option for those with morbid obesity (BMI≥40 or BMI≥35 with significant co-morbidities) is bariatric surgery. There are typically four surgical procedures available, which are categorised into ‘restrictive’ (1+2) and ‘mal-absorptive’ procedures (3,4+5). These include: (1) gastric banding, (2) gastroplasty (3) gastric bypass, (4) gastrectomy and (5) biliopancreatic diversion (aka. duodenal switch).

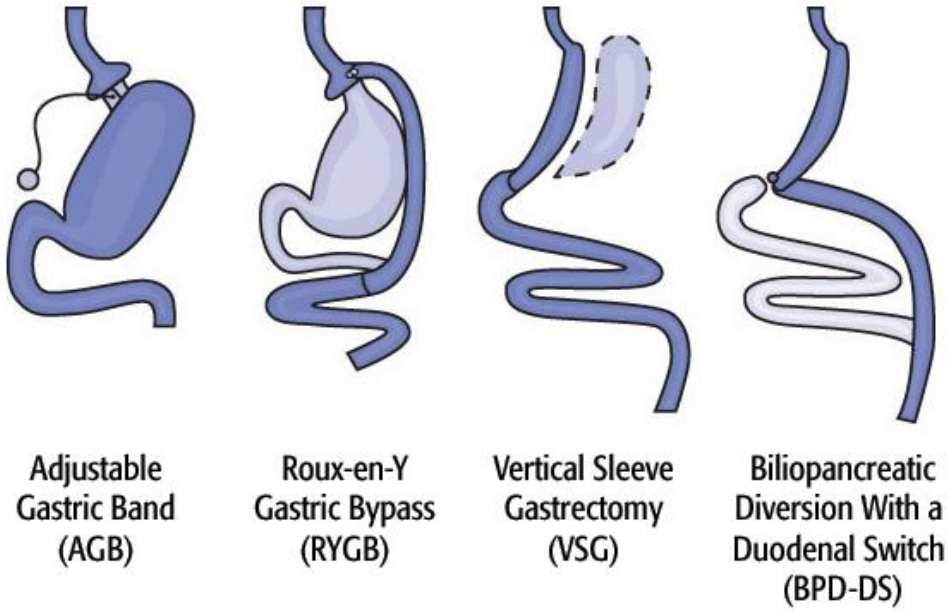


Figure 2-1: Diagram of Surgical Treatment Options

The diagram shows: (1) gastric banding, a small band is placed around the top of the stomach to restrict the size of the opening from the throat to the stomach; (2) gastric bypass; whereby food is sent directly into the small intestine affecting food absorption; (3) gastrectomy, whereby most of the stomach is removed; (4) bilopancreatic diversion, whereby food is re-routed away from the small intestine to limit how the body absorbs food (Pories, 2011).

The percentage of excess weight loss at the outcome point for surgery is much higher than those seen for lifestyle interventions. On average, a 61.2% reduction in excess weight, a 14.2 decrease in BMI and a 39.7kg absolute weight loss can be seen following a surgical procedure. More specifically, 47.5% of excess weight is lost after a gastric banding, 61.6% after a gastric bypass; 68.2% after a gastroplasty and 70.1% after a biliopancreatic diversion or duodenal switch (Buchwald et al., 2004).

Obesity interventions have traditionally focused on weight loss outcomes (Balsiger et al., 2000), but recently there has been a change of emphasis to the metabolic improvements, such as those seen post-surgery (Pories et al., 1995). Patients see improvements in diabetes (76.8% maintain a normal blood glucose level), hyperlipidemia (a total population decrease in cholesterol levels), hypertension (resolved in 61.7%), obstructive sleep apnea (resolved in 85.7%), and longevity (80% decrease in annual mortality; Buchwald et al., 2004; Sjostrom et al., 1998). Furthermore, these changes in metabolic profile can be seen in moderately obese

patients who exhibit little weight loss, suggesting that these metabolic changes have no direct link to weight loss (Kashyap et al., 2013).

However, there are a wide variety of risk factors associated with surgical interventions. The typical side-effects include; pain, wound infection, anxiety, complications and the risk of reoperation, due to the wearing away or breakdown of gastric bands or staple lines. In fact, 10-20% of patients who undergo weight-loss surgery require follow-up procedures to correct complications (Beyond, 2012). Such complications include gastrointestinal leaks and dumping syndrome (whereby stomach contents move too rapidly through the small intestine, producing symptoms such as nausea, weakness, faintness and diarrhoea). Nutritional deficiencies are also a common side-effect of mal-absorptive procedures, with up to 75% of patients who undergo bariatric surgery developing nutritional deficiencies such as anaemia, osteoporosis and metabolic bone disease (Davies et al., 2007; Xanthakos & Inge, 2006).

However, despite the risks, the outcomes for surgical procedures are generally more significant and enduring than with other interventions, not just for weight loss but for quality of life measures as well (Fontaine, 2001; Karlsson et al., 1998). Over a 10-year period, surgically-treated subjects exhibited greater weight loss, more physical activity, and lower energy intake than control subjects (Sjostrom et al., 2004). It is thought that the improved outcomes for bariatric surgery over the more traditional lifestyle interventions are associated with a change in gut hormone profiles (Mingrone et al., 2012; Schauer et al., 2012). More specifically, surgery is associated with decreased ghrelin and insulin levels and increased levels of PYY and GLP-1 (Chronaiou et al., 2012; le Roux et al., 2006; Thomas & Schauer, 2010), resulting in reduced appetite and adiposity signalling.

2.2.4 Pharmacological Interventions

Although lifestyle modifications remain the cornerstone of anti-obesity intervention, they only produce short-term weight loss, with patients experiencing significant weight regain after two years (Rossner et al., 2000; Wadden, 2004). Consequently, current guidelines advocate the use of adjunct pharmacotherapy (NHMRC, 2003).

2.3 An Historical Perspective

The guidelines for the development of anti-obesity drugs are issued by the FDA and EMEA (European Medicines Evaluation Agency). The clinical endpoint for approval of anti-obesity drugs is maintained weight loss for one year post-cessation of

treatment. To be clinically meaningful, weight reduction must be ≥5% (placebo subtracted; USA) and >10% of baseline weight which is also ≥5% than that of the placebo (Europe). Furthermore, 35% of patients must achieve this outcome in addition to improvements in secondary endpoints, such as cardiovascular factors; blood pressure, lipids, glycemia and, in Europe, improvements in waist-hip ratio, sleep apnea episodes and quality of life parameters (EMEA, 2006; FDA, 2007).

As early as the 19th century, **sheep thyroid** extract was used to increase metabolic rate and induce weight loss (Colon-Gonzalez et al., 2013). However, it was also associated with cardiac arrhythmias and cardiac arrest (Bhasin et al., 1981). Later, in the 1930s, **2,4-dinitrophenol** (an Adenosine-5'-triphosphate (ATP) inhibitor; ATP transports chemical energy within cells for metabolism) was introduced as a weight-loss drug, but was discontinued as it caused cataracts, dermatitis, and fatal hyperthermia (Valentino et al., 2010). Then, in the 1940s-1950s, **amphetamine**-like compounds were found to suppress appetite (Macphail & Gollub, 1974), and were approved in the USA. Amphetamines are sympathomimetics, increasing locomotor activity and decreasing food intake. However, the high incidence of tachycardia and hypertension, in addition to abuse potential led to the development of alternatives, continuing into the 1970s when **phentermine** (Achiphex®), **benzphetamine** (Didrex®), **phendimetrazine** (Bontril®, Plegine®; Statobex®), **mazindol** (Mazanor®, Sanorex®), **methamphetamine** (Desoxyn®), and **diethylpropion** (Tenuate®, Dospan®), were approved. See Table 2-1 for an overview of the drugs historically approved for obesity treatment, and Table 2-1 for a more detailed overview of the main anti-obesity drugs over the last 60 years.

Around the same time, a sympathomimetic amine **phenylpropanolamine** (PPA), present in cough and cold remedies, was commonly used as an appetite suppressant. However, this was linked to intracranial bleeding and strokes (Kernan et al., 2000) and, in 2005, the FDA removed it from all over-the-counter products (FDA, 2005). Interestingly, PPA is still available in prescription decongestants (Rinexin®) in Europe and its use is completely unrestricted in the United Kingdom.

The 1980-90s saw the development of serotonergic agents, such as **fenfluramine** (Pondimin®) and **dexfenfluramine** (D-fenfluramine; d-FEN; Redux®). These agents increased satiety by stimulating the release and inhibiting the reuptake of serotonin (5-HT; for a more detailed review of serotonergic agents see Chapter 6 and Halford et al., 2011; Rowland & Carlton, 1986). Although structurally similar to amphetamines, these monoamine-releasing drugs were predicted to have “lower liability for psycho-stimulant abuse” (Heal et al., 2009). However, there remained

the problem of adverse cardiovascular side-effects (Bays, 2004) such as pulmonary hypertension and cardiac valvulopathy (Abenhaim et al., 1996). Thus, despite the perceived success of phentermine and fenfluramine (so called 'Phen-fen'), a combination widely prescribed "off-label" for the long-term management of obesity (FDA, 1997b), the FDA announced their withdrawal from the market in 1997 (Connolly et al., 1997; Heal et al., 2009). Regardless of adverse side-effect profiles, these drugs clearly emphasised the importance of the serotonergic system in appetite regulation.

Table 2-1: History of drugs for the treatment of obesity

Adapted from: (Adan, 2013; Colon-Gonzalez et al., 2013; Powell et al., 2011). Off-Label Usage: prescribed for an alternative usage

Drug	FDA Approval	FDA Withdrawal
Phentermine	May-1959	Present (U.S only)
Amphetamine	Off-Label Usage	
Dextroamphetamine	Off-Label Usage	
Methamphetamine	Off-Label Usage	
Phenylpropanolamine	Off-Label Usage (Europe only)	
Benzphetamine	Oct-1960	
Deithlpropion	Aug-1959	
Phendimetrazine	Sep-1982	
Dexfenfluramine	Jun-1973	Sep-1997
Fenfluramine	Apr-1996	Sep-1997
Bupropion	Off-Label Usage	
Desvenlafaxine	Off-Label Usage	
Sibutramine	Nov-1997	Oct-2010
Rimonabant	01/06/2006	Oct-2008
Topiramate	Off-Label Usage	
Orlistat	Apr-1999	Present
Exenatide	Off-Label Usage	
Liraglutide	Off-Label Usage	
Alogliptin	Off-Label Usage	
Saxagliptin	Off-Label Usage	
Sitagliptin	Off-Label Usage	
Vildagliptin	Off-Label Usage	
Pramlintide	Off-Label Usage	
Lorcaserin	2012	Present (U.S only)
Qsymia (phentermine and topiramate)	2012	Present (U.S only)

Subsequently, **sibutramine** (see Chapter 6; Meridia®, Reductil®), a centrally-acting dual serotonin and noradrenaline reuptake inhibitor, was introduced to the market in 1997. In preclinical studies, it demonstrated its action to reduce food intake both acutely (Luque & Rey, 1999) and chronically (Wirth et al., 2001). In

chronic clinical studies, sibutramine reduced bodyweight by at least 5% in 56% of patients over a one year trial (Smith et al., 2001). However, data from the Sibutramine Cardiovascular Outcome Trial (SCOUT; James, 2005) showed an increased risk of serious, non-fatal cardiovascular events, such as stroke or heart attack (EMEA, 2010) and, in January 2010, sibutramine was suspended from the market (FDA, 2011).

More recently, a selective cannabinoid-1 (CB1) receptor inverse agonist, **rimonabant** (see Chapter 4; also known as SR141716; trade name: Acomplia®) was approved for the treatment of obesity in Europe. The placebo-subtracted weight loss for rimonabant ranged from 3.9 kg to 5.4 kg, with up to 58% of subjects losing ≥5% of their baseline bodyweight (Aventis, 2007). However, recommendations to the FDA suggesting a link with severe adverse psychiatric side-effects (MHRA, 2008a), such as depression and anxiety (Hill & Gorzalka, 2005), led to the withdrawal of rimonabant from the market in October 2008 (MHRA, 2008b). The prevalence of depression and suicide rose not only in patients already suffering mild symptoms, but also in patients with no history of mental illness (Gadde & Allison, 2006; Moreira & Crippa, 2009). These actions triggered Sanofi-Aventis to terminate studies on rimonabant. Nevertheless, the development of other CB1 receptor antagonists has continued (see reviews: Bermudez-Silva et al., 2010; Cluny et al., 2011; Kunos et al., 2009).

In view of these developments and until recently, there was only one drug approved for the long-term treatment of obesity; **Orlistat** (Xenical®, Alli™; WHO, 1999). Orlistat is an irreversible lipase inhibitor that prevents the absorption of up to 30% of digested fat (Bray & Greenway, 2007). A two-year placebo controlled randomised study found that doses of 60mg and 120mg per day reduced weight by 6.8kg and 7.6kg, respectively, compared to only 4.3kg in the placebo condition (Rossner et al., 2000). Recently, however, there have also been questions over the safety of orlistat. In addition to the undesirable gastrointestinal effect of steatorrhea, the FDA released a warning in May 2010 regarding the prevalence of liver injuries in patients taking orlistat. Furthermore, Public-Citizen (a consumer advocacy group and drug safety watchdog) requested the ban of orlistat in 2011. They cited data on increased incidence of liver toxicity, pancreatitis and liver stones in orlistat patients (Citizen, 2011; Weir et al., 2011).

In 2012, the FDA approved the marketing and distribution of two novel drug treatments for obesity. In late June, **lorcaserin** (see Chapter 6; Belviq®), a selective 5-HT_{2c} receptor agonist, was approved (as an adjunct to a reduced calorie

diet and exercise) for the management of chronic weight in obese and overweight adults with at least one weight related co-morbidity. Those receiving lorcaserin lost up to 6kg of bodyweight from baseline compared to placebo (~3kg), with 47% of subjects losing ≥5% bodyweight from baseline (Smith et al., 2010). This new drug had previously reached phase II/III trials but the FDA had initially denied it due to its association with tumour formation and the relatively low weight loss outcomes in rodent preclinical screeing (Arena, 2010). However, more recent trials demonstrate that the most common side-effects of lorcaserin in non-diabetic patients are headaches, fatigue, nausea, dry mouth, and constipation (FDA, 2012).

Then, in mid-July 2012, **Qsymia®** (a.k.a. Qnexa; Topiramate plus Phentermine) was approved. The exact mechanism by which it acts is not fully understood, but may involve GABA receptors, inhibition of carbonic anhydrase, and antagonism of glutamate (Vivus, 2008). The first "Equate" trial elicited an impressive 9kg weight loss using the full dose over 28 weeks (Vivus, 2008). The second "Equip" trial, achieved up to 12kg weight loss on the full dose over 56 weeks (Vivus, 2009), and the third "Conquer" trial produced similar weight loss, in addition to dose-dependent reductions in blood pressure, triglycerides and HbA1C (a measure of blood glucose). An average weight loss of 10% was seen in ≥60% of participants (Kennett, 2010). However, the highest dose was also associated with increased heart rate (Vivus, 2010). Although it had previously been rejected by the FDA due to concerns about birth defects, heart palpitations and suicidal thoughts (Vivus, 2010), the more accepted side-effects of Qsymia now comprise dry mouth, constipation, altered taste and insomnia (Powell et al., 2011).

Table 2-2: Summary of the main anti-obesity treatments developed over the past 60 years

Adapted from: (Adan, 2013; Dietrich & Horvath, 2012; McGavigan & Murphy, 2012)

Drug	Mechanism of Action	Side-effects	Mean Weight loss (Drug minus Placebo)
Phentermine	Noradrenaline releaser	Valvular heart disease and pulmonary hypertension	4.0kg
Fenfluramine	Serotonin releaser		2.4 kg
Sibutramine	Serotonin/noradrenaline reuptake inhibitor	Increased risk of stroke and myocardial infarction	4.2 kg
Rimonabant	CB1 receptor antagonist	Depression and Anxiety	4.7 kg
Orlistat	Gastric and pancreatic lipase inhibitor	Fatty and oily stools	3.0 kg
Lorcaserin	Serotonin 2C agonist	Dizziness, headache and insomnia	4.8 kg
Qsymia (phentermine and topiramate)	Noradrenaline releaser and anti-convulsant	Dizziness, headache and insomnia	12.2 kg

2.4 Recent developments of anti-obesity treatments (Monotherapy)

The above overview demonstrates that, despite the scale of the problem, the treatment options for obesity are limited. The global epidemic of obesity has outpaced the pharmaceutical industry's ability to develop new and safe drugs.

Before the FDA/EMEA can approve any drug treatments, they must have completed all the phases of clinical research. Firstly, potential compounds must go through preclinical testing which is done primarily, although not exclusively, in rodent models. The aim of this phase is to assess the pharmacodynamics, pharmacokinetics and toxicity of the compound. Further preclinical testing is then used to establish a behavioural profile and a safe dose range for development into clinical testing, of which there are four phases. In Phase I, the experimental treatment is given to a small sample (20-80 subjects) of healthy volunteers to assess safety, dose-range and side-effect potential. In phase II, the treatment is administered to a larger clinical sample (100-300 subjects) and, in Phase III, an even larger sample (1,000-3,000 subjects) with long-term follow up. These phases continually assess the safety profile and confirm its efficacy in reducing a variety of parameters including mortality rates. Phase IV is the final, post-marketing, phase which is used to confirm the risks and benefits of the drug and assess its optimal use (ABPI, 2013). See Table 2-3 for a summary of the potential treatments, currently undergoing clinical testing (see recent reviews; Adan, 2013; Carter et al., 2012; Colon-Gonzalez et al., 2013; Kennett & Clifton, 2010; Rodgers et al., 2012).

2.4.1 Central signalling

2.4.1.1 Melanocortin system

As discussed in Chapter 1, the melanocortin system consists of the primary anorectic and orexigenic centrally-acting neuropeptides, POMC and NPY. The use of **NPY antagonists** as anti-obesity drugs is in development. In view of the evidence implicating Y_1 and Y_5 receptors as critical to the orexigenic actions of NPY, NPY_5 antagonists, such as **velnepreit** (S-2367), have been found to produce significant weight loss in patients during phase II trials; ≥5% weight loss in 35% of subjects (Shionogi, 2011). These changes are accompanied by improvements in additional parameters such as waist circumference and lipid panels (Shionogi, 2011). In contrast, another NPY_5 receptor antagonist (**MK-0557**) has been abandoned due to the lack of clinically significant weight loss (Erondu et al., 2006). On the other hand, NPY receptor agonist and pancreatic polypeptide analogues;

obinepitide (Y_2/Y_4 receptor agonist) and **TM30339** (Y_4 agonist) are currently in phase II (Colon-Gonzalez et al., 2013).

A variety of drug discovery programmes have focused on the development of a MC4R agonist for the treatment of obesity. First in this series was **bremelanotide**, derived from melanocortin. However, this comound was largely associated with flushing, nausea, vomiting and, in some cases, hypertension (Wikberg & Mutulis, 2008). More recently, the MC4R agonist **MK-0493**, has been found to be effective in preclinical trials. However, it has failed to produce significantly relevant outcomes in phase II (Krishna et al., 2009). Another MC4R agonist, **RM-493**, is currently undergoing preclinical trials (Rhythm, 2011). It is pertinent to note that there are likely to be approval problems with compounds that affect cardiovascular and sexual function, such as MC4R agonists (Wikberg & Mutulis, 2008).

Similarly, MCH-1 antagonists (**BMS-830216** and **ALB-127158$_{(a)}$**) are currently undergoing clinical testing (see review: AMRI, 2011; BMS, 2011; Macneil, 2013). Although phase II results for BMS-830216 have not yet been released, findings from ALB-127158$_{(a)}$ phase I are positive. However, another MCH antagonist, **NGD-4715**, was withdrawn from clinical trials due to sleep disturbances and vivid dreams (Sargent & Moore, 2009).

AgRP and α-MSH play an integral role in food intake and bodyweight. Based on the principle that an imbalance of both these neuropeptides is seen in obese subjects, the inhibition of AgRP may be of therapeutic benefit in treating obesity. As such, the AgRP antagonist, **TTP435**, is currently undergoing phase II trials (ClinicalTrials.gov, 2013).

2.4.1.2 Monoamine systems

In the past, drugs targeting the serotonergic system (e.g. fenfluramine) have shown some success in the regulation of appetite (see Section 2.3 and Chapter 6). Of the fourteen currently recognised 5-HT receptor subtypes 5-HT_{1b}, 5-HT_{2c} and 5-HT_6 are currently considered viable targets for obesity drugs. The problem with the previously mentioned serotonergic drugs is their lack of selectivity, and side-effect profile. In addition to targeting receptors for weight modulation, they also produce cardiovascular problems via 5-HT_{2B} receptor mechanisms (Elangbam, 2010). **Lorcaserin** is an example of a more selective 5-HT_{2c} receptor agonist that doesn't exhibit side-effects such as valvulopathy. Additionally, a number of selective 5-HT_6 receptor agonists have been developed (Heal et al., 2008), although these are currently targeted at Alzheimer's disease.

Table 2-3: Summary of the monotherapies currently under investigation

Adapted from: (Adan, 2013; Chugh & Sharma, 2012; Colon-Gonzalez et al., 2013; Rodgers et al., 2012)

Drug	Company	Clinical Phase	Mechanism of Action
Tesofensine	NeuroSearch	Phase III	Serotonin/noradrenaline/dopamine reuptake inhibitor
Liraglutide	NovoNordisk	Phase III	GLP-1R agonist
Cetilistat	Alizyme	Phase III	Lipase inhibitor
Metreleptin	Amylin/Takeda	Phase III	Leptin receptor agonist
Velneperit	Shionogi	Phase II	Y_5 receptor antagonist
Pramlintide	Amylin	Phase II	Amylinomimetic
KRP-204	Kyorin	Phase II	Selective B3-adrenoceptor agonist
ATHX-105	Athersys	Phase II	5-HT2c receptor agonist
BMS-830216	Bristol Myers Squibb	Phase II	MCH1 receptor antagonist
MK-0493	Merck	Phase II	Selective MC4R agonist
TM30339	7TM	Phase II	Selective Y_4 receptor agonist
TTP435	TransTech Pharma	Phase II	AgRP inhibitor
MK-0557	Merck	Phase II	Y_5 receptor antagonist
HPP404	TransTech Pharma	Phase II	Histamine H3 receptor antagonist
SCH-497079	Schering-Plough	Phase II	Histamine receptor antagonist
Vanoxerine	ChanRx	Phase II	Dopamine reuptake inhibitor
Exenatide	Amylin	Phase II	GLP-1R agonist
Davalintide	Amylin	Phase II	Amylin mimetic
GI 181771X	GlaxoSmithKline	Phase II	CCK-A agonist
CYT009-GhrQb	Cytos	Phase II	Ghrelin vaccine
Cabergoline	Pfizer	Phase II	Long-acting D_2 receptor agonist
PYY_{3-36}	Merck/Pfizer/Nastech	Phase I/II	Y_4 and Y_2 receptor agonist
PYY_{3-36}/SNAC	Emisphere	Phase I/II	Y_4 and Y_2 receptor agonist
BVT.74316	Biovitrum	Phase I	$5\text{-}HT_{2C}$ receptor agonist
PRX-07034	EPIX Pharma	Phase I	$5\text{-}HT_6$ receptor antagonist
ALB-127158$_{(a)}$	AMRI	Phase I	MCH 1 antagonist
GSK598809	GlaxoSmithKline	Phase I	Dopamine (D3) antagonist
NOX-B11	Pfizer/Nozzon	Phase I	Bind and inhibit ghrelin
NN9924	Novo Nordisk	Phase I	GLP-1R agonist
Oxyntomoduline	Prolor	Phase I	GLP-1R agonist; OXM mimetic
TKS1225	Thiakis/Wyeth/Pfizer	Phase I	GLP-1R agonist; OXM mimetic
PP1420	Wellcome Trust	Phase I	Pancreatic polypeptide analogue
ZGN-433	Zafgen	Phase I	Methionine aminopeptidase 2 inhibitor
AZD7687	AstraZeneca	Phase I	Diglyceride acytransferase inhibitor
AZD8329	AstraZeneca	Phase I	llp-HSD1 inhibitor
INCB13739	Incyte	Phase I	llp-HSD1 inhibitor
AZD4017	AstraZeneca	Phase I	llp-HSD1 inhibitor
Maraviroc	Pfizer	Phase I	Selective chemokine receptor CCR5 antagonist
Betahistine	OBEcure Ltd	Phase I	Histamine H1 receptor agonist with partial H3 antagonistic activity
A331440	Abbott Labs	Preclinical	Histamine H3 receptor antagonist
AEZS-123	Aeterna Zentaris	Preclinical	Ghrelin receptor antagonist
Fumagillin	Zafgen	Preclinical	Methionine aminopeptidase 2 inhibitor
RM-0493	Merck	Preclinical	MC4R agonist
Ezlopitant	Pfizer	Preclinical	Neurokinin receptor-1 antagonist
STO-609	Merck	Preclinical	Calmodulin-dependent protein kinase 2 inhibitor

Dopamine stimulation is associated with increases in palatable food intake (see Chapter 5) and more recent research using fMRI methodology has assessed the effect of the selective D_3 antagonist, **GSK578809**, on food reward in obese and overweight individuals (Colon-Gonzalez et al., 2013).

As discussed above, the majority of drugs that act upon noradrenaline systems, such as amphetamine-related compounds, also have sympathomimetic and psychostimulant properties (Craddock, 1976). Therefore, the use of these drugs has been discontinued. More recently, compounds that utilise the noradrenergic system in combination with the other monoamine systems have been developed. Older, examples include **phentermine** (noradrenaline and dopamine reuptake inhibitor), **sibutramine** (noradrenaline and 5-HT reuptake inhibitor), and **bupropion** (noradrenaline and dopamine reuptake inhibitor; see Chapter 5). **Tesofensine** (NS2330) is a novel serotonin–noradrenaline–dopamine reuptake inhibitor. Originally developed for Alzheimer and Parkinson patients, phase II trials in predominantly non-obese patients found modest placebo-adjusted weight loss (Astrup, Meier, et al., 2008). Phase II trials have shown all doses to differ significantly on intake and bodyweight parameters compared to placebo after 24weeks (Astrup, Madsbad, Breum, Kroustrup, et al., 2008). The highest dose of tesofensine tested resulted in ≥10kg weight loss in 74% of patients compared to 7% in a dietary control group (Astrup, Madsbad, Breum, Kroustrup, et al., 2008). Adverse side-effects include dry mouth, nausea, dizziness, constipation, and abdominal pain. Another concern is that, in phase II clinical trials, there was a notable increase in heart rate and blood pressure at the highest dose (Astrup, Madsbad, Breum, Jensen, et al., 2008). Despite the latter reports, this compound has currently reached clinical trial phase III (Neurosearch, 2011).

Research has shown that histamine$_3$ receptor activation blocks histamine synthesis and release. Preclinical trials of histamine$_3$ receptor antagonists, such as **A331440**, have found reduced intake and bodyweight in mice on high-fat diets (Hancock et al., 2004; Hancock & Brune, 2005). Other histamine receptor antagonists, **HPP404** and **SCH497079** have reached phase I and phase II, respectively.

2.4.2 Peripheral peptide signals

As discussed in Chapter 1, diverse peptide and adiposity signals interact in the gut and the hypothalamus to control energy homeostasis and appetite. They modulate peripheral physiology (the storage and metabolism of digestive products), and signal nutritional status to the brain. Therefore, the manipulation of these signals may be the key to the effective treatment of obesity.

Ghrelin administration increases food intake. However, ghrelin neutralising RNA (**NOX-BII-2**) has provided non-significant results in the treatment of obesity (Moran & Dailey, 2009). Although prototype ghrelin vaccines (**CYT009-GhrQb**) have also shown non-significant effects, some second generation vaccines have demonstrated therapeutic potential (Zorrilla et al., 2006). The potency of endogenous ghrelin on appetite still provides hope, and some ghrelin antagonists, such as **AEZS-123**, are still in preclinical phases (Zentaris, 2009). Others, e.g. **BIM-28163**, have unexpectedly produced increases in bodyweight (Halem et al., 2005). **Ghrelin *O*-acyltransferase** (GOAT) is the active (octanoylated) form of ghrelin protein located in the stomach and pancreas, which acts on the GHSR receptor. Administration of this agent may also have potential (Yang et al., 2008).

The synthetic amylin analogue, **pramlintide**, is primarily used in the treatment of type-1 and type-2 diabetes (Hollander et al., 2003; Thompson et al., 1998). However, in a diabetes study, it has been shown to reduce bodyweight by up to 2.6kg over a 52week period (post-hoc data; Maggs et al., 2003). More recently, pramlintide has been assessed in non-diabetic obese individuals, with sufficient weight loss (3.5kg) at 4 months (Smith et al., 2008). **Davalintide**, a second generation amylin analogue, has completed phase II clinical trials. However, the weight loss efficacy and tolerability profile was not improved over that of pramlintide (Mack et al., 2010).

Anorectic mimetics, such as CCK-A agonists (**GI181771X**), which have previously been shown to slow gastric emptying in subjects, have failed to produce significantly meaningful weight loss at phase III (Jordan et al., 2008). However, aspects of the study design have been brought into question (Roses, 2009).

On the other hand, proteolysis-resistant GLP-1 analogues (see Chapter Seven) such as **liraglutide** (Victoza™) are currently in phase III. Liraglutide, currently approved for type-2 diabetes, can produce a 6kg weight loss, and ≥35% of subjects reduce their baseline bodyweight by ≥10% (Astrup et al., 2009). Other GLP-1 analogues, such as **exenatide** (Byetta™; see Chapter 7), have been reported to reduce bodyweight by only 5.8lbs over a 2 year period of weekly treatment exendin (Verdich et al., 2001). Another agent in this series, **NN9924**, is in phase I (Emisphere, 2011). However, the FDA have flagged concern about cardiac problems associated with these targets. Interestingly, inhibitors of dipeptidyl peptidase-4 (DPP-IV), the enzyme responsible for the degradation of GLP-1, appear to have little to no effect on weight loss (Amori et al., 2007; Fakhoury et al., 2010; Pratley & Gilbert, 2008; see Chapter 7).

PYY_{13-36}, a Y_2 analogue known as **PEG**, has reached phase I clinical testing and has been found to significantly reduce intake and bodyweight (Ortiz et al., 2007). However, oral forms of PYY have had a non-significant impact on clinically relevant parameters in phase II testing, producing only a 12kg reduction on bodyweight compared to the 6.7kg weight loss seen under placebo conditions (Gantz et al., 2007; Steinert et al., 2010). Furthermore, these compounds have been associated with a dose-dependent induction of vomiting and nausea (Degen et al., 2005). Pancreatic secretions have also been assessed as therapeutic targets, with synthetic PP analogues, such as **PP-1420**, entered into phase I trials in 2010 (Tan et al., 2012).

Long-lasting OXM analogues (**TKS1225** and **OXY-RPEG**) are currently in phase I. These agents not only have a longer half-life but also exhibit a greater potency compared to endogenous OXM. Weekly OXY-RPEG administration produces significantly greater effects on outcome measures compared to natural OXM (PROLOR, 2011).

2.4.3 Other Targets

A second generation lipase inhibitor, **cetilistat**, is currently in a phase III clinical trial (Kopelman et al., 2007; Kopelman et al., 2010). Although it produces outcomes similar to that of orlistat, it apparently has a better side-effect profile.

ZGN-433 is a methionine aminopeptidase 2 inhibitor. The mechanism through which this agent affects weight loss in obesity is unclear. It was found to produce weight loss in mice and, in dogs, was associated with weight loss and improved glycaemic control (Chugh & Sharma, 2012; Colon-Gonzalez et al., 2013). In human trials, it produced reductions of 1 kg of bodyweight per week, alongside improved lipid profiles beyond that expected from weight loss alone (Powell et al., 2011). Phase II testing is due to begin in the very near future.

Ezlopitant is a neurokinin receptor-1 antagonist that reduces sucrose intake, and the consumption of sweetened foods and drinks (Steensland et al., 2010). This profile supports the involvement of the neurokinin-receptor system in reward-related behaviours, and provides a therapeutic target for obesity induced by the over-consumption of positive reinforcers, such as high sugar/fat foods.

2.5 Recent development of anti-obesity treatments (Combination Therapies)

Clearly food intake and bodyweight are controlled by multiple mechanisms. Problems arise when homeostatic responses counter the effects of manipulating any one of these mechanisms. History demonstrates that few “single mechanism” approaches to obesity have been successful in achieving the FDA and EMEA criteria of ≥5% weight loss. Advantages of polytherapy, which actually began some 20 years ago with the phentermine/fenfluramine combination, include the use of lower drug doses, possible synergistic but at least additive weight loss, fewer and less serious side-effects and reduced potential for counter-regulation (Greenway, Whitehouse, et al., 2009; Padwal, 2009; Rodgers et al., 2012; Roth et al., 2010). Table 2-4 summarises the main combination therapies currently in late phase clinical testing for the treatment of obesity.

2.5.1 Clinical Phase

There are currently two combinations seeking FDA approval: Contrave™ and Empatic™.

Contrave™ (see Chapter 5) is the combination of bupropion and naltrexone (Greenway, Dunayevich, et al., 2009; Greenway, Whitehouse, et al., 2009; Inc, 2007). Naltrexone doesn’t significantly produce weight loss on its own (Atkinson et al., 1985; Malcolm et al., 1985), but is currently approved for treatment of alcoholism (Volpicelli et al., 1992). It acts by blocking the μ-opioid receptor, augmenting the release of POMC in the ARC and suppressing the release of α-MSH, hence increasing the weight loss effect of the noradrenaline and dopamine reuptake inhibitor, bupropion (Bray & Greenway, 2007; Greenway, Dunayevich, et al., 2009). During phase III, Contrave™ showed significant weight loss and reduced HbA1C (a measure of blood glucose) after 56 weeks (6.1kg vs. 1.4kg placebo; Orexigen Theapreutics, 2010). As patients on Contrave™ experienced an increased heart rate of 1bpm (Orexigen Theapreutics, 2010), it was rejected by FDA in 2011 pending a larger cardiovascular trial (Orexigen Theapreutics, 2011). Despite this, there has been progress with the FDA using a faster path to resubmission of the Contrave™ New Drug Application (NDA).

Table 2-4: Summary of the combination therapies currently under investigation

Adapted from: (Adan, 2013; Rodgers et al., 2012). NDA: New Drug Application

Drug	Company	Status	Mechanism of Action
Contrave™ (bupropion and naltrexone)	Orexigen	Declined 2011 (NDA submission expected soon)	Noradrenaline/dopamine reuptake inhibitor and opioid receptor antagonist
Empatic (bupropion and zonisamide)	Orexigen	Phase III (NDA submission expected soon)	Noradrenaline/dopamine reuptake inhibitor and anti-convulsant
Pramlintide and Metreleptin	Amylin	Phase II	Amylinomimetic and leptin
Dov 21947	Dov Pharmaceutical	Phase II	Serotonin/noradrenaline/dopamine reuptake inhibitor
Obinepitide	7TM	Phase II	Neuropeptide Y_2 and Y_4 agonist
Vytorin	Merck	Phase III	Inhibition of cholesterol synthesis and absorption

Empatic™ is the combination of sustained release formulations of bupropion and zonisamide, an antiepileptic drug that affects serotonergic and dopaminergic activity, in addition to inhibiting sodium and calcium channels. It was proposed that the addition of one would offset the adverse side-effects of the other; i.e. the depressive and sedative issues associated with zonisamide and the seizures associated with bupropion (Gadde, 2007). In phase II, Empatic™ showed a 7.5% weight reduction and, after 24 weeks, patients had not yet reached a plateau suggesting that greater levels of weight loss could be possible (Orexigen Theapreutics, 2009). Although reported adverse events were primarily headaches, insomnia and nausea, zonisamide has been associated with cognitive impairment, mood disorders and potentially, teratogenicity (Kennett GA, 2010; Mula & Sander, 2007; Rosenstock et al., 2007). Therefore, further testing is required. However, the FDA stated that Phase III data may be sufficient to support submission of an NDA without data from a cardiovascular outcomes trial.

Recently, there has been considerable interest in **Pramlintide-and-metreleptin**, which uses a neurohormonal strategy to combine a synthetic analogue of amylin with an analogue of human leptin (Roth, Roland, et al., 2008; Trevaskis, Lei, et al., 2010; Turek et al., 2010). Low dose ranges of amylin (0, 10, and 50µg/kg^{-d}) and leptin (0, 5, 25, and 125µg/kg^{-d}), synergistically reduced food intake and bodyweight after continuous infusion over 28 days (Trevaskis, Coffey, et al., 2008). In fact, the co-administration of amylin and leptin can further reduce food intake and weight gain compared to control by an additional 16% and 4%, respectively (Roth, Roland, et al., 2008). The basis of this combination is that amylin agonism can restore leptin responsiveness in preclinical models and obese humans (Trevaskis, Coffey, et al.,

2008; Trevaskis, Lei, et al., 2010; Turek et al., 2010). A 24 week trial found that after a 4 week lead-in period with pramlintide alone, the combination treatment led to significantly greater weight loss (12.7%) than treatment with pramlintide or metreleptin alone (8.4% and 8.2%, respectively; Ravussin et al., 2009). Furthermore, following a continued 52 week extension, subjects maintained their weight loss post-treatment (Takeda, 2010). An interesting point to note is that the weight loss in the combination treatment did not plateau post-experiment, as is typical following cessation of monotherapies. Despite this profile, however, the combination was suspended in March 2011 due to safety concerns (Takeda, 2011).

DOV 21,947, is a triple monoamine reuptake inhibitor ("TRIP") primarily developed for the treatment of depression. Phase I trials demonstrated a bodyweight loss of ≥4.6lb after 8 weeks. Interestingly, the study did not include dietary restrictions or exercise programs that are often incorporated in obesity trials. Therefore, it is remarkable that DOV 21,947 produced such a robust reduction in bodyweight. Due to the significant co-morbidity of major depressive disorder with obesity, this particular combination treatment could not only manage depression but also impact weight gain (Sargent & Moore, 2009; Tizzano et al., 2008).

2.5.2 Preclinical Phase

Due to the current issues (such as cardiovascular risk) associated with gaining FDA and EMEA approval for anti-obesity drugs, a huge variety of polytherapies are in early stage (typically preclinical) development (see Table 2-5). Four research strategies can be identified in the current polytherapy literature (see review: Roth et al., 2010); (1) satiety peptides plus satiety peptides, (2) adiposity signals plus satiety peptides, (3) small molecule agents plus adiposity signals or satiety peptides, and (4) small molecule agents plus small molecule agents.

2.5.2.1 Satiety Peptides + Satiety Peptides

Successful combinations of satiety peptides include **CCK** with either **bombesin** and **glucagon** (Gibbs & Smith, 1982; Hinton et al., 1986; Stein & Woods, 1981) or **amylin** (Bhavsar, Watkins, et al., 1998a). **Amylin** has also been successfully combined with **PYY** (Roth et al., 2007).

GLP-1 receptor agonists, including Exendin-4 (for a full review of GLP-1 combinations, see Chapter 6) have been successfully combined with; **glucagon** (Day, 2009), **GIP** (Finan, in submission), **calcitonin** (Bello et al., 2010), **PYY_{3-36}** (Paulik et al., 2011; Reidelberger et al., 2011b; Steinert et al., 2010; Talsania et al., 2005) and **amylin** (Bello et al., 2010; Roth et al., 2012). Combining an amylin

agonist with exenatide in non-human primates, has revealed a synergistic anorectic effect at 1–4 hours and an additive anorectic profile at 5-6 hours (Bello et al., 2010).

2.5.2.2 Adiposity signals + Satiety Peptides

In addition to the leptin-amylin combination (see Section 1.5.1, above), **amylin** has been co-administered with **phentermine** and **sibutramine** (Roth, Trevaskis, et al., 2008). **Leptin** has also been combined with a variety of other anorectic agents such as; **$PYY_{3–36}$** (Trevaskis, Lei, et al., 2008), **GLP-1** analogues AC3174 (Roth, Roland, et al., 2008) and **exendin-4** (Bojanowska & Nowak, 2007; Mueller et al., 2012), and **CCK** (Emond et al., 1999; Matson et al., 2000; Trevaskis, Turek, et al., 2010). However, none of these combinations has resulted in the synergistic weight loss observed with the amylin-leptin combination.

2.5.2.3 Small Molecule Agents + Adiposity/Satiety peptides

Simultaneous targeting of both central and peripheral mechanisms has also been investigated. For example, the combination of **leptin** with; **rimonabant** (Boustany-Kari et al., 2011), **FGF21** (fibroblast growth factor gene; Mueller et al., 2012), **sibutramine** (Boozer et al., 2001) and **topiramate** (Lalonde et al., 2004).

Furthermore, **sibutramine** and **phentermine**, have been combined with **pramlintide** (amylin analogue; Aronne, Halseth, et al., 2010). Here, the combination treatments were significantly different from any treatments given alone, and the percentage of subjects achieving ≥5% and ≥10% weight loss was significantly higher in the combination treatments compared to that of the monotherapies and placebo (28 and 3%, respectively; Roth, Trevaskis, et al., 2008). In addition, **amylin** has been co-administered with **bupropion/naltrexone** treatment (Clapper et al., 2013) revealing additive effects with the combination of the catecholaminergic and opioidergic systems. Opioid antagonists, such as **naltrexone** (Liang et al., 2013; for a full review of GLP-1 combinations see Chapter 7) have also been combined with peripheral peptides such as **GLP-1** receptor agonists.

Table 2-5: Summary of treatment combinations under preclinical investigation

The efficacy of these combination are categorised as: Sub-additive, whereby the combination treatment produced intake/bodyweight data lower than the sum of the individual drug effects; Additive, whereby the combination treatment produced intake/bodyweight data equivalent to the sum of the individual drug effects; and Synergistic, whereby the combination treatment produced intake/bodyweight data greater than the sum of the individual drug effects.

	Combination		Action	Measures			References
				Food Intake	Weight loss	Cardiovascular parameters	
Satiety Peptides + Satiety Peptides	**CCK**	Bomesin	Additive	X			Stein and Woods (1981)
		Glucagon and Bomesin	Synergistic	X			Hinton et al. (1986)
		Amylin	Synergistic	X			Bhavsar, Watkins, et al. (1998a)
	Exendin-4 / GLP-1	Glucagon	Additive	X	X	X	Day, 2009
		GIP	Additive	X	X	X	Finan (in submission)
		Glucagon, GIP	Additive	X	X	X	Finan (unpublished)
		Amylin	Additive	X	X		Bello et al. (2010); Roth et al. (2012)
		$PYY_{3–36}$	Synergistic	X	X		Paulik et al. (2011); Reidelberger et al. (2011b); Steinert et al. (2010); Talsania et al. (2005)
	Amylin	$PYY_{3–36}$	Additive	X	X		Roth et al. (2007)
Adiposity Signals + Satiety Peptides	**Amylin**	Leptin	Synergistic	X	X		Ravussin et al. (2009); Roth, Roland, et al. (2008); Trevaskis, Coffey, et al. (2008)
	Leptin	$PYY_{3–36}$	Sub-additive	X	X		Trevaskis, Lei, et al. (2008); Trevaskis, Coffey, et al. (2008)
		Exendin-4 / GLP-1	Additive	X	X		Bojanowska & Nowak (2007); Mueller et al. (2012)
		CCK	Synergistic	X	X		Emond et al. (1999); Matson et al. (1997); Trevaskis, Turek, et al. (2010)

Small Molecule Agents + Adiposity/Satiety Peptides	**Amylin**	Bupropion/ Naltrexone	Additive	X	X		Clapper, 2013 Clapper et al. (2013)
		Sibutramine	Synergistic	X	X	X	Aronne, Halseth, et al. (2010); Roth, Trevaskis, et al. (2008)
		Phentermine	Synergistic	X	X	X	Aronne, Halseth, et al. (2010); Roth, Trevaskis, et al. (2008)
	Leptin	Rimonabant	Additive	X	X		Boustany-Kari et al. (2011)
		Topiramate	Additive	X	X	X	Lalonde et al. (2004)
		FGF21	Additive		X		Mueller et al. (2012)
		Sibutramine	Synergistic	X	X		Boozer et al. (2001)
	PYY	NPY_2 antagonist	Additive	X	X		Moriya et al. (2009)
	Rimonabant / AM259	Exendin-4 / GLP-1	Sub-additive	X	X		Bojanowska & Radziszewska (2011)
		Oleoyl-estrone	Sub-additive	X		X	Ferrer-Lorente et al. (2007)
		Oleoylethanolamide	Sub-additive	X			Serrano et al. (2008)
		GIP antagonist	Sub-additive	X	X	X	Irwin et al. (2008)
		mGlu(5) antagonist	Synergistic	X			Varga et al. (2012)
	Naltrexone	Exendin-4	Additive	X			Liang et al. (2013)
Small Molecule Agents + Small Molecule Agents	**NPY_1**	NPY_5	Supra-additive	X	X		Mashiko et al. (2009)
	Topiramate	Metaformin	Sub-additive		X	X	Toplak et al. (2007)
	Rimonabant / AM256	Sibutramine	Sub-additive	X	X		Tallett et al. (2010a)
		mCPP	Synergistic	X			Ward et al. (2008)
		Naloxone	Synergistic	X	X		Kirkham & Williams (2001); Rowland et al. (2001); Tallett et al. (2008b, 2009a)
		Dexfenfluramine	Additive	X			Rowland et al. (2001)
		Nalmefene	Additive	X			Chen, Huang, et al. (2004)
		MCH agonists	Synergistic	X	X	X	Verty et al. (2013)
	Phentermine	Fenfluramine	Sub-additive		X		Li et al. (2003)
	Naloxone	Sibutramine	Sub-additive	X	X		Tallett et al. (2010b)

Interactions between the **cannabinoid system** (for a full review of CB1 antagonist combinations, see Chapter 4) and peripherally acting peptides is seen with combinations such as CB1 receptor antagonist/inverse agonists with; **exendin-4** (Bojanowska & Radziszewska, 2011), **oleoyl-estone** (Ferrer-Lorente et al., 2007), **oleoylethanolamide** (Serrano et al., 2008), **GIP antagonists** (Irwin et al., 2008) and **mGlu(5)** antagonists (Varga et al., 2012).

Furthermore, **PYY_{3-36}** in combination with **GLP-1** (Steinert et al., 2010) and peripherally administered **NPY Y_2** receptor agonists produces an additive effect on bodyweight, adiposity and food intake in DIO mice over 14 days (Moriya et al., 2009).

2.5.2.4 Small Molecule Agents + Small Molecule Agents

Combinations of two centrally-acting agents, such as **NPY_1 and NPY_5** antagonists, are currently undergoing preclinical testing. Results demonstrate a greater suppression of 24 hour food intake and bodyweight gain compared to that of either mono-therapy in DIO mice, thus demonstrating a supra-additive effect (Mashiko et al., 2009). However, NPY_1 receptor antagonists are known to have cardiovascular side-effects (Gullestad et al., 2003; Malmstrom, 2002), and may also impact processes such as affect, mood and reproductive function (Eva et al., 2006).

Interaction between two centrally acting systems, for example, the **cannabinoid system** (for a full review of CB1 antagonist combinations, see Chapter 4), the **monoaminergic** system (for a full review of 5-HT agonist combinations, see Chapter 6) and/or the **opioid system** (for a full review of opioid antagonist combinations, see Chapter 4), has also been assessed. Combinations include, **rimonabant** with serotonogeric agents: ***m*CPP,** a 5-HT_{2C} receptor agonist (Ward et al., 2008), **sibutramine**, a 5-HT and noradrenaline reuptake inhibitor (Tallett et al., 2010a), **dexfenfluramine,** a serotonin releaser (Rowland et al., 2001) **MCH** agonists (Verty et al., 2013), and **opioid** receptor antagonists (Chen, Huang, et al., 2004; Rowland et al., 2001; Tallett et al., 2008b). These opioid antagonists have also been assessed in combination with **sibutramine** (Tallett et al., 2010b).

Topiramate has also been combined with **metformin**, an anti-diabetic drug that enhances insulin sensitivity (Toplak et al., 2007). This combination produced significant weight loss at both doses tested (96mg/day and 192mg/day), and an improvement in glycaemic control in obese subjects with type 2 diabetes. This is in line with the recent trend towards drug development that acts to reduce the severity of metabolic disorders rather than having a sole focus on bodyweight reduction.

2.6 Summary

Cannabinoid, serotonergic, dopaminergic, opioid and peptidergic receptors offer new targets for the modulation of eating behaviour and hence the induction of weight loss. Drugs that can affect adipogenesis, thermogenesis and energy expenditure also hold considerable potential as effective agents. More importantly, drugs not originally developed for obesity, such as simvastatin and ezetimibe (developed for hyperlipidaemia), maraviroc (developed for HIV treatment) and DOV 21,947 (developed for major depressive disorder), are now being considered as potential options for obesity treatment.

Encouragingly, some combination therapies are in late phase clinical trials after demonstrating superior efficacy and an improved safety profile compared with the single agents (for example Contrave™ and Empatic™). However, a huge range of combinations shown to be effective in preclinical research remain to be assessed in human trials. The most recent development is the increased focus on improvements in the metabolic syndrome and not just bodyweight parameters.

2.7 Overall Thesis Aims

Neurobiological research over the past decade has identified a large number of biological (peripheral & central) signalling pathways involved in appetite regulation and energy homeostasis. Consistent with this multiplicity of mechanism, drug monotherapies for obesity and related disorders, although initially achieving weight loss, more often than not are subject to counter-regulation. As such, current interest in this field is focusing on the therapeutic potential of drug polytherapy or combination treatment. It is argued that combination treatments, comprising pharmacological agents acting on different but related pathways, may ultimately be more effective in producing sustained weight loss and improvements in co-morbidity. In principle, polytherapy also permits the use of lower (sub-anorectic) doses of individual agents, thereby opening up the possibility of (i) additive/synergistic effects on food intake and weight gain as well as (ii) the reduction/elimination of side-effects normally associated with higher (individually anorectic) doses of the constituent agents.

The current thesis aims to expand our understanding of the interacting systems that contribute to the regulation of appetite and bodyweight. In this context, the vast majority of behavioural research on appetite tends to naively focus on simple outcome measures or endpoints e.g. a reduction in food intake and/or a reduction in

bodyweight. However, as such outcomes can be reached either directly by an action on the normal physiological regulation of appetite or indirectly via a host of non-specific mechanisms, this thesis will be as much concerned with process as with outcome (Blundell & Latham, 1978; Halford et al., 1998; Rodgers et al., 2010). Therefore, the current research will employ a continuous behavioural monitoring technique (typical of BSS methodology; see Chapter 3) to assess the extent to which given pharmacological manipulations suppress food intake in a behaviourally-selective and physiologically-relevant manner.

Several recent reviews in the area, (Adan, 2013; Rodgers et al., 2012) have emphasised that, of the novel therapies in the anti-obesity drug development pipeline, very few (if any) have been researched beyond the level of outcome measures. Thus, the overarching objective of the current thesis is to provide the missing 'process' detail for at least some of these treatments and treatment combinations, using the dependent measures of intake, feeding and non-feeding behaviour and the BSS during one-hour tests with palatable food. The potential effects of these manipulations on weight gain for up to 7 days following acute treatment will also be explored.

2.7.1 Chapter Four Aims

The first three experiments of the present series further address the nature of the interaction/s between the endocannabinoid and endogenous opioid systems. Although previous research has demonstrated the effectiveness of cannabinoid CB1 receptor antagonist/inverse agonists in the suppression of food intake and/or bodyweight, it is unclear whether this is a selective action. Preclinical research suggests that the acute anorectic effect of the cannabinoid CB1 receptor antagonist/inverse agonists rimonabant in rats may occur as an indirect consequence of response competition from compulsive scratching and grooming (Tallett et al., 2007b). As rimonabant's pruritic effects can be attenuated by low doses of the opioid receptor antagonist naloxone (Tallett et al., 2008b), this hypothesis is to be further investigated.

The specific aim of Chapter 4 is therefore to directly test the response competition hypothesis of acute rimonabant anorexia in male rats using the co-administration of two sub-anorectic doses of naloxone with a moderate rimonabant dose (Experiment 1). Due to the somewhat ambiguous outcome of Experiment 1, two further experiments (Experiment 2 and 3) were conducted on the identical theme.

2.7.2 Chapter Five Aims

Chapter 5 focuses on interactions between the endogenous opioid and monoamine (noradrenaline & dopamine) systems, such as those that underpin the recent development of Contrave™. This novel agent is a combination of naltrexone and bupropion, whereby naltrexone acts to inhibit the μ-opioid receptor, augmenting the activity of POMC neurons, thus increasing the anorectic effect of bupropion (Bray & Greenway, 2007; Greenway, Dunayevich, et al., 2009; Greenway et al., 2010; Greenway, Whitehouse, et al., 2009).

The three experiments that form Chapter 5 examine the individual anorectic effects in male rats of acute treatment with (i) the general opioid receptor antagonist naltrexone (Experiment 4) and (ii) the noradrenaline and dopamine reuptake inhibitor bupropion (Experiment 5), and (iii) their combination (Experiment 6). The dose-response information provided by Experiments 4 and 5 informed the design of the interaction experiment (Experiment 6).

2.7.3 Chapter Six Aims

It was initially intended that we follow the previous thread of research and focus on interactions between the constituents of other combination “anti-obesity” drugs that are in the process of gaining FDA approval, e.g. Empatic® (bupropion and zonisamide) and Qnexa® (aka Qsymia™; phentermine and topiramate). However, limitations in commercial availability and/or funding meant this would not be a feasible avenue of research for the current thesis. As such, our revised strategy maintained a focus on opioid receptor antagonists in combination with other anorectic agents. Chapter 6 assesses opioid-serotonergic interactions through a combination of naltrexone with the preferential 5-HT_{2C} receptor agonist *m*CPP.

The two studies reported in Chapter 6 examine the anorectic effects of acute treatment with the serotonin 5-$HT_{1B/2C}$ receptor agonist *m*CPP in male rats. Although quality behavioural research with this compound has previously been reported, it was felt essential to obtain detailed dose-response data under present conditions (Experiment 7). Those data informed the design of Experiment 8, which explored the acute anorectic effects of co-treatment with a sub-anorectic dose of *m*CPP and two low doses of naltrexone.

2.7.4 Chapter Seven Aims

Chapter 7 investigated the anorectic efficacy of endogenous peptide exendin-4, alone and in combination with naltrexone. In view of the limited behavioural research available, Experiment 9 assessed the dose-response anorectic effects of acute treatment with the naturally-occurring GLP-1R mimetic exendin-4 in male rats. These data, in turn, informed the design of Experiment 10 which examined the acute anorectic effects of co-treatment with a low dose of exendin-4 and naltrexone (two doses).

2.7.5 Summary: Thesis Aims

To summarise, the overall aims of the current thesis were to characterise:

- the acute anorectic effects in male rats of individual treatment with the general opioid receptor antagonist naltrexone, the noradrenaline and dopamine reuptake inhibitor bupropion, the serotonin 5-$HT_{1B/2C}$ receptor agonist *m*CPP, and the naturally-occurring GLP-1R mimetic exendin-4;
- the acute anorectic effects in male rats of co-treatment with rimonabant and naloxone, bupropion and naltrexone, mCPP and naltrexone, and exendin-4 and naltrexone.

Chapter 3 General Methodology

The general methodology for all experiments is summarised in this chapter. Any deviations are specified within the individual empirical chapters.

3.1 Ethics

All experimental procedures were licensed by the Home Office, and conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986. Experiments were conducted under my own Personal Licence (PIL: 40/9977) and Project Licences held by Professor R.J. Rodgers (40/3014 January 2011 – December 2011 inclusive, and 40/3547 January 2012 – April 2013 inclusive)

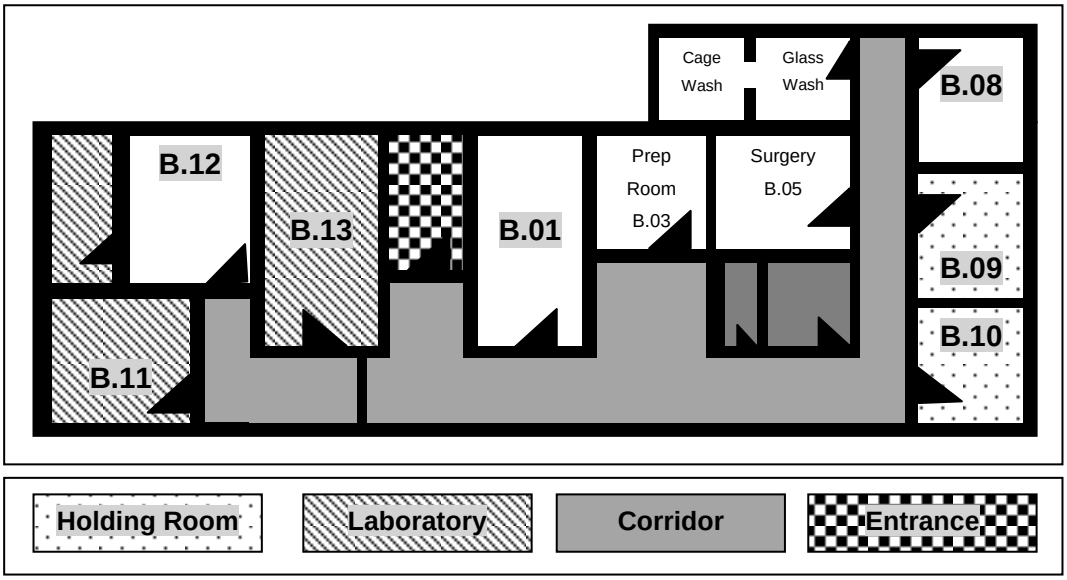


Figure 3-1: Laboratory Floor Plan

(Adapted from Ishii, 2003; Tallett, 2009) White areas signify those used during the current research. B.08, holding room; B.12, testing room; B.01/B0.05, injection rooms. Note: Floor plan is not to scale.

3.2 Subjects, and Housing

All studies used adult male Lister hooded rats, obtained from Charles River (Manston, Kent, UK), weighing between 190.3g and 233.3g on arrival. The animals were initially pair-housed (46 x 26.5 x 26 cm) for 7 days, following which they were transferred for the remainder of the study to individual cages (45 x 20 x 20 cm) containing environmental enrichment (polycarbonate rat tunnels; Datesand Ltd,

Manchester, UK). Single housing facilitated initial familiarisation with the test diet as well as daily bodyweight tracking. Subjects were housed within visual, auditory and olfactory range of conspecifics held in adjacent cages.

Animals were normally held in B.08 with the exception of Experiments 7 and 8 in which, to avoid any disruption to animals currently undergoing testing, new animals were briefly housed in B.01 (see Figure 3-1).

Animals were maintained on a 12-h normal light cycle (lights on: 0700h; lights off 1900h) in an environment controlled for temperature (21 ± 1ºC) and humidity (50 ± 2%). A normal light cycle was employed in view of the much clearer BSS profiles seen when animals are tested during the light phase of the light/dark cycle (Tallett et al., 2009b).

Animals were handled on a daily basis for routine husbandry, habituation to handing and recording of body weights (daily between 09.00-10.00h for the duration of the study). Animal cages were cleaned out on a bi-weekly basis. Although no animals were removed due to signs of ill health, one animal was removed from Experiment 5 due to an inconsistent baseline.

3.3 Diet

Food (standard pelleted chow; BK No.1 Rodent Breeder and Grower, Special Diets Services, UK; digestible energy value = 13.62 KJ/g or 3.25 kcal/g) and tap water were generally available ad libitum in the animals' home cages throughout the study. The only exception was during the injection-test interval when both chow and enrichment were removed.

The test diet (mash) consisted of a hydrated mash made freshly each morning by adding water to the powdered form of the diet (BK No.1 Rodent Breeder and Grower, Special Diets Services, UK; 1g dry powder = 3.125g mash; digestible energy value = 4.48 KJ/g). Portions of mash were then evenly distributed to glass pots, covered and refrigerated until required. Mash has the advantage of being more palatable than the standard chow (Ishii, Blundell, Halford, Rodgers, et al., 2003), increasing baseline intake levels and eliminating the need for prior food deprivation to motivate feeding. Furthermore, its consistency minimises spillage and the likelihood of being removed from the pot for hoarding and/or consumption elsewhere (Halford et al., 1998).

3.4 Drugs & Administration

Drugs employed in the current thesis were rimonabant (Experiments 1 - 3), naloxone (Experiments 1 - 3), bupropion (Experiments 4 + 5), naltrexone (Experiments 5, 8 + 10), *m*CPP (*meta*-chlorophenylpiperazine; Experiments 7 + 8) and exendin-4/exenatide (Experiments 9 + 10).

Details of the compounds, their sources, injection-test intervals and corresponding vehicle solutions are summarised in Table 3-1. All drugs were administered via the intraperitoneal (i.p.) route, in a volume of 1 ml/kg body weight in either the Surgery (B.05) or B.01 dependent upon room availability (see Figure 3-1).

For Experiments 1-8, test solutions were freshly prepared in the morning of each test day in the Prep Room (B.03; see Figure 3-1). For Experiments 9 and 10, exenatide was prepared to required concentrations, dispersed into individual aliquots (0.7ml) and frozen at -20ºC until shortly before use.

Drug doses were selected on the basis of previous research or from dose-response studies during the current work; details may be found in individual experimental chapters. All doses cited are expressed as the salt.

3.5 Apparatus

Daily bodyweights were recorded using Mettler Toledo (VIPER SW 6) weighing scales. Feeding tests were conducted in glass arenas (60 x 30 x 45 cm) large enough to allow animals the freedom to engage in a variety of behaviours (see Figure 3-2; for review see: Rodgers et al. 2010). The arena floor was lightly covered in clean wood shavings for each subject, and a water bottle suspended from one of the end walls. A glass (Pyrex) pre-weighed food pot filled with hydrated mash was placed in the centre of the arena and fixed to the floor using Velcro™ attached on the base of the food pot. An annular metal mounting was then placed over the food pot.

Two video cameras (JVC TK-S530 and Vista VPC520CM) recorded the test sessions for subsequent behavioural analysis. One camera was positioned above the arena and the other placed horizontal to the front wall (see Figure 3-2). This multi-angled view facilitates behavioural analysis and enhances scoring accuracy by minimising any coding ambiguity arising from a single angle (Halford et al., 1998).

Table 3-1: Details of Drugs Administered

Drug	Doses Used	Supplier	Vehicle Solution	Injection-Test Interval
Rimonabant (SR141716A; [N-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide])	1.5mg/kg	Sanofi-Aventis (Chilly-Mazarin, France; Exps: 1 + 2) Cambridge Bioscience, (Cambridge, UK; Exp 3)	Rimonabant was initially dissolved in 3 drops (0.42ml) of dimethyl-sulfoxide (DMSO; Sigma-Aldrich, Poole, UK) and then made up to required concentration in 0.5% methylcellulose (Sigma-Alrich, Poole, UK). A methylcellulose/DMSO mixture was used for control injections.	30 minutes
Bupropion hydochloride	10mg/kg 20mg/kg 40mg/kg	Sigma-Aldrich (Poole, UK)	Bupropion, Naloxone, Naltrexone, and m-CPP were dissolved in physiological saline (0.9%), which alone acted as the control injection	15 minutes
Naloxone hydrochloride	0.01mg/kg 0.05mg/kg 0.1mg/kg	Sigma-Aldrich (Poole, UK)		30 minutes
Naltrexone hydrochloride	0.1mg/kg 1mg/kg 3mg/kg	Sigma-Aldrich (Poole, UK)		15 minutes
***m*CPP** hydrochloride	0.1mg/kg 1mg/kg 3mg/kg	Tocris Bioscience (Bristol, Avon, UK)		30 minutes
Exendin-4 (exenatide)	0.025µg/kg 0.25 µg/kg 2.5 µg/kg	Tocris Bioscience (Bristol, Avon, UK)	Exendin-4 was dissolved in distilled water, which alone acted as the control	30 minutes

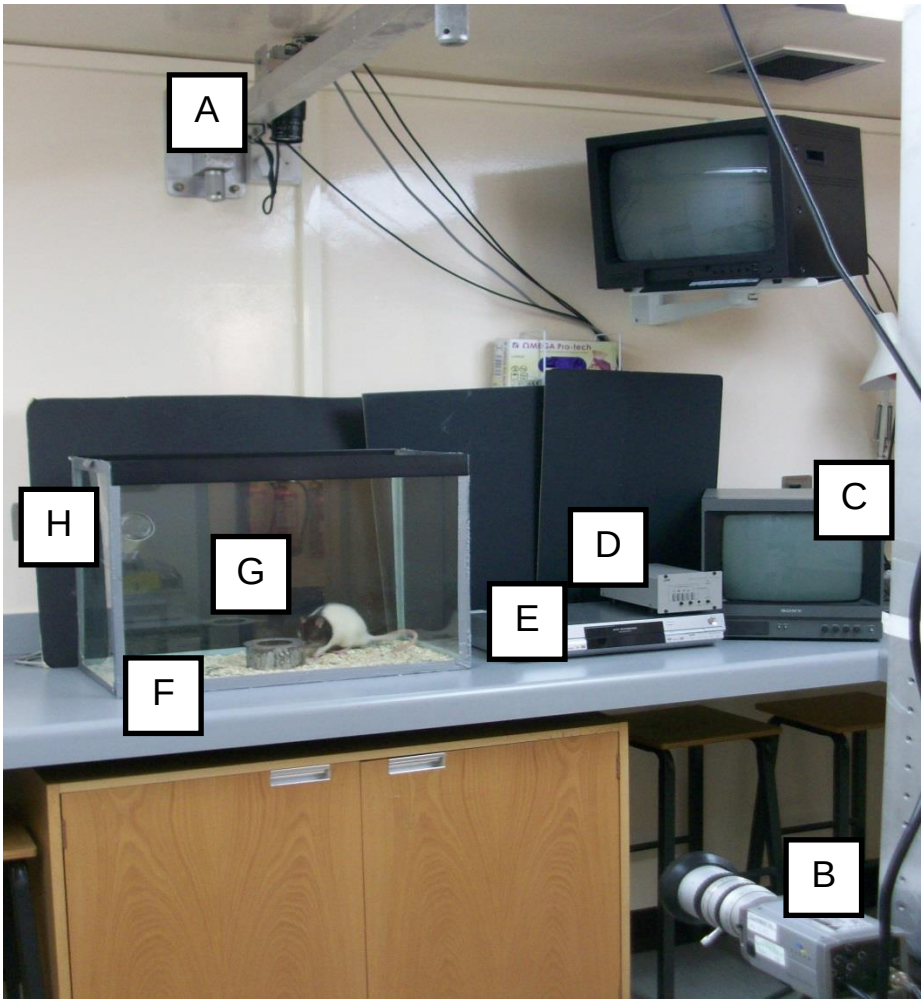


Figure 3-2: Photograph of the lab layout

A. Top view camera, B. Side view camera, C. Television, D. Merger, E. DVD recorder, F. Glass tank, G. Food pot, H. Water Bottle

The video-cameras were linked to a DVD recorder (Panasonic DMR-E55) and monitor (SONY black and white video monitor PVM-145E) via and image merger (JVC video effector; TK-C50E)

Five arenas were simultaneously utilized for habituation. However, due to limited availability of recording equipment, only one test arena could be used for experimental sessions. Observation arenas were emptied and wiped down between each subject and thoroughly cleaned in a cage-wash at the end of each experiment.

3.6 Design

All studies used a within-subjects design with a Latin Square method employed to counterbalance the different dose conditions. A between-treatment washout period

of seven days was used in Experiments 1-3 and 7-10. However, a three day wash-out period was adequate for Experiments 4, 5 and 6. To ensure the elimination of the test compound's and any active metabolites, each experiment carefully compared individual animal data for those subjects receiving the highest dose followed by the vehicle condition. Any unusual behaviour seen within the vehicle condition could then be attributed to carry-over effects. However, no carry-over effects were found in any of the present experiments.

3.7 The Behavioural Satiety Sequence (BSS)

The vast majority of preclinical behavioural research on appetite still naively focuses on simple outcome measures (i.e. reductions in food intake and/or bodyweight gain). However, such outcomes can be reached either directly by an action on the normal physiological regulation of appetite or indirectly via a host of non-specific mechanisms. As research should be as much concerned with process as with outcome (Rodgers et al., 2010), a continuous behavioural monitoring technique (typical of Behavioural Satiety Sequence [BSS] methodology) was employed to assess the extent to which currently-used test compounds (alone and/or in combination) suppress food intake in a behaviourally-selective manner (Halford et al., 1998).

BSS methodology profiles changes in behavioural structure by plotting the time course of three primary behavioural parameters; eat duration, groom duration and rest duration. The model is a reliable means of differentiating primary anorectic drug effects (whereby drugs act via normal physiological mechanisms of appetite control) from secondary anorectics (whereby any anorectic effect is mediated by some non-specific mechanism such as, increased or decreased locomotion or malaise; Rodgers et al., 2010). This methodology has advantages over techniques which purely concentrate on food intake and/or changes in body weight. Behavioural analysis can identify drugs that affect components of eating motivation by acting on the processes that initiate, sustain and terminate feeding behaviour.

Initially documented 40years ago (Bindra & Blond, 1958; Bolles, 1960) and further developed in the 1970s (Antin et al., 1975; Blundell JE, 1979), the BSS has been used to characterize the effects both of pharmacological and non-pharmacological manipulations on the predictable behavioural pattern associated with satiation in rats (Halford et al., 1998; Rodgers et al., 2010). The BSS methodology is based upon the principle that, as rats eat to satiety, they exhibit a characteristic sequence

of the three primary elements, i.e. progression from feeding, through exploratory active behaviours such as locomotion and grooming, to resting/sleeping. The transition to resting is indicative of post-absorptive satiety (Antin et al., 1975). Anorexia not only affects food intake and feeding behaviours, it can also impact upon other aspects of the behavioural repertoire.

The complete BSS profile is initiated by stomach distension and/or movement of food through the intestine, but is not seen in sham-feeding animals (Antin et al., 1975). As the structural integrity of the BSS can be preserved by some anorectic agents but disrupted by others, the BSS reflects the satiety process rather than the simple cessation of feeding (Antin et al., 1975; Blundell et al., 1985). A normal/control BSS profile (as seen in Figure 3-3) should display a peak feeding response at the start of the session followed by a reduction in eating, bouts of grooming and a gradual increase in resting. Drugs that act specifically by inducing satiety should therefore produce an orderly sequence of post-consumptive behaviours with a premature termination of eating and premature onset of resting, i.e. an acceleration (or shift to the left) in the sequence (as seen in Figure 3-3).

Specific behavioural recording allows for the distinction between several different natural behavioural signatures. For example, pre-feeding induced anorexia is coupled with increased latency to eat and a reduced eating period alongside an acceleration in the eat-rest transition, whereas prior fasting results in a shift to the right (a delay) alongside increased food intake and duration of feeding behaviour (Ishii, Blundell, Halford, & Rodgers, 2003). The administration of lithium chloride (LiCl) or diet adulteration with quinine also reduce food intake. However, in contrast to the behavioural signature of free feeding, LiCl anorexia is a result of reduced eating bouts and reduced eat rate, and quinine-induced anorexia is characterized by highly atypical intermittent food sampling/digging, a large number of short eating bouts, a slow eating rate, and a reduction in practically all active behaviours in the first half of the session (Blundell et al., 1985; Ishii et al., 2004; Rodgers et al., 2010). The behavioural signatures of LiCl and quinine are therefore useful for the identification of appetite suppressants that induce malaise or which alter taste sensitivity and/or palatability (Ishii, Blundell, Halford, & Rodgers, 2003).

The variation in behavioural profiles demonstrates the utility of the BSS in identifying primary and secondary reductions in food intake such as nausea (LiCl) or food contamination (quinine). Other anorectics also act non-specifically, disrupting the structural integrity of the BSS by the induction of hyperactivity/stereotypy as seen with amphetamine (Blundell & McArthur, 1981;

Halford et al., 1998) and rimonabant (Tallett et al., 2007b). Additionally, the BSS has successfully defined the effects of serotonergic drugs, such as fenfluramine and fluoxetine (Hewitt et al., 2002; Lee et al., 2004), as well as the role of orexin, cannabinoid and opioid systems on the expression of feeding behaviour (Ishii, Blundell, Halford, Upton, Porter, Johns, Jeffrey, et al., 2005; Ishii et al., 2004; Ishii, Blundell, Halford, Upton, Porter, Johns, & Rodgers, 2005; Rodgers et al., 2000; Rodgers et al., 2001; Tallett et al., 2007b, 2008a).

This preclinical model of feeding behaviour is a powerful diagnostic tool allowing for early insight into the behavioural specificity and therapeutic potential of the anti-obesity agents. However, it has yet to be fully utilized in the study of combination therapies. Following BSS analysis, promising treatment combinations should ideally be subject to more thorough behavioural assessment such as meal patterning analysis, macronutrient preference and dietary-induced hyperphagia in addition to studies investigating hedonics and motivation. More thorough preclinical work should result in a greater understanding of how agents produce the endpoints outlined by the FDA and EMEA (Rodgers et al., 2010).

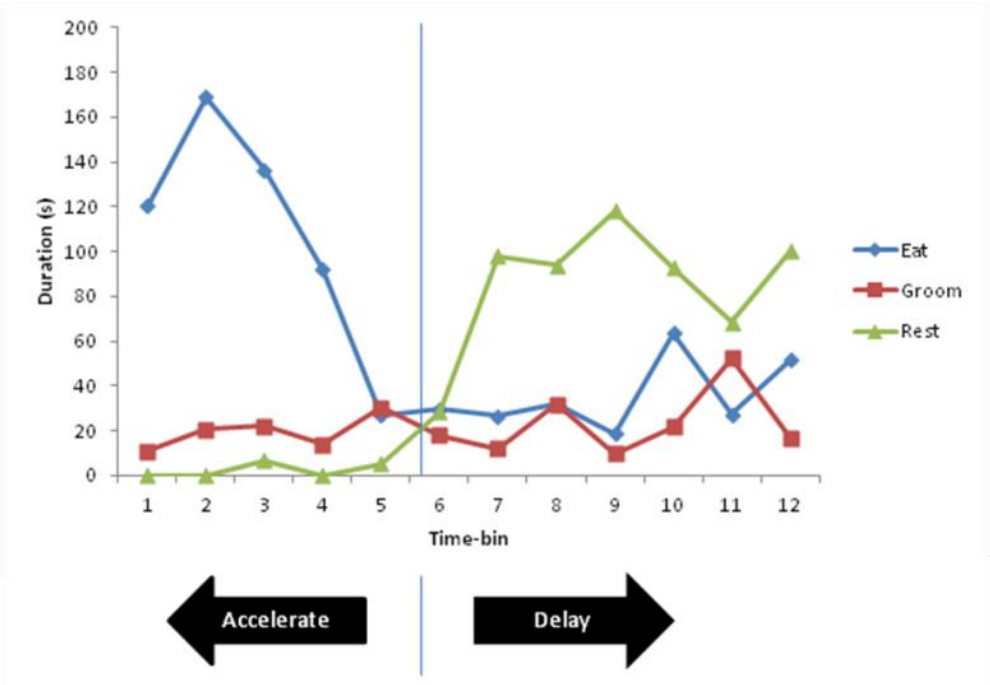


Figure 3-3: A 'typical' BSS profile (Adapted from Experiment One, Current Thesis)

The behavioural satiety sequence (BSS) in non-deprived adult male Lister hooded rats tested for 1 h with palatable mash (n=10). Data shown are mean duration (s) scores for the three component behaviours (eat, groom, rest) in each of 12×5 min timebins comprising the 1 h test. As shown by the horizontal arrows, the vertical line (representing the transition from eating to resting i.e. behavioural satiety) would shift to the left (accelerate) with an anorectic agent and to the right (delay) with an appetite stimulant. Behavioural selectivity of treatment would be indicated by preservation of the sequence despite acceleration or delay.

3.8 Procedures and Behavioural Testing

All habituation and test sessions were conducted during the light phase of the light/dark cycle (1000-1600h) under normal laboratory illumination (265 lux). See Figure 3-4 for a flow chart summary of the current protocol.

3.8.1 Acclimatization Period

The body weight of each animal was recorded daily between 09.00 and 10.00h. The acclimatisation period lasted three weeks following the arrival of animals from the commercial supplier. During the first week, the animals were housed in pairs and then individually for the remainder of the study. This period allows for habituation to laboratory conditions, including the light cycle, personnel and handling. At the end of the final week of the acclimatization period, subjects were exposed to the mash diet in the home cage for 3h on 2 consecutive days.

3.8.2 Habituation Phase

Over the week following acclimatisation, animals were habituated to pseudo-experimental conditions daily for 5 days. The exception to this protocol was Experiment 10 in which subjects underwent an additional habituation day due to timetabling issues (see Chapter 7, Methodology). Habituation sessions involved the removal of home cage chow and enrichment, and i.p. injection of vehicle prior to a one-hour exposure to the behavioural test arena (including mash). For combination studies, the subjects were left in their home cage in the preparation room for the required time before administration of the second i.p. injection (vehicle). This process was run concurrently with five animals due to the availability of five identical test arenas. The food pot was weighed before and after the one-hour exposure allowing for calculation of consumption, while accounting for any evaporation and spillage. Tweezers were used to extract any stray wooden shavings or fecal matter from the food pot before weighing. An extra portion of pre-weighed mash positioned beside the test arena during testing allowed accurate assessment of loss of food mass through evaporation. This evaporation control occurred during two test sessions a day; test session two (in the morning) and test session four (in the afternoon). This daily habituation process familiarised animals with the test environment and ensured the development of a stable food intake pattern prior to the experimental phase. Any animals not displaying a steady baseline food intake of palatable mash towards the end of the habituation week were excluded from the experiment (see Experiment 5).

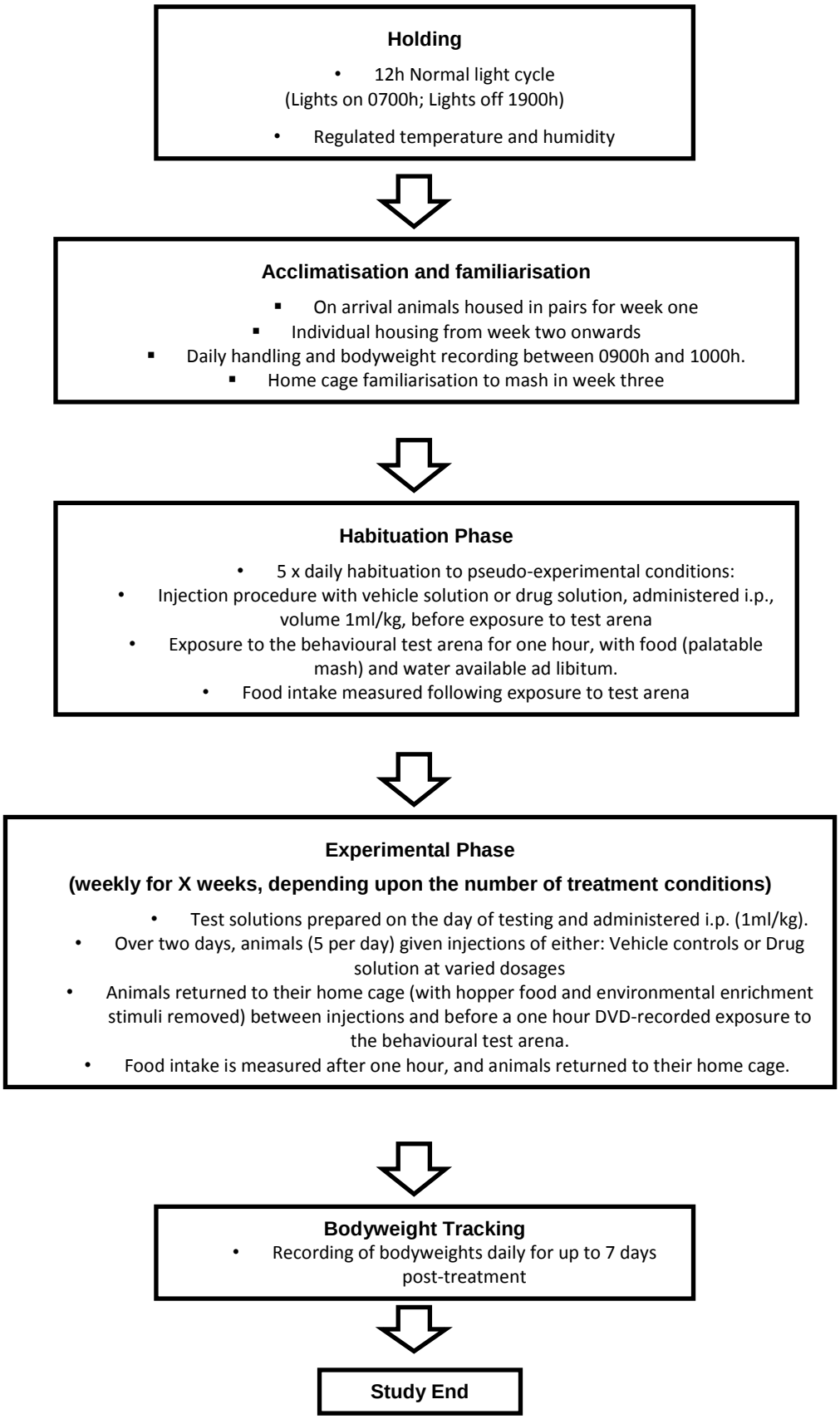


Figure 3-4: Summary of Experimental Procedure

(Adapted from Rodgers, et al, 2010)

3.8.3 Experimental Phase

The experimental weeks followed on directly and consecutively from the habituation period. Each subject was individually transported to a preparation room (Surgery/B.01; see Figure 3-1) and administered the appropriate agent (vehicle or drug; as indicated by the study-specific Latin Square) via i.p. injection, before being returned to the home cage (food and environmental enrichment stimuli removed). For combination studies, the subjects were then left in their home cage in the preparation room for the required time before administration of the second i.p. injection (vehicle or drug). Following the appropriate total injection-test-interval, subjects were then transported to the test laboratory (B.12; Figure 3-1) and placed in the test arena (with pre-weighed mash and water) for the one hour DVD-recorded test session (Figure 3-2). As only one animal could be tested at any one time, injections and testing were staggered throughout the light phase, with five animals tested daily. After the test session, subjects were returned to their home cages in which standard chow diet and environmental enrichment had been reinstated. Food pots were re-weighed, accounting for any spillage, and the test food intake calculated. During each session, two control food pots (positioned adjacent to the test arena) were used to assess loss of food mass simply through evaporation: these measurements confirmed minimal evaporation loss. Body weights were then tracked for either 3 or 7 days (dependent upon experimental design) to determine whether there were any prolonged acute treatment effects on weight gain.

3.8.4 Error Protocol

Any errors occurring throughout the 10 experiments underwent the same error protocol: (i) If the error was identified before the end of an experiment, the individual animal was re-run on its allocated day in the week following the predicted end of study; (ii) If the error was identified after the end of an experiment, the data points for the specific dose for that individual animal were statistically calculated using mean substitution; replacing missing data points with the mean of the variables within that dose condition. This procedure avoided removal of the entire dataset for an individual animal. This would have been unethical, and would have reduced the number of subjects thus increasing variance in the dataset.

3.8.4.1 Types of Error

For specific details refer to individual experimental chapters.

3.8.4.1.1 Electronic Failure

Cases where any electronic equipment malfunctioned during data collection; e.g. a DVD player error or lighting fault.

3.8.4.1.2 Animal Illness

Cases where an animal exhibited unusual behaviour before, during, or following testing; e.g. high levels of resting or awkward walking.

3.8.4.1.3 Data loss

Cases where data points were lost due to a computer or DVD error.

3.9 Behavioural Analysis

DVDs were scored blind by a highly trained observer (intra-rater reliability > 0.8), using ethological analysis software (‘Hindsight’; Weiss, 1995) that permits real-time scoring of behaviour via direct keyboard entry to a PC. The continuous monitoring methodology used, to record the duration and frequency of behaviour throughout the 1h test session, is particularly time consuming. The scoring of test DVDs for the whole thesis required a total of 480 hours (Experiments 2, 3, 4, 5, 7 and 9: 40hours each; Experiments 1, 6, 8 and 10: 60hours each). Although this is a labour-intensive method, the alternative options such as time-sampling techniques are associated with observer bias and typically overestimate the true duration and frequency of behaviour (Halford et al., 1998). Based on previous research (for recent review see: Rodgers et al., 2010), measures recorded from DVD were: latency to locate food source, and latency to eat (see Table 3-3), together with the frequency and duration of the following behaviours: eating; drinking; grooming; scratching; sniffing; locomotion; rearing; and resting (see Table 3-2 and Figure 3-5). It should be noted that, in all studies, levels of drinking were generally very low, undoubtedly due to the use of hydrated test food; as such, these data are not reported. Two further measures of feeding behaviour were derived from the recorded parameters: average duration of eating bouts, and average eating rate.

In addition to analysing treatment effects on total scores, each 60-minute test period was divided into 12 x 5-min timebins, thus permitting analysis of treatment effects on behaviour over the session. For these timecourse analyses, specific attention was paid to the behavioural satiety sequence (BSS), i.e. the temporal relationship between eating, grooming, and resting (Rodgers et al., 2010).

Table 3-2: Definitions of behavioural variables scored during the one-hour test session

Adapted from Tallett (2009)

Behaviour	Description
Eating	Biting, gnawing, or swallowing wet mash from food pot or directly from front paws.
Grooming	Licking of the body, feet and genitals. Stroking whiskers with paws. Biting of tail.
Scratching	Use of the hind legs to scratch the coat or head.
Locomotion	Walking around the cage or circling; movements that involve all four limbs.
Rearing	Front paws raised from the tank floor, either supported against the tank wall or free standing in front of the body.
Sniffing	Rapid wrinkling of the nose (twitching of vibrissae) at an aspect of the environment. Head movements with rear limbs immobile.
Resting	Lying in a relaxed position with head curled to the body or resting n the bottom of the tank; animal inactive.
Drinking	Licking the spout of the water bottle.

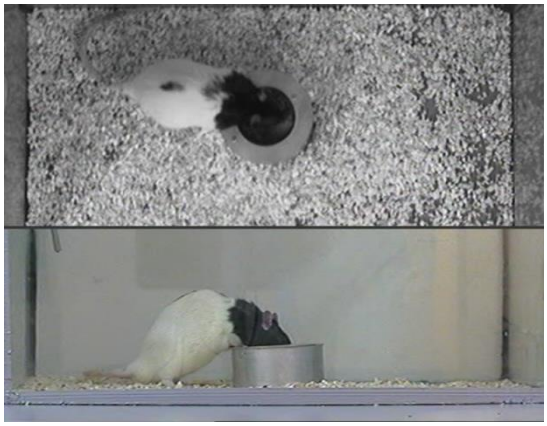
Table 3-3: Definitions of additional feeding variables scored during the one hour test session

Adapted from Tallett (2009)

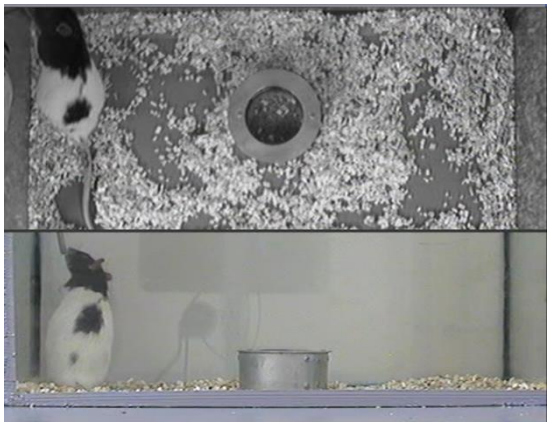
Measure	Description
Food Intake (g)	The calculated difference between pre-test and post-test food pot weight, accounting for spillage
Eat Bout Length (s)	Average duration of feeding bout calculated by division of total eating duration (s) by total eating frequency
Eat Rate (g/minute)	Average eating rate calculated by division of total food intake (g) by total eat duration (m)
Food Identification Latency (s)	Time in seconds from the start of the test session to first contact with the food pot
Eat Latency (s)	Time in seconds from the identification of the food source to the first bout of feeding

Figure 3-5 (overleaf): Scored Behaviours

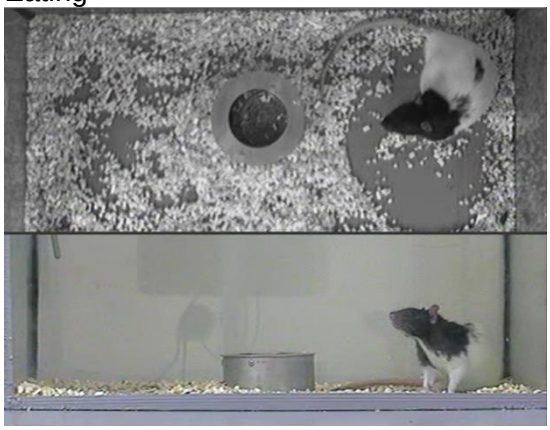
From top left to bottom right; overhead and side views of eating, drinking, sniffing, rearing, locomotion, resting, scratching and grooming.



Eating

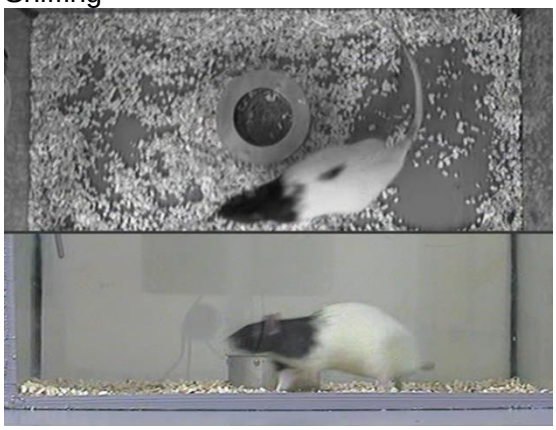


Drinking

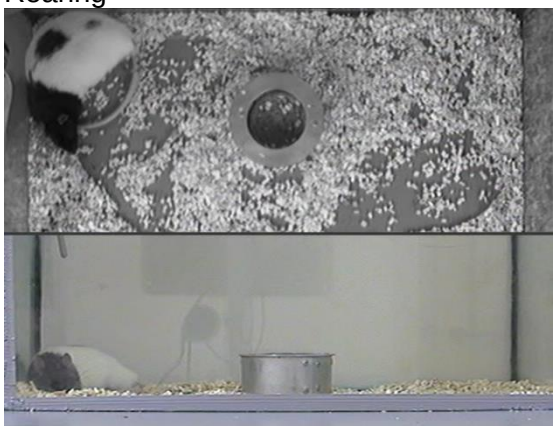


Sniffing

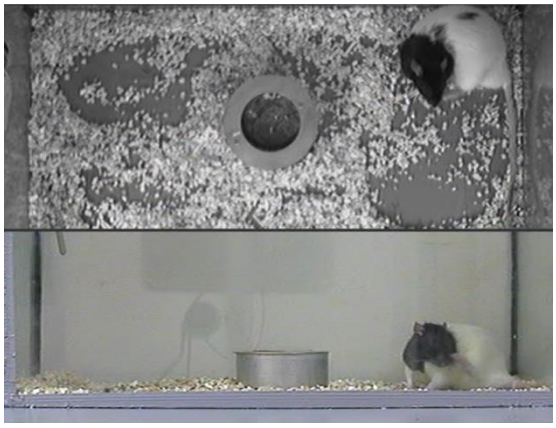
Rearing



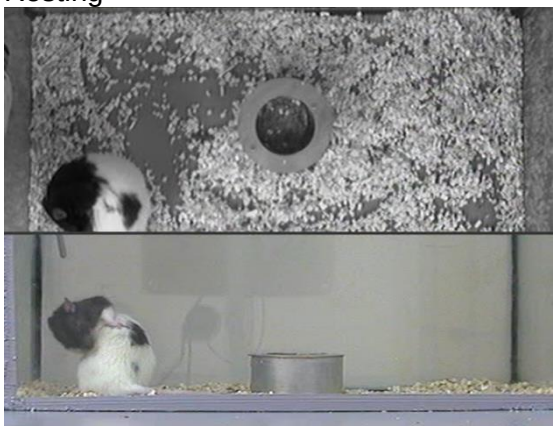
Locomotion



Resting



Scratching



Grooming

3.10 Statistical Analysis

Following Hindsight scoring, data were transferred into Microsoft Excel where it could be suitably organized for export into SPSS (Statistical Package for the Social Sciences; v16-20) for statistical analysis. For combination studies (Experiments 1, 2, 3, 6, 8 and 10) Bonferroni post-hoc analyses were conducted in STATISTICA 7 (StatSoft, Tulsa, USA). In all cases, where datasets failed Mauchly's Test of Sphericity, Greenhouse-Geisser significance levels are reported. For the purposes of brevity, all degrees of freedom reported within the text refer to the 'sphericity assumed' values. The Greenhouse-Geisser altered degrees of freedom can be found in the relevant appendices. Findings were accepted as significant when $p \leq 0.05$

3.10.1 Habituation Phase Data

To confirm the development of stable levels of intake before commencement of the experimental phase, food intake during the habituation phase was analysed using a one-way repeated measures ANOVA (analysis of variance), followed by Bonferroni comparisons.

3.10.2 Experimental Phase Data

The raw data produced by Hindsight was organised by treatment condition before drug effects on one-hour food intake and one-hour behavioural measures (frequency and duration) were analysed. One-way repeated measures ANOVAs were used for dose-response studies. Combination studies underwent two-way repeated measures ANOVA. In each case, significant ANOVAs were followed by the appropriate Bonferroni comparisons.

To analyse changes over time, the 12 x 5 minute timebins for each behaviour (frequency and duration) were subject to repeated measures ANOVA: treatment x time, for dose-response studies, and treatment x treatment x time, for combination studies. Any significant treatment x time interactions were followed up by one-way ANOVAs for each timebin. Where the data failed Mauchly's test of sphericity, corrected Greenhouse-Geisser values are reported.

3.10.3 Bodyweight Data

Treatment effects for test day bodyweight, and 7-day absolute weight gain (3-day absolute weight gain where necessary) were analysed by one-way repeated measures ANOVA for dose response studies, and two-way repeated measures

ANOVA for combination studies. Percentage bodyweight gain over the 7 day post-treatment (3-day where necessary) were analysed by two-way repeated measures ANOVA (drug x day) for dose-response studies. For combination studies three-way repeated measures ANOVA was used. Significant interactions were initially explored using one-way ANOVA (two-way ANOVA for combination studies) for each day followed by, where significant, Bonferroni tests.

3.11 Data Reporting

Statistical details from individual experiments in the current thesis will be reported using a common format (see Table 3-4). At the end of each Chapter, the main findings from the constituent experiments will be briefly outlined in bullet point format. The main discussion issues from each empirical chapter are reserved for Chapter 8.

Table 3-4: An outline of the template used to report experimental results.

n/a: not applicable

Heading	Purpose	Figures and Tables
Habituation Phase Food Intake	To confirm the development of stable intake prior to experimental testing	n/a
Test Day Bodyweight	To confirm the comparability of bodyweight across treatment conditions	n/a
Test Day Food Intake	To emphasise minimal evaporation loss, and outline treatment effects on food intake	A figure will summarise the effect of acute treatment on mash intake
Total (one-hour) Behavioural Analyses	To outline treatment effects on feeding related parameters (Table 3-3) as well as frequencies and durations of the behavioural variables (Table 3-2)	A table will summarise the acute effects of treatment on eating-related parameters and a figure will show the effects of treatment on the duration (upper panel) and frequency (lower panel) of ingestive and non-ingestive behaviours
Timebin Behavioural Analyses	To confirm expected behavioural time effects and outline main effects and/or interactions of behaviours within specific timebins	A figure will illustrate the effects of treatment on timecourses for any relevant behaviours.
Behavioural Satiety Sequence (BSS)	To visually assess the eat-to-rest transition and the structural integrity of the BSS following treatment	A figure will show effects of treatment on the BSS
Bodyweight Gain	To confirm normal growth patterns and reveal any post-treatment effects on bodyweight gain	n/a
Summary of Main Findings	To summarise the findings and conclusions	n/a

Chapter 4 Cannabinoid and Opioid System Interactions

4.1 The Cannabinoid System

The past decade has witnessed substantial advances in our understanding of the neurobiology of appetite and energy homeostasis (Halford, Boyland, Blundell, et al., 2010; Kennett, 2010; Rodgers et al., 2012; Vickers et al., 2011). Of major significance in this regard is the endocannabinoid system, with particular emphasis on the anti-obesity potential of cannabinoid CB1 receptor antagonist/inverse agonists such as rimonabant (for reviews see: Cota et al., 2006; DiMarzo, 2008; Kirkham, 2009; Marco et al., 2012).

The cannabinoid system is a lipid signalling system (Bermudez-Silva et al., 2010; Matias & Di Marzo, 2007), based on several endogenous cannabinoids including anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG; Sugiura et al., 1995). Anandamide and 2-AG act at two cannabinoid receptor subtypes CB1 (Devane et al., 1988; Matsuda et al., 1990) and CB2 (Howlett, 2002; Munro et al., 1993), both of which are distributed widely throughout brain (including the olfactory bulb, hippocampus, amygdala, thalamic nuclei, cerebellar cortex, and brainstem) and periphery (Piomelli, 2003). Although CB1 receptor expression in the hypothalamus is surprisingly low, the presence of the cannabinoids in this region nonetheless suggests important functions relating to appetite regulation (Marsicano & Lutz, 2006). Peripherally CB1 receptors are present in adipose tissue, liver cells, skeletal muscle and the pancreas (Cota, Marsicano, Lutz, et al., 2003; Osei-Hyiaman et al., 2005). CB2 receptors are also peripherally located and primarily play a role in immunological functions (Howlett, 2002).

4.1.1 Cannabinoids & Appetite Regulation

It has long been established that cannabis administration increases food intake (Abel, 1975). The active ingredient, Δ^9-tetrahydrocannabinol (THC), is responsible for this action and has been shown to increase feeding both in animal models (Brown et al., 1977; Koch, 2001; Williams et al., 1998) and humans (Foltin et al., 1988; Hart et al., 2002; Hollister, 1971). Similarly, the administration of endocannabinoids has also been shown to produce hyperphagia (Hao et al., 2000; Jamshidi & Taylor, 2001; Kirkham et al., 2002; Williams & Kirkham, 1999).

These cannabinoid effects on food intake are mediated via the CB1 receptor. Thus, THC-induced hyperphagia is reversed by CB1 (but not by CB2) receptor

antagonists (Williams & Kirkham, 2002). Similarly, CB1 KO mice exhibit a lean and hypophagic phenotype (Di Marzo et al., 2001; Ravinet Trillou et al., 2004). Furthermore, research has repeatedly confirmed that selective CB1 receptor antagonist/inverse agonists, such as rimonabant (SR141716A; Rinaldicarmona et al., 1994), dose-dependently reduce intake of a variety of diets (see below).

4.1.2 Cannabinoid Receptor Antagonists & Appetite Regulation

Rimonabant, the first selective CB1 receptor antagonist/inverse agonist (Rinaldicarmona et al., 1994) has been found to significantly and dose-dependently decrease intake of sucrose (Freedland et al., 2001) and ethanol (Higgs et al., 2003; see Section 4.1.2). It has therefore been suggested as a target for the treatment of alcohol, nicotine and marijuana abuse (Colombo et al., 2007; Le Foll & Goldberg, 2005). However, evidence has also shown reductions in normal lab chow consumption (Colombo et al., 1998; De Vry & Jentzsch, 2004; Freedland et al., 2001; Gomez et al., 2002; Hildebrandt et al., 2003; Hodge et al., 2008; McLaughlin et al., 2006; McLaughlin et al., 2003; McLaughlin et al., 2005; Rowland et al., 2001; Simiand, Keane, Keane, & Soubrié, 1998; Thornton-Jones, 2005; Verty, McGregor, et al., 2004a; Verty et al., 2003; Vickers, Webster, et al., 2003; Werner & Koch, 2003; Wiley et al., 2005; Williams & Kirkham, 2002), suggesting that there may be no real difference in effect between different types of test diet (McLaughlin et al., 2003; Verty, McGregor, et al., 2004a).

Other CB1 receptor antagonists have also been found to reduce food intake, including: AM251 (Chambers et al., 2006; Chambers et al., 2004; Chen, Huang, et al., 2004; Hildebrandt et al., 2003; McLaughlin et al., 2003; McLaughlin et al., 2005; Rutkowska, 2004; Shearman et al., 2003; Tallett et al., 2007a); AM281 (Werner & Koch, 2003); AM1387 (McLaughlin et al., 2006); LH21 (Pavon et al., 2006); taranabant (MK-0364; Addy et al., 2008; Fong et al., 2007); AM4113 (Hodge et al., 2008; Järbe et al., 2008; Sink et al., 2008; Sink et al., 2010; Sink KS, 2009); otenabant, surinabant (SR147778; Lamota et al., 2008), Ibipinabant (SLV319) and LY320135 (see Di Marzo, 2008).

The short-term effects of cannabinoid receptor antagonists on appetite regulation are thought to be mediated centrally in the basal forebrain (Cota, Marsicano, Tschop, et al., 2003; Jamshidi & Taylor, 2001; Kirkham et al., 2002) and peripherally via capsaicin-sensitive, CCK-1 receptor vagal afferents in the stomach and duodenum (Coutts & Izzo, 2004; Gomez et al., 2002). Centrally, the cannabinoid system is thought to regulate the expression of various orexigenic and anorexigenic neuropeptides. In this context, CB1 receptors are co-localised with

CRH in the PVN, MCH in the LH and with pre-pro-orexin in the VMH (Cota, Marsicano, Tschop, et al., 2003). The cannabinoid system has also been found to interact with NPY, with cannabinoid receptor agonists stimulating and cannabinoid receptor antagonists inhibiting NPY expression (Gamber et al., 2005). Additionally, the orexigenic effects produced by NPY can be blocked by rimonabant pre-treatment and by CB1 receptor KO (Di Marzo et al., 2001; Poncelet et al., 2003). However, rimonabant administration in NPY-null mice still produces an anorectic effect (Di Marzo et al., 2001). The lack of co-localisation of the two neuropeptides in the ARC (Cota, Marsicano, Tschop, et al., 2003) suggests that the cannabinoid system acts downstream of NPY. Evidence also demonstrates a central cannabinoid interaction with dopamine (Duarte et al., 2004; Verty, McGregor, et al., 2004b) and opioid (see section 4.3; Chen, Huang, et al., 2004; Rowland et al., 2001; Tallett et al., 2008b, 2009a; Verty et al., 2003; Williams & Kirkham, 2002) systems. Peripherally, evidence suggests that CB1 receptor antagonism is associated with reduced plasma ghrelin levels (Cani et al., 2004) and blockade of ghrelin-induced hyperphagia (Tucci et al., 2004).

Interestingly, significant effects of CB1 receptor antagonist on bodyweight often outlast those seen on food intake, suggesting the development of tolerance to the acute anorectic effect of these agents (Colombo et al., 1998; Vickers, Webster, et al., 2003). CB1 receptor KO mice exhibit resistance to high-fat diets and do not become as obese as their wild-type littermates (Cota, 2003; Ravinet Trillou et al., 2003). These findings suggest that CB1 receptor antagonists may have actions additional to appetite suppression, e.g. increasing energy expenditure (Liu et al., 2005) or acting peripherally on adipocytes to inhibit lipogenesis (Bermudez-Silva et al., 2008; Cota, Marsicano, Tschop, et al., 2003; Osei-Hyiaman et al., 2005; Pagano et al., 2008). Such possibilities are supported by evidence showing that blockade of CB1 receptors increases the levels of adiponectin, an enzyme that blocks lipogenesis and which is therefore inversely associated with adiposity levels (Bensaid et al., 2003; Poirier et al., 2005). It is suggested that rimonabant mediates its effects on bodyweight directly on adipose tissue, potentially via the enhancement of the adiponectin gene (Bensaid, 2003; however see: Thornton-Jones & Clifton, 2006). The peripheral effect of the cannabinoid system on bodyweight is further supported by evidence that CB1 receptor antagonists that cannot cross the BBB have produced similar effects on bodyweight suppression to those seen with brain-penetrant CB1 receptor antagonists (Wu et al., 2011). This suggests a dissociative effect of CB1 receptor antagonists on intake and bodyweight, dependent upon site or sites of action.

Despite the apparent success of CB1 receptor antagonists, they may have a floor effect. The administration of rimonabant (Freedland et al., 2000; McLaughlin et al., 2003), AM251 (Hildebrandt et al., 2003; McLaughlin et al., 2003) and taranabant (Aronne, Tonstad, et al., 2010) has failed, even at high doses, to completely abolish feeding. Whereas fenfluramine and dexfenfluramine can completely abolish lever-pressing for food, the maximum suppression seen with rimonabant and AM251/1387 is only 60-70% (McLaughlin et al., 2006; McLaughlin et al., 2003; McLaughlin, 2010).

4.1.3 Cannabinoids and the Rewarding Aspects of Appetite Regulation

As discussed in Chapter 3 (Section 3.7), food intake can be reduced by drug actions unrelated to the normal physiological regulation of appetite (Halford et al., 1998; Rodgers et al., 2010). In this context, it is pertinent to note that CB1 receptor antagonists also influence food reward and the rewarding effects of drugs of abuse (Carai et al., 2005; Cota et al., 2006), anxiety (Griebel et al., 2005), and learning and memory (Marsicano et al., 2002).

Although, THC increases food intake in humans (Hart et al., 2002), more detailed findings suggest that this is specific to increases in palatable foods (Foltin et al., 1986). Similarly, the CB1 receptor agonist, Arachidonyl-2-chloroethylamide (ACEA), also produces increases in specific macronutrient (carbohydrate) intake (Erick Escartin-Perez et al., 2009). Furthermore, monkeys and mice have been found to self-administer endocannabinoids and cannabinoid receptor agonists, an effect blocked by rimonabant (Justinova et al., 2005; Martellotta et al., 1998). Therefore, it is thought that the cannabinoid system acts, at least in part, to enhance the rewarding aspects of food, thereby increasing the motivation to eat (Kirkham & Williams, 2001). This aspect of cannabinoid-induced hyperphagia is thought to be mediated by dopamine. CB1 receptors are co-localised with dopamine D_2 receptors in the nucleus accumbens (Pickel et al., 2004), and rimonabant attenuates dopamine release following the consumption of palatable food (Melis et al., 2007).

Reciprocally, CB1 receptor antagonist/inverse agonists, such as rimonabant, have been found to selectively reduce the intake of palatable foods (Arnone et al., 1997; Simiand, Keane, Keane, & Soubrie, 1998). This effect is thought to be a result of reduced palatability (exemplified with sucrose licking) and reward value (exemplified with break points; Maccioni et al., 2008; Rasmussen & Huskinson, 2008; Sanchis-Segura et al., 2004; Ward et al., 2008), rather than induction of satiation (Higgs et al., 2003).

4.1.4 Side-Effects of CB1 receptor Antagonists

4.1.4.1 Scratching and Grooming (Pruritus)

It had been established that changes in feeding produced by cannabinoid receptor antagonists are not due to sedation (Gardner & Mallet, 2006; Jarbe et al., 2006; McLaughlin et al., 2005). However, nausea and pruritus (an itching syndrome; Hodge et al., 2008; Tallett et al., 2007b, 2007c) were found in preclinical testing in rodents.

Of particular relevance to the present thesis is the observation that CB1 receptor antagonists reliably induce cannabinoid withdrawal symptoms (Aceto et al., 1996; Cook et al., 1998; Navarro et al., 1997; Rubino et al., 1998), and/or motor effects (Bass et al., 2002; Compton et al., 1996; Pavon et al., 2006). More specifically, this can be characterised as a syndrome of compulsive grooming and scratching in rodents (e.g. Janoyan et al., 2002; Jarbe et al., 2002; Jarbe et al., 2003; Jarbe et al., 2004; Jarbe et al., 2006; Tallett et al., 2007a, 2007b, 2007c). In humans, this itching of the skin is known as pruritus and was reported in human clinical trials as a significant adverse effect both of rimonabant (MHRA, 2008b) and taranabant (Addy et al., 2008; Aronne, Tonstad, et al., 2010). In fact, intense scratching/grooming is even seen in rodents treated with the newer neutral CB1 receptor antagonist (see later, Section 4.1.3.3) such as AM4113 (Hodge et al., 2008; Järbe et al., 2008). The latter finding implies that not all of the adverse effects of rimonabant and similar agents can be attributed to their inverse agonist properties. Interestingly, rimonabant-induced scratching is not seen following intracerebroventricular administration (Schlosburg et al., 2011), suggesting a peripheral site of action.

Although the grooming and scratching syndrome is now a well-established feature of rimonabant (Schlosburg et al., 2011; Tallett et al., 2007b; Ward et al., 2008) and AM251 (Hodge et al., 2008; Tallett et al., 2007a) treatment, it was infrequently reported in feeding studies with this class of agent. Recently, work from The University of Leeds showed that the CB1 receptor antagonist/inverse agonist-induced scratching and grooming severely disrupts the BSS in rats, leading to the suggestion that the acute anorectic effect of such agents may simply be due to response competition (Tallett et al., 2007a, 2007b). However, this hypothesis has been challenged by Hodge and colleagues (2008) whereby the pattern of drug-induced grooming was yoked to forced locomotion in an un-drugged group of rats fed in a modified running wheel. As this particular form of disruption did not affect food intake, it was concluded that simple response competition could not account for the anorectic response to CB1 receptor antagonist/inverse agonists. However, it

is debatable whether locomotion-induced disruption is equivalent to the behavioural disruption caused by continuous drug-induced itching, scratching and grooming. An alternative approach to testing the response competition hypothesis would be to identify a means of attenuating the scratching/grooming syndrome and to assess the effects of this manipulation on food intake and feeding behaviour.

4.1.5 Therapeutic potential for the Treatment of Obesity

4.1.5.1 Rimonabant

In addition to the extensive preclinical work with rimonabant and related compounds, CB1 receptor antagonist/inverse agonist administration has been shown to reduce food intake, bodyweight gain, waist circumference, cardiovascular risk factors and hunger ratings in humans over periods of up to 12 weeks (Despres et al., 2009; Heshmati et al., 2001; Scheen et al., 2006; Van Gaal et al., 2005).

More specifically, the preclinical data on rimonabant was confirmed in obese humans by four separate phase III clinical trials known as; the Rimonabant in Obesity (RIO) - Europe (Van Gaal et al., 2005), RIO – Lipids (Despres et al., 2005), RIO – Diabetes (Scheen et al., 2006) and RIO – North America (Pi-Sunyer et al., 2006) trials. All four studies produced similar results indicating that, after 1-year of treatment, >60% of patients achieved weight loss of ≥5%, and >30% of patients achieved weight loss of ≥10%. However, five meta-analyses of these clinical trials brought to light major safety concerns regarding rimonabant treatment (Chavez-Tapia et al., 2009; Christensen et al., 2007; Curioni & Andre, 2006; Johansson et al., 2009).

4.1.5.2 Depression, Anxiety and Suicidal Thoughts

On top of the already increased risk of depression in obese patients (Luppino et al., 2010), the most common complaint from patients undergoing rimonabant treatment was depression (Johansson et al., 2009; Nissen et al., 2008; see Serra & Fratta, 2007). In fact, CRESCENDO, a rimonabant cardiovascular trial, was halted due to five suicides (Topol et al., 2010). Furthermore, pooled data from a number of trials revealed that while on rimonabant, the risk of depression is double that of the placebo group (Rumsfeld & Nallamothu, 2008). Considering the ‘reward’ components of rimonabant anorexia and the role of the cannabinoid system in motivated behaviours, such as sex and social interaction (Melis et al., 2007), it is unsurprising that rimonabant is found to block reward and lead to mood disorders such as depression (Bermudez-Silva et al., 2010).

Additionally, 1% of patients discontinued rimonabant treatment due to feelings of anxiety (Pi-Sunyer et al., 2006), an effect also seen with taranabant treatment (Proietto et al., 2010). Interestingly, administration of CB1 receptor agonists in animals has been found to exert both anxiogenic (anxiety inducing; Arevalo et al., 2001; Navarro et al., 1997; Onaivi et al., 1990) and anxiolytic (anxiety inhibiting; Haller et al., 2004; Kinsey et al., 2011; Patel & Hillard, 2006; Sciolino et al., 2011) actions. Similarly, CB1 receptor antagonist/inverse agonists have also been found to exert both anxiogenic (Rodgers et al., 2005) and anxiolytic actions (Degroot & Nomikos, 2004; Rodgers et al., 2003). These conflicting findings may relate to variations in dose level and hence the affected neural circuits.

4.1.5.3 Second Generation CB1 Antagonists

Enthusiasm for CB1 receptor antagonist/inverse agonists as a potential obesity treatment waned as a result of the 2008 suspension (psychiatric risk) of marketing authorisations for Acomplia® (rimonabant; see Chapter 2), and the subsequent termination of several commercial CB1-receptor drug development programmes, including Merck abandoning the development of alternative CB1 receptor antagonists such as taranabant (Aronne, Tonstad, et al., 2010). Nevertheless, a number of findings would support retention of the CB1 receptor as a molecular target relevant to the regulation of appetite and energy homeostasis (Bermudez-Silva et al., 2010; Kunos et al., 2009; McLaughlin, 2012; Rodgers et al., 2012; Vemuri et al., 2008). These include growing evidence that: **(i)** neutral or 'silent' CB1 receptor antagonist (such as AM4113) retain the anorectic and weight loss advantages of rimonabant but with an improved side-effect profile, and **(ii)** peripherally-restricted CB1 receptor antagonists (such as AM6545, LH-21, MJ15, URB 447) significantly reduce both intake and weight gain, thereby possibly avoiding the psychiatric complications associated with CNS-penetrant compounds. Additional hope rests in related research avenues including the development of CB1 receptor partial agonists, allosteric modulators of CB1 receptors, and agents that alter endocannabinoid levels (Bermudez-Silva et al., 2010).

Furthermore, recent work has emphasised the positive effect of rimonabant in producing metabolic improvements such as decreased coronary artery atherosclerosis (Nissen et al., 2008), improved glycemic control (Rosenstock et al., 2008), and reduced accumulation of intra-abdominal fat and liver fat (Despres et al., 2009). Such findings suggest that rimonabant may still be a beneficial obesity treatment with improved patient selection.

4.1.5.3.1 Neutral CB1 Receptor Antagonists

Drugs such as rimonabant are inverse agonists/antagonists at the CB1 receptor; this means that in addition to blocking the receptor site, the inverse agonists simultaneously induce a pharmacological response opposite to that of an agonist (see Figure 4-1). The poor side-effect profile of rimonabant is thought to be related to blocking the constitutive CB1 activity. On the other hand, the use of neutral CB1 receptor antagonist that lack intrinsic activity at the receptor (Pertwee, 2005), provide the potential for weight loss without the adverse side-effects (Greasley & Clapham, 2006).

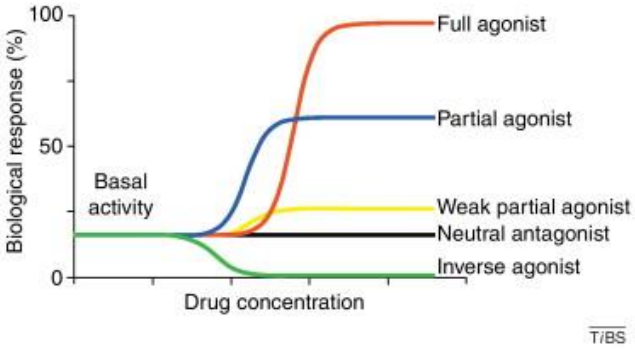


Figure 4-1: Diagram demonstrating the pharmacological effects of different ligands.

Full agonist: a ligand that binds to and activates a receptor and elicits a physiological response; Partial agonist: ligand that elicits only a partial response when compared to a full agonist; Neutral Antagonist: any ligand that blocks binding of endogenous agonists to the receptor; Inverse agonist: a ligand that binds to a receptor and inhibits the basal or constitutive activity of a receptor. Source: Tate (2012)

These include: O-2050 (Gardner & Mallet, 2006; Higuchi et al., 2010); LH-21 (Alonso et al., 2012; Chen et al., 2008; Pavon et al., 2006); AM4113 (Cluny et al., 2011); PSNCBAM-1 (Horswill et al., 2007; Wang, Horswill, et al., 2011); and NESS 0327, a rimonabant analogue (Ruiu et al., 2003).

Generally, it is considered that these ‘neutral’ antagonists exhibit a safer side-effect profile. For example, neutral CB1 receptor antagonist, AM4113, does not induce nausea (Järbe et al., 2008; Salamone et al., 2007). However, as previously mentioned, some of the newer CB1 receptor antagonist (AM4113 and AM251) still induce pruritus (Hodge et al., 2008; Järbe et al., 2008). Furthermore, they may not be as effective as appetite suppressants. For example, O-2050 fails to block the hyperphagic effects of THC (Wiley et al., 2011). Since LH-21 is able suppress intake in CB1 knockout mice (Chen et al., 2008), some of these newer compounds may not be selective CB1 receptor antagonists but, instead, elicit their effects on food intake via a different system.

4.1.5.3.2 Peripherally-restricted CB1 Receptor Antagonists

In view of the psychiatric nature of the risks associated with drugs like rimonabant, an alternative route would be to target CB1 receptor antagonists that cannot cross the BBB. Peripherally-restricted CB1 receptor antagonists thus far developed include: MJ15 (Chen et al., 2010); URB-447 (DiPatrizio et al., 2011; Ward & Raffa, 2011); AM6546 (Cluny et al., 2010; Randall et al., 2010) and LH-21, described as a poor CNS penetrant (Alonso et al., 2012; Pavon et al., 2006). These compounds elicit a similar effect on bodyweight to that seen with rimonabant, supporting the hypothesis that the bodyweight effects of these drugs are peripherally mediated. However, it is interesting to note that these compounds have failed to produce comparable effects on metabolic parameters (Pavon et al., 2006).

4.1.5.3.3 Other CB1 Receptor Strategies

Alternative CB1-related strategies include the use of CB1 receptor partial agonists and manipulation of endocannabinoid levels. Partial agonists can also act as partial antagonists when levels of endogenous agonists are elevated, as seen with endocannabinoids and obesity (Matias & Di Marzo, 2007). Therefore, by blocking the elevated levels of obesity-induced endocannabinoids, such compounds could potentially prevent the psychiatric side effects. Similarly, drugs that target the enzymes responsible for synthesis and degradation of endocannabinoids may counteract the dysregulation. Target enzymes include: NAPE-PLD, for the production of anandamide; DAGL, for the production of 2-AG; FAAH for the breakdown of both endocannabinoids; and MAGL, for the breakdown of 2-AG (Di Marzo, 2008; DiMarzo, 2008; DiMarzo, 2009). Recent developments include O-5596, which inhibits the biosynthesis of 2-AG and has been found to decrease food intake in mice (Bisogno et al., 2009).

The final option available is an adjunctive strategy (Pertwee, 2009). As discussed in Chapter 2, the combination of two drugs at sub-anorectic dose levels can significantly improve the side-effect profile compared to the individual compounds.

4.1.5.4 Potential Combinations (preclinical)

Although there are no current cannabinoid combinations in clinical testing, known cannabinoid interactions with other systems, including the serotonergic and opioid systems (see later, Section 4.3), have encouraged preclinical interest in cannabinoid combinations.

The co-localisation of CB1 receptors and 5-HT transporters in the brain (Ashton et al., 2006; Hermann et al., 2002) suggests the existence of potentially important cannabinoid-serotonergic interactions. In this context, CB1 receptor agonists

reduce 5-HT expression (Molina-Holgado, 1993; Nakazi et al., 2000; Sagredo, 2006) through binding to 5-HT receptors (Kimura, 1998), while CB1 receptor antagonism increases serotonin release (Tzavara et al., 2003). In feeding research, CB1 receptor-induced alcohol intake is prevented by 5-HT_{1A} receptor blockade (Kelai, 2006). These findings would suggest that the administration of CB1 receptor antagonist with compounds that enhance serotonergic activity should produce an enhanced suppression of intake. Co-treatment studies have assessed the effect of rimonabant with serotonergic agents, such as d-fenfluramine (a serotonin releaser; Rowland et al., 2001), *m*CPP (serotonin 2c receptor agonist; Ward et al., 2008), and sibutramine (serotonin and noradrenaline reuptake inhibitor; Tallett et al., 2010a). However, each study found differing results (Ward, Synergistic; Rowland, Additive; Tallett, Sub-additive).

In the same vein, evidence has shown that CB1 receptors inhibit the anorectic effects of downstream melanocortin-4 receptors (Verty, McFarlane, et al., 2004). Further investigation confirmed that the combination of sub-anorectic doses of rimonabant and α-MSH synergistically attenuated feeding in rats (Verty, McFarlane, et al., 2004). It is also known that leptin administration decreases cannabinoid levels (Di Marzo et al., 2001), whilst leptin deficiency up-regulates CB1 levels (Di Marzo et al., 2001; Thanos et al., 2008). Furthermore, CB1 receptor KO mice display an increased sensitivity to leptin (Ravinet Trillou et al., 2004). These data suggest that the co-administration of leptin and CB1 receptor antagonists has potential for synergy in the treatment of obesity and related co-morbidities such as diabetes (Tam et al., 2012).

4.1.6 Summary; The Cannabinoid System

Cannabinoid CB1 receptor antagonist/inverse agonists, such as rimonabant, suppress food intake and weight gain in both rodents and humans. However, a variety of side-effects, such as mood disorders and pruritus, have thus far undermined full clinical exploitation of the therapeutic potential of such agents. Nevertheless, a range of novel CB1 research strategies is currently being explored, including drug polytherapy.

4.2 The Opioid System

There are four families endogenous opioid peptides; β-endorphin, enkephalin, dynorphin and endomorphin (Mansour et al., 1988; Snyder, 1977; Waldhoer et al., 2004), derived respectively from the prohormones; POMC, proenkephalin, prodynorphin. The precursor to endomorphin has yet to be identified.

There are now known to be multiple opiate receptor sites (mu: μ; delta: δ; kappa: κ) throughout CNS and peripheral tissues (Goldstein & Naidu, 1989; Magnan et al., 1982; Raynor et al., 1994). All receptor sub-types are expressed in the VTA, and VMH. Additionally, κ-receptors are expressed in the PVN, and μ-receptors in the LH (Adan, 2013). Interestingly, κ-receptors are expressed on AgRP releasing neurons, while μ-receptors are expressed on POMC neurons, where they act as inhibitory auto-receptors blocking the release of β-endorphin. Therefore, μ-receptor antagonists would increase POMC release (Greenway, Whitehouse, et al., 2009).

4.2.1 Opioid Receptor Antagonists & Appetite Regulation

The role of opioids in appetite (see reviews; Bodnar, 2004; Cooper, 1993; Yeomans & Gray, 2002) was initially highlighted in patients with eating disorders. Underweight anorexic (Kaye et al., 1987) and bulimic patients (Brewerton et al., 1992) exhibit suppressed levels of various endogenous opioid peptides, which normalise upon weight gain.

The administration of plant-derived opioids (Martin et al., 1963) and other opioid receptor agonists (Bodnar, 2004; Grandison & Guidotti, 1977; Leibowitz & Hor, 1982; McCarthy et al., 1981; McKay et al., 1981; Morley & Levine, 1981; Sanger et al., 1981) induce hyperphagia, an effect blocked by opioid receptor antagonists (Sanger et al., 1981). When given alone, broad spectrum antagonists, such as naloxone and naltrexone, significantly inhibits food intake in food-deprived animals (Barbano & Cador, 2006; Brands et al., 1979; Brown & Holtzman, 1979; Carey et al., 1981; Cooper, 1980; Glass et al., 2001; Hadjimarkou et al., 2004; Holtzman, 1974; Kirkham & Blundell, 1984, 1987; Koch & Bodnar, 1994; Lowy & Yim, 1981; Markskaufman & Balmagiya, 1985; Markskaufman & Kanarek, 1981; McCarthy et al., 1981; Sanger & McCarthy, 1981b, 1982). Opioid receptor antagonists also suppress hyperphagic responses to: glucoprivation (Koch & Bodnar, 1994; Ostrowski et al., 1981), stress (Morley et al., 1980; Teskey et al., 1984), stimulation of the LH (Carr & Simon, 1983), and lesions of the VMH (King et al., 1979). The anorectic effects of opioid antagonists are also more pronounced in obese vs. lean animals (Margules et al., 1978; McLaughlin & Baile, 1984) and in satiated vs. deprived animals (Barbano & Cador, 2006; Brown & Holtzman, 1979). Moreover, opioid receptor antagonism is more effective in reducing the intake of palatable foods compared to regular chow (see Section 4.2.2).

Interestingly, selective opioid receptor antagonists, such as: the κ-opioid receptor antagonist, nor-binaltorphamine (Arjune & Bodnar, 1990; Carr et al., 1989); the μ-receptor antagonists, β-funaltrexamine (Arjune & Bodnar, 1990; Ukai & Holtzman,

1988) and GSK1521498 (Giuliano, 2012); and the δ-receptor antagonist, naltrindole (Arjune et al., 1991; Jackson & Sewell, 1985a), elicit differential effects on feeding. Evidence suggests that there may be a more dominant role for μ-receptors. For example, μ-receptor KO mice display a diminished anorectic response to naloxone/naltrexone (Zhang et al., 2006), and μ-receptor antagonism elicits a similar potency to general opioid antagonism (Simone et al., 1985), whereas κ-opioid receptor and δ-receptor antagonists often fail to alter feeding (Koch & Bodnar, 1994).

When assessing macronutrient intake (Koch & Bodnar, 1994; Markskaufman & Kanarek, 1981) and meal patterns (Glass et al., 2001; Kirkham & Blundell, 1987), evidence shows that opioid antagonists only suppress intake following the consumption of food. Therefore, opioid antagonism does not delay the latency to approach or initiate feeding (Beczkowska, Bowen, et al., 1992; Beczkowska, Koch, et al., 1992; Glass et al., 2001; Kirkham & Blundell, 1986; Tallett et al., 2008a) but, instead, elicits its anorectic effects through the acceleration of meal termination (Kirkham & Blundell, 1987) via reductions in eat duration and increased resting behaviour (Kirkham & Blundell, 1984; Tallett et al., 2008a). In other words, opioid receptor blockade elicits the early onset of satiety, and is involved in the maintenance rather than initiation of feeding (Levine & Billington, 2004).

It is generally considered that the opioid antagonist-induced anorexia is centrally mediated (Carr & Simon, 1983; Markskaufman & Kanarek, 1981; Sanger & McCarthy, 1981a). POMC neurons are not only involved in the synthesis of endogenous opioids (Pennock & Hentges, 2011) but also express opioid receptors, activation of which hyperpolarise the neurons and inhibit firing (Ibrahim et al., 2003; Kelly et al., 1990); thus acting as auto-inhibitory receptors. Unsurprisingly then, morphine reduces POMC and α-MSH in the hypothalamus (Wardlaw et al., 1996), while μ- and κ- receptor blockade prevents AgRP-induced hyperphagia (Brugman et al., 2002). Conversely, there is an increase in NPY mRNA following naltrexone treatment (Kotz et al., 1996), and NPY-induced feeding is attenuated by naloxone and naltrexone (Kotz et al., 1995; Rudski et al., 1996; Schick et al., 1991) but not by δ- receptor antagonism (Kotz et al., 1993). Opioids may also produce anorectic effects by blunting melanocortin and oxytocin signalling (Olszewski & Levine, 2007). Naltrexone administration blocks the orexigenic effect of Orexin A (Sweet, 2004), while butorphanol (a μ- and κ- agonist) reduces c-Fos-positive oxytocin cells in the PVN (Olszewski & Levine, 2007). Conversely, orexin administration increases enkephalin levels in the VTA, PVN and amygdala (Karatayev et al., 2009). Interestingly, galanin hyperphagia is also attenuated by naloxone (Dube et al.,

1994) while μ-receptor, but not κ-receptor, antagonism abolishes galanin-induced hyperphagia (Barton et al., 1996).

Despite this strong support for central mechanisms of action, there is still some evidence for peripheral mediation of opioid-induced anorexia via insulin. For example, insulin administration attenuates the increase in sucrose intake (Sipols et al., 2002) and conditioned place preference (Figlewicz et al., 2004).

4.2.2 Opioids & the Rewarding Aspects of Appetite Regulation

Opioids are thought to be involved in the hedonics of feeding (Berridge, 2000; Cooper, 2004; Hayward & Low, 2007; Yeomans & Gray, 2002). As previously mentioned, opioid receptor antagonism is more effective at reducing the intake of palatable foods compared to regular chow (Apfelbaum & Mandenoff, 1981; Barbano & Cador, 2006; Cleary et al., 1996; Cooper et al., 1985; Cooper & Turkish, 1989; Giraudo et al., 1993; Glass et al., 1999; Glass et al., 2001; Hayward et al., 2006; Islam & Bodnar, 1990; Levine & Billington, 2004; Markskaufman et al., 1984; Sanger & McCarthy, 1981a, 1982), effects thought to be mediated via μ- and κ-receptors. Evidence shows that agonism of these receptors preferentially increases the consumption of energy dense food but not normal chow (Cooper et al., 1985; Zhang et al., 1998). Conversely, the novel selective μ-receptor opioid antagonist, GSK1521498, has been shown to elicit a dual effect on motivation for and hedonic impact of food, supressing intake via reductions in food seeking and binge-eating (reward seeking) behaviours (Giuliano et al., 2012). In overweight patients, this novel agent has been shown to reduce intake and pleasurable responses to high fat/sugar foods (Nathan et al., 2012).

There is an on-going debate (see: Taha, 2010) as to whether opioid receptor agonism increases intake (and opioid antagonism decreases intake) of preferred foods independent of macronutrient intake (Giraudo et al., 1993; Gosnell et al., 1990; Leventhal et al., 1995; Zhang et al., 1998) or if this effect is preferential to fat consumption (Markskaufman & Balmagiya, 1985; Markskaufman et al., 1984; Markskaufman & Kanarek, 1981). On the other hand, some have argued that the opioid system affects feeding driven by sweet taste (Apfelbaum & Mandenoff, 1981; Cooper et al., 1985; Giraudo et al., 1993), as demonstrated with sucrose and saccharin solutions (Beczkowska et al., 1993; Gosnell & Majchrzak, 1989; Kirkham, 1990). However, due to the natural baseline of fat preference, it is important to dissociate fat/sugar content from preferred foods, which can be done by establishing baseline preferences for fat/carbohydrate. Data can be further confounded by total caloric intake; for example, while naltrexone administration

decreases cookie consumption, it actually increases total chow consumption (Cooper & Turkish, 1989).

Interestingly, Glass (2000) found that intake of preferred/non-preferred foods was dependent upon the site of administration. For example, naltrexone administered into the PVN reduced the intake of both foods, potentially targeting energy regulation, whereas, naltrexone administered into the amygdala reduced intake of preferred foods only, targeting hedonic or affective processes. As expected from a compound targeting hedonic feeding, opioid receptor antagonism is also found to decrease animal and human food preferences (Bertino et al., 1991; Fantino et al., 1986; Yeomans & Gray, 2002; Yeomans & Wright, 1991) and reduce sham feeding (Cooper, 2004; Cooper & Kirkham, 1993; Kirkham & Blundell, 1987; Kirkham & Cooper, 1988). This is thought to be mediated via opioid receptor activation stimulating an increase in NAcc dopamine release (Devine et al., 1993; Hirose et al., 2005), thereby increasing food intake and hedonic taste reactivity (Zhang et al., 1998; Zhang & Kelley, 2000), both of which can be blocked by opioid receptor antagonism (Sahr et al., 2008). These findings suggest that opioid-induced food intake is mediated via the mesolimbic dopamine pathway, a perspective strongly supported by the discovery of ‘hot-spots’ for μ-opioid enhancement of taste hedonics in the ventral forebrain (Berridge, 2009; Berridge et al., 2010; Nathan & Bullmore, 2009).

4.2.3 Opioids and Non-Appetite Regulatory Behaviours

In addition to appetite regulation, opioids are involved in a range of psychological and physiological processes including; analgesia, stress, learning and memory, alcohol and drug abuse, sexual activity, mood and mental illness (see recent review: Bodnar, 2012). Of primary interest within the context of this thesis are its effects on mood and pruritus.

4.2.3.1 Pruritus

One of the most common adverse effects of morphine administration in humans is pruritus (Ballantyne et al., 1988; Cousins & Mather, 1984). Conversely, opioid receptor antagonists, such as naloxone and naltrexone, are among the most effective treatments for various human skin disorders, characterised by pruritus (Bernstein et al., 1982; Cies & Giamalis, 2007; Phan et al., 2010; Terra & Tsunoda, 1998). It is interesting the note the efficacy of methyl naltrexone (Friedman & Dello Buono, 2001) and topical naltrexone (Bigliardi et al., 2007) in the treatment of human pruritus, which suggests a peripheral site of action (Schlosburg et al., 2011).

4.2.3.2 Depression and Anxiety

Opioid δ-receptor agonists dose-dependently induce anxiolytic effects, while antagonism or genetic deletion of these receptors produces an anxiogenic profile (Filliol et al., 2000; Perrine et al., 2006). Interestingly, δ-receptor deletion or KO also produces a depressive behavioural profile (Filliol et al., 2000) whereas agonism reduces depression-like behaviour (Broom et al., 2002). This is of particular relevance when considering the therapeutic potential of opioid receptor antagonists for the treatment of obesity, a condition known to already be associated with high levels of depression and anxiety.

4.2.4 Therapeutic Potential for the Treatment of Obesity

4.2.4.1 Given Alone

Naloxone and naltrexone have been shown to acutely suppress intake and cravings in lean, bulimic and obese humans (Atkinson et al., 1985; Cohen et al., 1985; Drewnowski et al., 1995; Fantino et al., 1986; Marrazzi et al., 1995; Trenchard & Silverstone, 1983; Wolkowitz et al., 1988). However, naltrexone is less effective than naloxone in humans (Maggio et al., 1985; Malcolm et al., 1985; Mitchell et al., 1987). This short-term suppressant effect of the opioid antagonists on food intake (~11-29%; Yeomans & Gray, 2002) does not result in a subsequent reduction in bodyweight (Atkinson et al., 1985; Malcolm et al., 1985; Mitchell et al., 1987). This lack of impact on bodyweight parameters means that there is little potential for the therapeutic exploitation of opioid receptor antagonism when administered alone.

4.2.4.2 Given in Combination

Despite the minimal weight loss seen with naloxone alone, the combination of broad spectrum opioid antagonists with other appetite suppressants has produced additive and supra-additive effects on food intake and bodyweight reduction. For example, Contrave™; the combination of bupropion and naltrexone (Greenway Whitehouse, et al., 2009; see Chapter 5), has reached phase III clinical trials. As discussed in Chapter 2, the naltrexone element acts by inhibiting the μ-opioid receptor, augmenting the release of POMC in the ARC and supressing the release of α-MSH, hence increasing the weight loss effect of bupropion (Bray & Greenway, 2007; Greenway, Whitehouse, et al., 2009).

Preclinical animal studies demonstrated a fully additive interaction in lean mice, and a synergistic action in DIO mice (Greenway, Whitehouse, et al., 2009). It is pertinent to note the degrees of drug action varied dependent upon the animal model used (Lalonde et al., 2004; Serrano et al., 2008). However, clinical studies

have suggested a sub-additive effect with the majority of the anorectic action produced by bupropion (Greenway et al., 2010). In 2010, the Contrave® Obesity Research I (COR-I) study was conducted (Greenway et al., 2010). Evidence demonstrates clinically meaningful reductions in bodyweight; 48% of the naltrexone (32mg) plus bupropion group lost 5% of their body weight while 25% lost 10% of their baseline bodyweight (Greenway et al., 2010; Makowski et al., 2011).

Additive and synergistic interactions on food and alcohol intake have also been reported for co-treatment with opioid receptor antagonists and drugs that enhance 5-HT activity (Beczkowska, 1991; Fernandez-Tome, 1988; Gardell, 1997; Hagan et al., 1997; Johnson, 2000; Le & Sellers, 1994; Rezvani et al., 2000). Disappointingly however, a more detailed assessment of feeding behaviours with the sub-anorectic combination of naloxone and sibutramine produced effects that were substantially lower than those predicted on the basis of the sum of the individual drug effects (Tallett et al., 2010b).

There have also been numerous reports of additive and/or supra-additive interaction between sub-anorectic doses of opioid receptor antagonists (naloxone, nalmefene) and CB1 receptor antagonist/inverse agonist (rimonabant, AM251; Chen, Huang, et al., 2004; Pietras & Rowland, 2002; Rowland et al., 2001; Tallett et al., 2008b, 2009a; Williams, 2001; see Section 4.3).

The manipulation of both the satiety and reward systems simultaneously (as discussed in Chapter 2) has the potential to produce additive actions on food intake. Therefore, the opioid antagonist, naltrexone has also been assessed in combination with the GLP-1 agonist, Exendin-4 (Liang et al., 2013; see also chapter 7), with the combination producing a greater suppression of short-term food intake than that seen with either drug individually.

4.2.5 Summary; The Opioid system

Broad spectrum opioid receptor antagonists, such as naloxone and naltrexone, suppress food intake and weight gain in both rodents and humans. It is now generally considered that the opioid system plays a prominent role in the hedonics of feeding. Research also points to the therapeutic potential of opioid receptor antagonists in combination with manipulations of the cannabinoid and 5-HT systems.

4.3 Cannabinoid-Opioid System Interactions

There is substantial evidence for biologically-relevant molecular cross-talk between the endocannabinoid and endogenous opioid systems (Rios et al., 2006; Schoffelmeer et al., 2006). In behavioural research, such interactions have been reported for nociception (Manzanares et al., 1999), drug reward (Tanda & Goldberg, 2003) and appetite (Gallate et al., 1999; Lockie et al., 2011; Skelly et al., 2010; Trojniar & Wise, 1991; Williams & Kirkham, 2002).

4.3.1 System Interactions in Appetite Regulation

Early studies demonstrated that naloxone administration blocked the orexigenic (Trojniar & Wise, 1991; Williams & Kirkham, 2002) and antinociceptive (Pietras & Rowland, 2002) effects of THC. Conversely, rimonabant was found to block morphine-induced hyperphagia (Verty et al., 2003). Evidence that opioid antagonist administration blocks cannabinoid-induced feeding has also been shown in regards to the hedonic aspects of feeding. Naloxone blocks the CB1 receptor agonist-induced increase in breakpoints in operant responding for palatable food (Gallate et al., 1999). Conversely, opioid-induced food-reinforced responding can be blocked by CB1 receptor antagonism (Gallate et al., 1999; Solinas & Goldberg, 2005; Trojniar & Wise, 1991; Verty et al., 2003; Williams & Kirkham, 2002). Similarly, naloxone blocks the ability of CB1 receptor agonists to stimulate alcohol intake (Colombo et al., 2005).

Of primary relevance to this thesis, is the finding that the co-administration of sub-anorectic doses of naloxone and rimonabant (or AM251) suppresses food intake (Kirkham & Williams, 2001; Rowland et al., 2001; Tallett et al., 2008b, 2009a). Additional evidence shows that sub-anorectic co-treatment with nalmefene (another opioid receptor antagonist) and AM251 (Chen, et al., 2004) also produces a synergistic effect on food intake. These reports suggest that there may be potential for the combination of these systems in the treatment of obesity.

4.3.2 System Interactions in Non-Appetite Regulatory Behaviours

4.3.2.1 Reward

Genetic deletion and pharmacological receptor blockade demonstrate that CB1 receptors modulate both opiate reward and motor functions (Braida et al., 2001; Ledent et al., 1999; Navarro et al., 2001) and, conversely, that opioid receptors are implicated in the motivational effects of cannabinoids (Ghozland et al., 2002). Within the NAcc, stimulation of both opioid and cannabinoid CB1 receptors

enhances food reward, whereas their antagonism reduces palatability (Mahler, 2007). This is unsurprising as both systems stimulate dopamine-containing neurons in the VTA (Johnson & North, 1992; Pickel et al., 2004; Rodriguez et al., 2001; Tanda et al., 1997), which may act as the downstream mediator for their hedonic effect on food intake.

4.3.2.2 Scratching and Grooming

Of particular relevance in regards to scratching and grooming are the previously mentioned studies by Tallett et al. (Tallett et al., 2008b, 2009a). The studies explored opioid-cannabinoid system interaction in appetite regulation using combination treatment with sub-anorectic doses of a CB1 receptor antagonist/inverse agonist (rimonabant or AM-251) and the opioid receptor antagonist, naloxone. The combination treatments produced additive effects on food intake and ingestive behaviour. Furthermore, both CB1 receptor antagonists induced significant amounts of scratching (Tallett et al., 2007a, 2007b). However, most interestingly, the authors demonstrated that naloxone markedly attenuate the compulsive scratching response seen in response to low doses of CB1 receptor antagonists.

4.4 Rationale: Chapter Four

The ‘response competition’ hypothesis holds that the acute anorectic response to conventional CB1 receptor antagonist/inverse agonist (e.g. rimonabant, AM-251) is due primarily (if not exclusively) to the induction of an intense scratching and grooming syndrome that interferes with feeding behaviour during tests of finite duration (Tallett et al., 2007a, 2007b).

As scratching and grooming are also features of the newer ‘silent’ or neutral CB1 receptor antagonist (Hodge et al., 2008; Järbe et al., 2008), the aim of studies reported in Chapter 4 was to test a clear prediction of the response competition hypothesis; namely, that low dose naloxone treatment should attenuate both the pruritic and anorectic responses to a moderate dose of rimonabant.

4.5 Experiment One; Rimonabant (1.5mg) and Naloxone (0.1 and 0.01mg/kg) Interaction

4.5.1 Method

All procedures were conducted under Home Office licence in accordance with the UK Animals (Scientific Procedures) Act 1986. For the main methodological details, please refer to Chapter 3 (General Methods).

4.5.1.1 Subjects and Design

10 adult male Lister hooded rats (216.09 ± 2.75g on arrival from Charles River, U.K and 483.00 ± 10.10g by the end of the study) were used. A within-subjects design was adopted whereby each subject received all six treatments according to a Latin Square (with a 7 day wash out period): Vehicle + Vehicle (VV); Vehicle + Naloxone 0.01mg/kg (VNL); Vehicle + Naloxone 0.01mg/kg (VNH); Rimonabant 1.5mg/kg + Vehicle (RV); Rimonabant 1.5mg/kg + Naloxone 0.01mg/kg (RNL); or Rimonabant 1.5mg/kg + Naloxone 0.1mg/kg (RNH).

4.5.1.2 Drugs

Naloxone hydrochloride (Sigma-Aldrich, Poole, UK) was dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. Rimonabant ([N-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide]), kindly donated by Sanofi-Aventis (Chilly-Mazarin, France), was suspended in a small volume of dimethyl sulfoxide (DMSO; Sigma-Aldrich) and subsequently made up to required concentrations in 0.5% methylcellulose (Sigma-Aldrich). Doses of naloxone (0.01 mg/kg and 0.1 mg/kg) and rimonabant (1.5 mg/kg) were selected on the basis of recent dose-response studies in our laboratory (Tallett et al., 2008b and references therein). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg 30 minutes (rimonabant/vehicle) or 15 minutes (naloxone/vehicle) prior to testing.

4.5.1.3 Procedure

Testing occurred over six weeks, with two test days per week and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square

4.5.2 Results

Full statistical details can be found in Appendix 1.

4.5.2.1 Habituation Phase Food Intake

As is typical of the current protocol, mash consumption differed significantly over the course of habituation (trial 1: 10.7 ± 1.5g; trial 2: 14.8 ± 1.4g; trial 3: 16.9 ± 1.5g; trial 4: 17.8 ± 2.1g; trial 5: 19.2 ± 1.8g ($F(4,36) = 11.04$, $p < 0.001$). Trial 1 intake was significantly lower than on trials 3 and 5 ($p \leq 0.05$), and trial 2 intake lower than on trial 5 ($p < 0.01$). The lower intake scores on the first two habituation trials was probably due to environmental novelty. However, the development of stable consumption was confirmed by the lack of significant differences across trials 3-5 ($p > 0.05$), as well as the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (16.56 ± 1.22g).

4.5.2.2 Test Day Bodyweight

Test-day bodyweights were comparable across treatment conditions (VV: 412.6 ± 11.3g; VNL: 412.5 ± 17.0g; VNH: 420.4 ± 17.1g; RV: 421.5 ± 14.3g; RNL: 427.3 ± 14.5g; RNH: 424.6 ± 11.2g (rimonabant: $F(1,9) = 0.65$, $p > 0.05$; naloxone: $F(2,18) = 0.16$, $p > 0.05$; rimonabant x naloxone: $F(2,18) = 0.07$, $p > 0.05$).

4.5.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.30% throughout the experiment (range = 0.13-0.52%). Treatment effects on food intake are summarised in Figure 4-2. There was a significant main effect of rimonabant ($F(1,9) = 58.49$, $p < 0.001$), but no significant main effect for naloxone ($F(2,18) = 2.28$, $p > 0.05$) or a drug interaction ($F(2,18) = 1.46$, $p > 0.05$).

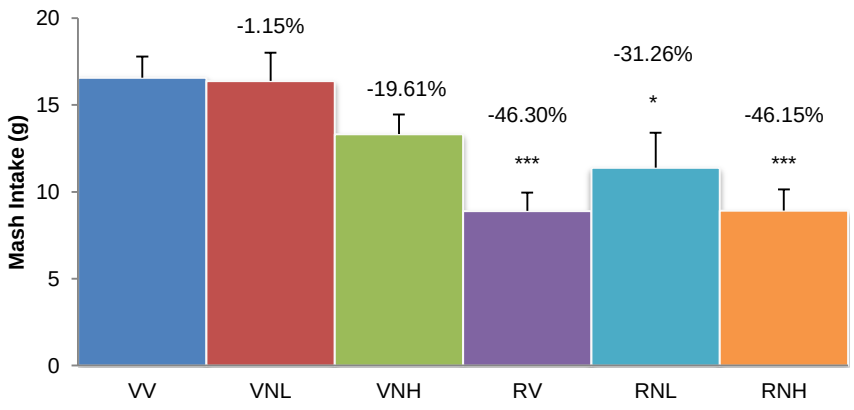


Figure 4-2: Experiment One: Effects of acute rimonabant and naloxone, alone and in combination, on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = vehicle, R = rimonabant 1.5 mg/kg; NL = naloxone 0.01 mg/kg; NH = naloxone 0.1 mg/kg. See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV

Bonferroni comparisons confirmed that, whereas neither dose of naloxone alone altered intake compared with VV control, significant suppression was evident in all conditions receiving rimonabant (RV: $p = 0.001$; RNL: $p = 0.032$; RNH: $p = 0.001$). Despite the apparently reduced anorectic effect of rimonabant when co-administered with the lower dose of naloxone (see Figure 4-2), the fact that RNL remained significantly different from VV, but did not differ from rimonabant given alone (RV), suggests only a weak low dose naloxone attenuation of rimonabant anorexia.

4.5.2.4 Total (one-hour) Behavioural Analyses

Data for eating-related parameters are summarised in Table 4-1, while treatment effects on total 1-h frequency and duration scores for ingestive and non-ingestive behaviours are illustrated in Figure 4-3.

Table 4-1: Experiment One. Acute effects of rimonabant and naloxone, alone and in combination, on eating-related parameters

Data are mean values (± S.E.M). V = vehicle; R = rimonabant 1.5 mg/kg; NL = naloxone 0.01 mg/kg; NH = naloxone 0.1 mg/kg (Mean + SE)

Measure	VV	RV	VNL	VNH	RNL	RNH
Latency to locate food (s)	10.45 ± 2.73	12.43 ± 3.64	7.47 ± 2.49	9.18 ± 3.19	12.98 ± 4.18	6.38 ± 2.24
Latency to eat (s)	22.13 ± 8.64	36.05 ± 15.62	21.64 ± 7.91	24.50 ± 7.81	13.48 ± 2.77	57.48 ± 13.92
Eat bout duration (s)	20.99 ± 3.46	12.88 ± 1.08	20.84 ± 2.91	18.98 ± 1.75	13.94 ± 1.57	14.49 ± 1.87
Eating Rate (g/min)	1.31 ± 0.12	1.37 ± 0.22	1.44 ± 0.23	1.41 ± 0.20	1.86 ± 0.78	1.78 ± 0.48

There were no rimonabant x naloxone interactions for any of the test variables ($F(2,18) \leq 3.42$, $p > 0.05$), although the F-value for groom duration closely approached significance ($p = 0.055$). Significant main effects of rimonabant were revealed for eat bout duration (Table 4-1) as well as the duration (Figure 4-3) of eating and locomotion, and both the frequency and duration of grooming, scratching and sniffing ($F(1,9) \geq 7.27$, $p \leq 0.05$). Significant main effects of naloxone were found only for the frequency and duration of grooming ($F(2,18) \geq 5.45$, $p \leq 0.02$), although the main effect for eat latency closely approached significance ($F(2,18) = 3.51$, $p < 0.052$).

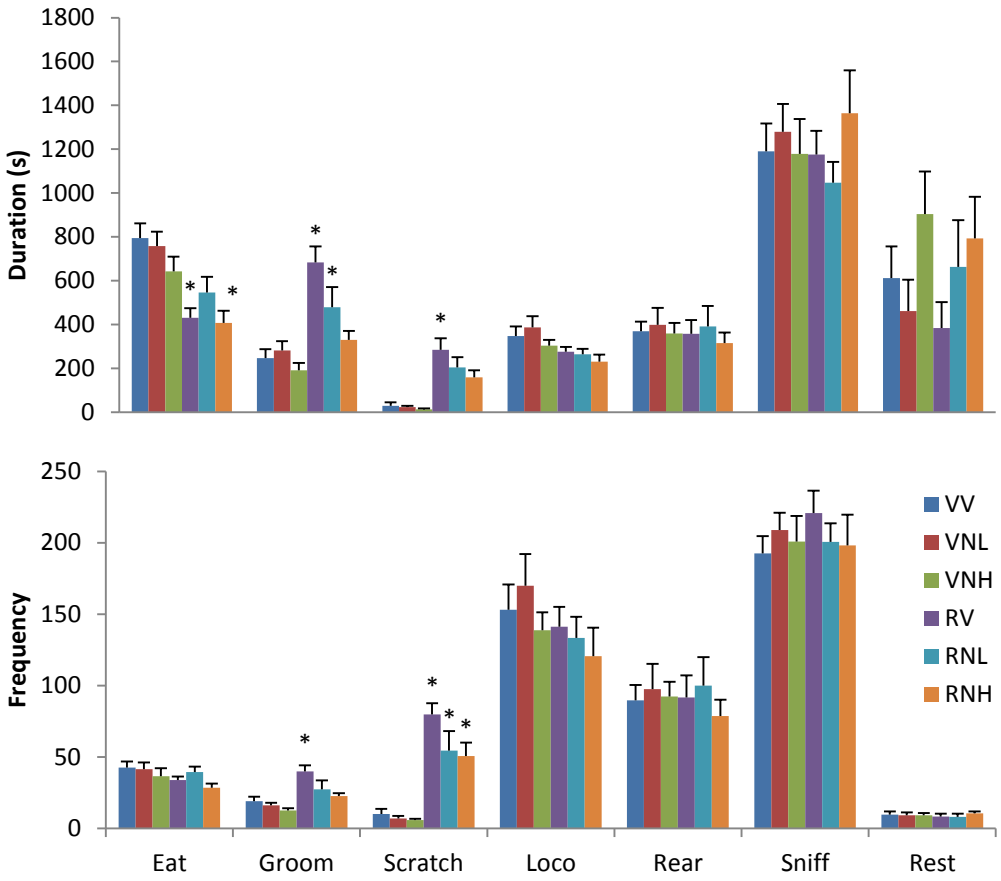


Figure 4-3: Experiment One. Effects of rimonabant and naloxone, alone and in combination, on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = vehicle, R = rimonabant 1.5 mg/kg; NL = naloxone 0.01 mg/kg; NH = naloxone 0.1 mg/kg. See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV

Post-hoc analyses showed that, relative to VV control, neither dose of naloxone alone significantly influenced feeding duration (Figure 4-3). In contrast, rimonabant alone (RV, p = 0.003) and in conjunction with the higher dose of naloxone (RNH, p = 0.001) significantly reduced time spent feeding. Co-administration of rimonabant and the lower dose of naloxone (RNL) just failed to reach an acceptable level of statistical significance (p = 0.076 vs VV) and, since this combination did not differ significantly from either drug given alone, this profile (like that for mash intake) would be consistent with a weak low dose attenuation of rimonabant's inhibitory effect on time spent feeding. As post-hoc analysis of the data for eat bout and locomotion duration failed to reveal any significant differences between drug treatment groups and VV control, the significant main effect for rimonabant most likely reflects relatively minor influences of the compound that emerge only as a

result of large sample sizes. The absence of significant pairwise contrasts for eat latency suggests that the main effect of naloxone on this measure can similarly be attributed to the enhanced power of main effects analysis.

Neither the duration nor frequency of grooming or scratching was significantly altered by either dose of naloxone given alone. However, while the duration of grooming was markedly enhanced by rimonabant given alone (RV vs VV; $p = 0.001$), this measure was not significantly increased when the CB1 receptor antagonist/inverse agonist was given in combination with either dose of naloxone (RNL, RNH). While a lack of difference between RNL and RV would be consistent with partial low dose attenuation of rimonabant-induced grooming, the significant difference between RV and RNH ($p < 0.01$) is indicative of full high dose reversal this aspect of the compulsive syndrome. Although groom frequency was also significantly increased only by rimonabant given alone (RV vs VV; $p < 0.02$, incomplete naloxone attenuation was suggested by the lack of difference between rimonabant alone and when given together with either dose of naloxone (RNL, RNH). The duration of scratching was significantly increased by rimonabant given alone ($p = 0.001$) and in combination with the lower ($p = 0.03$), but not higher, dose of naloxone. Again, however, the absence of a significant difference between RV and either RNL or RNH would support a pattern of partial attenuation. Naloxone had a lesser effect on the frequency of rimonabant-induced scratching which, despite a clear dose-dependent trend towards naloxone attenuation (Figure 4-3), remained elevated relative to VV control in all conditions receiving rimonabant (RV: $p < 0.001$; RNL: $p < 0.03$; RNH: $p < 0.05$).

4.5.2.5 Timebin Behavioural Analyses

ANOVA revealed a strong main effect of time for all behavioural measures ($F(11, 99) \geq 1.98$, $p \leq 0.04$), confirming the characteristic temporal decline in active behaviours and increase in inactive behaviours associated with this paradigm (Rodgers et al., 2010). There were no significant three-way interactions (rimonabant x naloxone x time; $F(22,198) \leq 1.48$, $p > 0.05$) or any significant naloxone x time interactions ($F(22,198) \leq 1.21$, $p > 0.05$). However, significant rimonabant x time interactions were found for the duration of eating and locomotion, as well as the frequency and duration of grooming, scratching and rearing ($F(11,99) \leq 1.98$, $p \leq 0.05$). Although the rimonabant x time interactions for other measures failed to reach significance ($F(11,99) \leq 1.19$, $p > 0.05$), the F-value for locomotion frequency closely approached significance ($F(11,99) = 1.87$, $p = 0.053$).

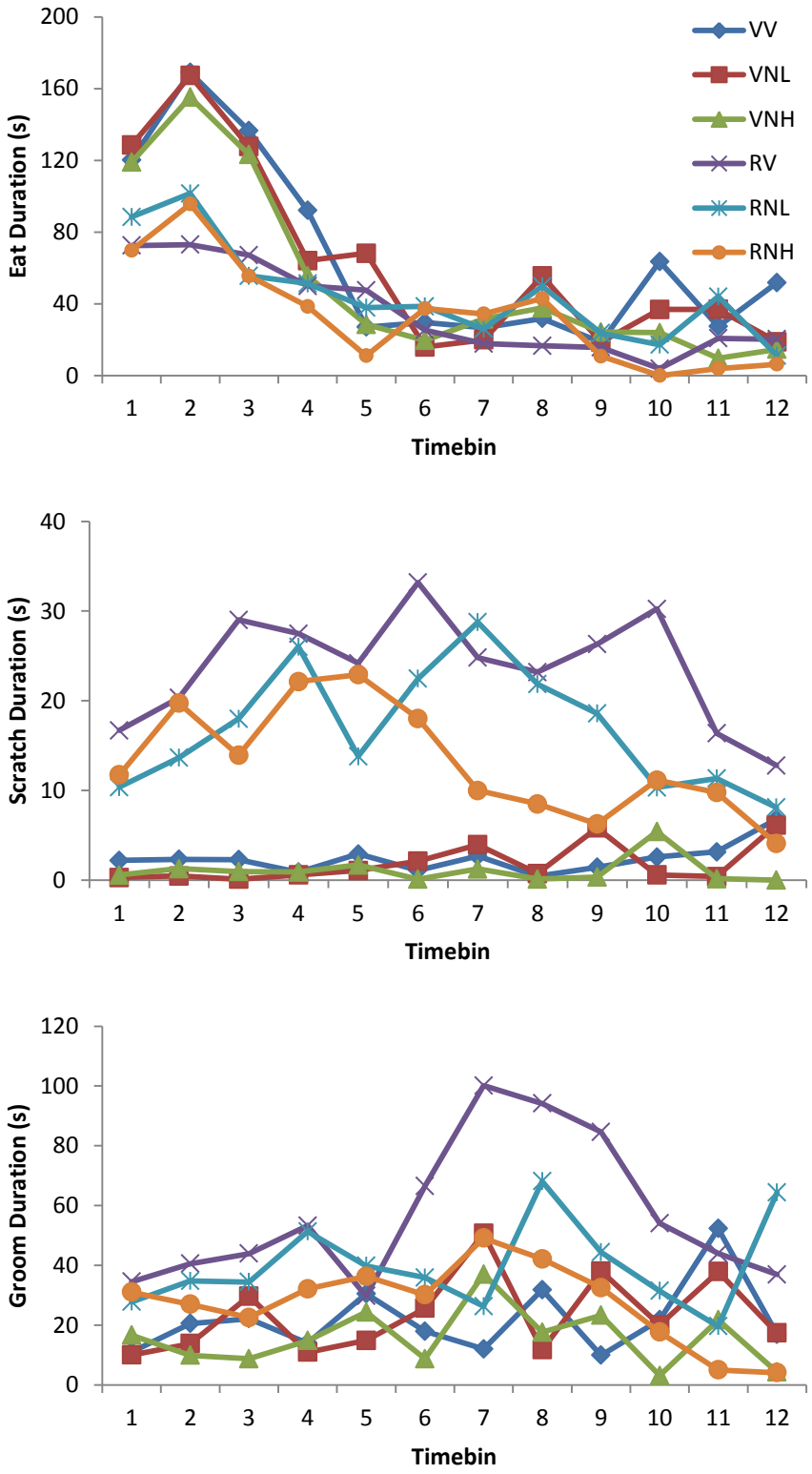


Figure 4-4: Experiment One. Effects of rimonabant and naloxone, alone and in combination, on the timecourses of eating, scratching and grooming.

Data are expressed as the mean duration of each behaviour in 12 x 5-min timebin. V = vehicle, R = rimonabant 1.5 mg/kg; NL = naloxone 0.01 mg/kg; NH = naloxone 0.1 mg/kg. See text for further details.

A series of 2-way ANOVAs within each timebin subsequently revealed that rimonabant significantly reduced eat duration in timebins 1-3 and 10 ($p < 0.02$), locomotion duration in timebins 4-6, 8 and 11 ($p \leq 0.05$) and both rear frequency and duration in timebins 1, 3, 4, and 8 ($p \leq 0.052$). The frequency of grooming was significantly increased in timebins 1, 2, and 5-10 ($p \leq 0.04$) while its duration was enhanced in timebins 1, 2, 4, 6 and 8 ($p \leq 0.05$). Most dramatically of all, both the frequency (timebins 1-12 inclusive, $p \leq 0.05$) and duration (timebins 1-11 inclusive, $p \leq 0.04$) of scratching was effectively increased throughout the entire test session.

Treatment effects on the temporal profiles of eating, scratching and grooming (Figure 4-4) clearly illustrate that rimonabant (i) suppressed the peak feeding response, an effect unaltered by naloxone co-administration, (ii) induced compulsive scratching throughout the session, an effect dose-dependently attenuated by naloxone co-administration, and (iii) induced compulsive grooming behaviour, particularly over the second half of the session, an effect again dose-dependently attenuated by the opioid receptor antagonist.

4.5.2.6 Behavioural Satiety Sequence (BSS)

Treatment effects on the behavioural satiety sequence (BSS) are summarised in Figure 4-5. The control condition (VV, top left panel) shows that the peak feeding response (first 20 mins) gradually waned as the test session progressed. A transition between eating and resting occurred at approximately 30min, with resting then becoming the predominant behaviour for the remainder of the session. This pattern was essentially unaltered by the lower dose of naloxone given alone (VNL; centre left panel), but with some evidence of a modest acceleration of the sequence (eat – rest transition around 25 min) in the higher naloxone condition (VNH; bottom left panel). This profile is consistent with the trend (albeit non-significant) towards a weak anorectic effect of 0.1 mg/kg naloxone (Figure 4-5).

Rimonabant by itself (RV; top right panel) not only markedly suppressed the peak feeding response, but also disrupted the normal BSS with grooming dominating the profile particularly over the second half of the test session. Co-administration of naloxone (RNL; centre right panel, and RNH; bottom right panel) dose-dependently reduced this intense grooming response thereby tending to ‘normalise’ the behavioural profile between 30 and 60min. Although the data for the first half of the test session showed eating to once again be the prepotent behaviour in RNL and RNH conditions, the peak feeding response in these treatment conditions remained much lower relative to VV, VNL and VNH (Figure 4-5). Nevertheless, compared

with RV profile, eat-rest transitions were more clearly evident in both RNL (30-35min) and RNH (20-25min) conditions.

4.5.2.7 Bodyweight Gain

Data not shown. ANOVA on 7-day absolute bodyweight gain failed to reveal a significant rimonabant x naloxone interaction ($F(2,18) = 1.15$, $p > 0.05$) or significant main effects for either rimonabant ($F(1,9) = 2.56$, $p > 0.05$) or naloxone ($F(2,18) = 1.41$, $p > 0.05$). Nevertheless, all animals in treatment conditions involving rimonabant generally gained a few grams less (mean: 17.2g) than those in the other conditions (mean: 20.8g: see Tallett et al, 2000a). This lack of a significant effect on post-treatment weight gain was confirmed by analysis of percent weight gain, which also failed to reveal a three-way interaction ($F(12,108) = 0.97$, $p > 0.05$), any two-way interactions (rimonabant x naloxone: $F(2,18) = 0.88$, $p > 0.05$; day x rimonabant: $F(6,54) = 0.56$, $p > 0.05$; day x naloxone: $F(12,108) = 0.89$, $p > 0.05$), or any drug main effects (rimonabant: $F(1,9) = 1.77$, $p > 0.05$; naloxone: $F(2,18) = 1.53$, $p > 0.05$). The significant main effect for day ($F(6,54) = 108.74$, $p < 0.001$) confirms treatment-independent growth patterns.

4.5.3 Summary of Main Findings

In Experiment 1, the effects of combined treatment with rimonabant and naloxone differed slightly depending upon the dose of the opioid receptor antagonist. Thus, while dose-dependently attenuating both the frequency and duration of scratching and grooming, the anorectic response to rimonabant appeared totally unaffected by the higher dose (0.1 mg/kg) of the opioid antagonist. In fact, treatment with the lower naloxone dose (0.01 mg/kg) appeared to attenuate the effects of rimonabant on intake and time spent feeding, although these effects were statistically weak. Thus, while apparently reducing the degree of anorexia, combined treatment with rimonabant and the low dose of naloxone nevertheless still reduced intake to a significant level versus vehicle control. Furthermore, there was no difference in intake scores between rimonabant given alone and when co-administered with 0.01 mg/kg naloxone. For time spent feeding, although statistical significance was lost (vs vehicle) in the rimonabant/low naloxone treatment condition, once again the combination did not differ significantly from rimonabant when given alone. In view of these factors, and the results obtained with the higher naloxone dose, it would be difficult to argue for any truly meaningful impact of naloxone on rimonabant-induced anorexia.

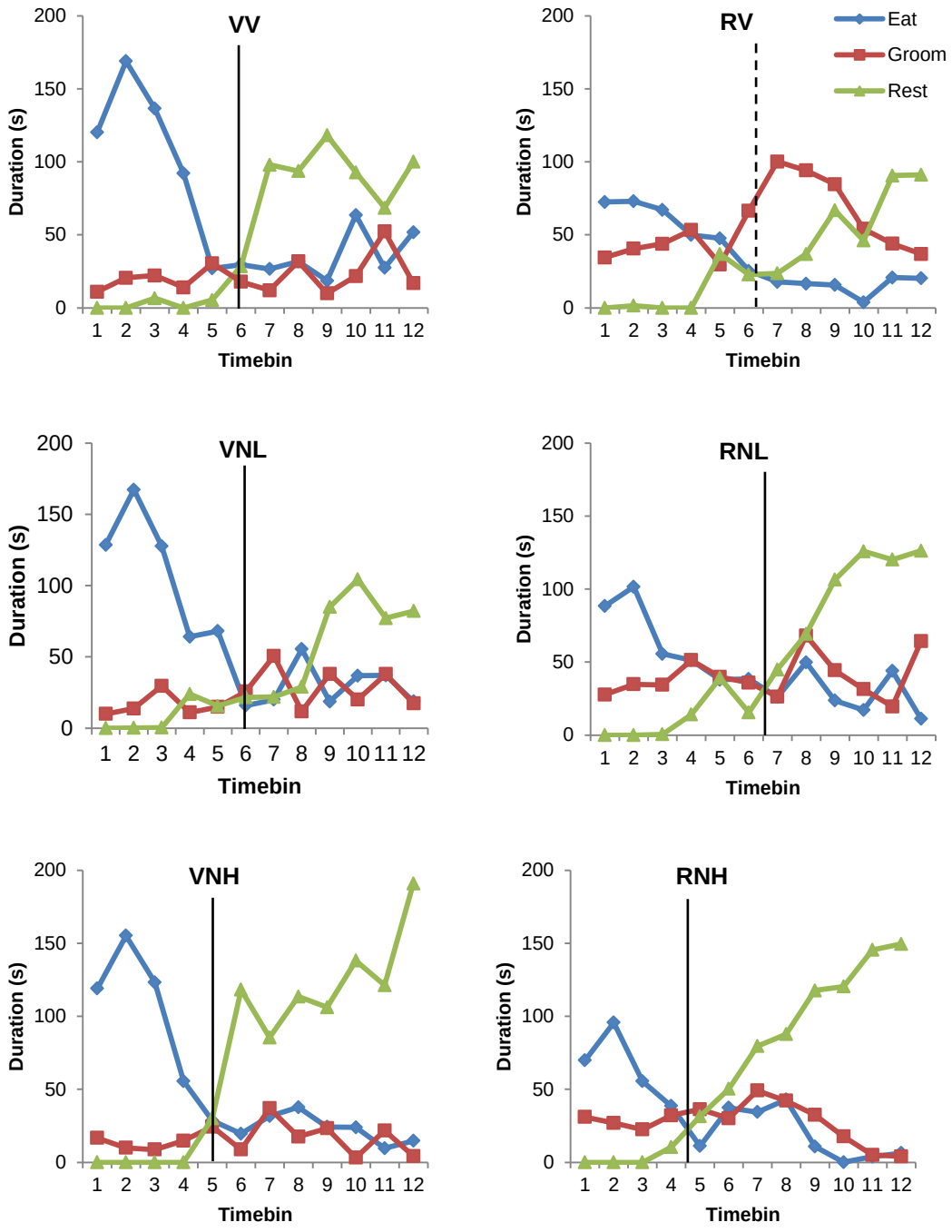


Figure 4-5: Experiment One. Effects of rimonabant and naloxone, alone and in combination, on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting; this line is dashed for the RV treatment condition in view of the lack of a clear transition; V = vehicle, R = rimonabant 1.5 mg/kg; NL = naloxone 0.01 mg/kg; NH = naloxone 0.1 mg/kg. See text for further details.

4.6 Experiment Two; Rimonabant (1.5mg/kg) and Naloxone (0.5mg/kg) Interaction

Due to the somewhat unclear nature of Experiment 1, it seemed prudent to verify its conclusions in a follow-up experiment which assessed the effects of an intermediate dose (0.05 mg/kg) of naloxone on rimonabant-induced behavioural changes.

4.6.1 Method

4.6.1.1 Subjects and Design

10 adult male Lister hooded rats (216.60 + 2.79 g on arrival and 480.98 ± 11.42g by the end of the study) were used. A within-subjects design was adopted whereby each subject received all four treatments according to a Latin Square (with a 7 day wash out period): Vehicle + Vehicle (VV); Vehicle + Naloxone 0.05mg/kg (VN); Rimonabant 1.5mg/kg + Vehicle (RV); or Rimonabant 1.5mg/kg + Naloxone 0.05mg/kg (RN).

4.6.1.2 Drugs

Naloxone hydrochloride (Sigma-Aldrich, Poole, UK) was dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. Rimonabant ([N-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide]), kindly donated by Sanofi-Aventis (Chilly-Mazarin, France), was suspended in a small volume of dimethyl sulfoxide (DMSO; Sigma-Aldrich) and subsequently made up to required concentrations in 0.5% methylcellulose (Sigma-Aldrich). The doses of naloxone (0.05 mg/kg) and rimonabant (1.5 mg/kg) were selected on the basis of Experiment 1 (see Section 4.5). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg 30 minutes (rimonabant/vehicle) or 15 minutes (naloxone/vehicle) prior to testing.

4.6.1.3 Procedure

Testing occurred over four weeks, with two test days per week, and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square

4.6.2 Results

Full statistical details can be found in Appendix 2.

4.6.2.1 Habituation Phase Food Intake

As expected, mash consumption differed significantly over the course of habituation (trial 1: 9.7 ± 0.78g; trial 2: 13.04 ± 1.71g; trial 3: 13.98 ± 1.45g; trial 4: 19.08 ± 1.43g; trial 5: 17.71 ± 2.25g ($F(4,36) = 11.493$, $p < 0.001$). Although food intake on trial 1 was significantly lower than most other trials (3-5; $p \leq 0.05$), the development of stable intake was confirmed by the lack of difference across trials 4-5 ($p > 0.05$), as well as the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (18.39 ± 1.57g).

4.6.2.2 Test Day Bodyweight

Consistent with the Latin Square design, test-day bodyweights were comparable across the various treatment conditions (VV: 418.76 ± 13.45g; VN: 419.91 ± 15.48g; RV: 430.58 ± 14.66g; RN: 425.56 ± 12.47g (rimonabant: $F(1,9) = 0.45$, $p > 0.05$; naloxone: $F(1,9) = 0.05$, $p > 0.05$; rimonabant x naloxone: $F(1,9) = 0.07$, $p > 0.05$).

4.6.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.28% throughout the experiment (range 0.12 - 0.38%). Treatment effects on food intake are summarised in Figure 4-6. There was a significant main effect of rimonabant ($F(1,9) = 43.335$, $p \leq 0.001$), but no significant main effect for naloxone ($F(1,9) = 1.447$, $p > 0.05$) or a significant drug interaction ($F(1,9) = 0.03$, $p > 0.05$). Bonferroni comparisons confirmed that, although naloxone given alone did not alter intake compared with VV control, significant suppression was evident in both conditions receiving the CB1 receptor antagonist/inverse agonist (RV: $p = 0.003$; RN: $p = 0.001$; RNH). The apparently weaker anorectic response to rimonabant alone compared to co-administration with naloxone (see Figure 4-6) was not objectively confirmed by a significant difference between the RV and RN conditions ($p > 0.05$).

4.6.2.4 Total (one-hour) Behavioural Analyses

Treatment effects on the total frequency and duration of ingestive and non-ingestive behaviours are illustrated in Figure 4-7, while data for feeding-related parameters are summarised in Table 4-2. It should be noted that levels of drinking were extremely low in this study and are not reported.

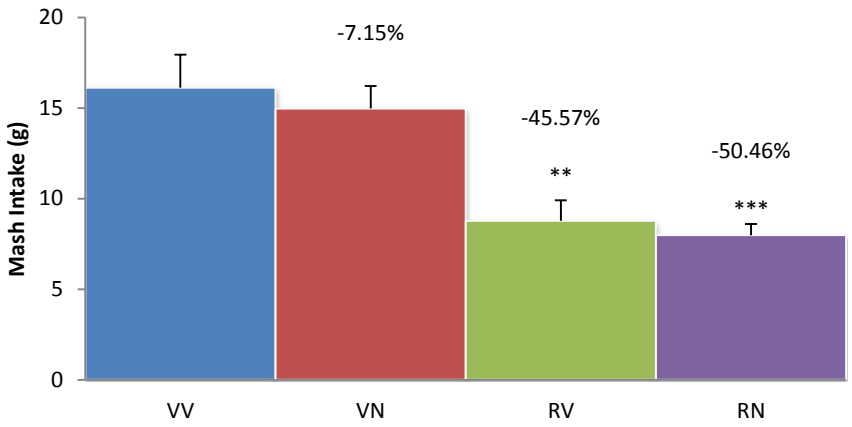


Figure 4-6: Experiment Two. Effects of rimonabant and naloxone, alone and in combination, on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = vehicle, R = Rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg; See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV

There were no significant rimonabant x naloxone interactions for any of the test variables ($F(1,9) \geq 2.32$, $p > 0.05$). Significant main effects of rimonabant were found for eat bout (Table 4-3) as well as the frequency and duration (Figure 4-7) of feeding, locomotion, grooming, sniffing, scratching, and rear frequency ($F(1,9) \leq 3.66$, $p \leq 0.05$). In contrast, there were no significant main effects of rimonabant on eat rate, the latency to identify the food source or to commence eating, or the duration of rearing ($F(1,9) \geq 3.08$, $p > 0.05$). Significant main effects of naloxone were not seen for any of the behavioural measures ($F(2,18) \geq 2.95$, $p > 0.05$).

Table 4-2: Experiment Two. Acute effects of rimonabant and naloxone, alone and in combination, on eating-related parameters

(Mean ± SE); V = vehicle, R = Rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV. See text for further details.

Measure	VV	VN	RV	RN
Latency to locate food (s)	45.88 ± 20.36	21.71 ± 8.33	18.26 ± 6.24	15.61 ± 4.96
Latency to Eat (s)	57.81 ± 19.60	32.17 ± 7.22	39.68 ± 12.40	50.11 ± 13.61
Eat bout Duration(s)	14.58 ± 2.36	15.41 ± 1.81	13.10 ± 2.25	10.12 ± 1.08
Eating rate (g/min)	1.53 ± 0.19	1.55 ± 0.15	1.57 ± 0.28	1.41 ± 0.15

Post-hoc analyses showed that, relative to VV control, naloxone when given alone, failed to significantly influence feeding duration. In contrast, rimonabant alone (RV,

p = 0.003) and in conjunction with naloxone (RN, p < 0.005) significantly reduced time spent feeding. In addition, rimonabant alone did not produce a significant reduction in food intake compared to naloxone alone (p = 0.117) whereas the combination was significantly different (p < 0.05), demonstrating that the combination reduced time spent feeding more so than rimonabant alone.

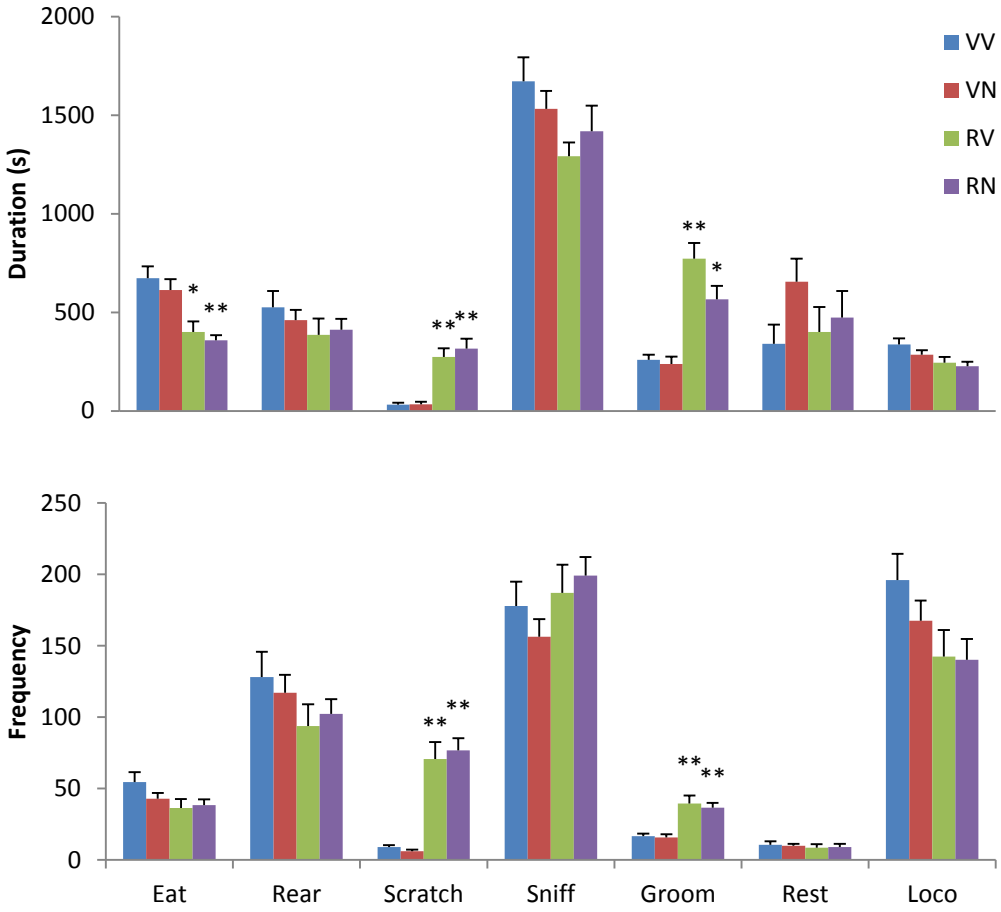


Figure 4-7: Experiment Two. Effects of rimonabant and naloxone, alone and in combination, on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

(Mean ± SE); V = vehicle, R = Rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 versus VV. See text for further details.

Post-hoc analyses of the data for eat frequency, eat bout, rear frequency and frequency and duration of sniff and locomotion failed to reveal any significant differences between drug treatment groups and VV control. Therefore, the significant main effect for rimonabant must reflect relatively weak inhibitory effects of the CB1 receptor antagonist/inverse agonist that emerge only as a result of large sample sizes. Neither the duration nor frequency of grooming or scratching was

significantly altered by naloxone given alone or in combination. All measures of grooming and scratching were significantly increased with the administration rimonabant alone and in combination. There is a trend for naloxone in combination to enhance scratching duration (RV, $p = 0.01$; RN, $p > 0.005$) compared to vehicle (VV). Whereas the opposite can be said for grooming behaviours (Frequency; RV, $p > 0.005$; RN, $p > 0.05$; Duration; RV, $p > 0.001$; RN, $p > 0.05$). This suggests that naloxone, somewhat unexpectedly, is having minimal effects upon the rimonabant-induced grooming and scratching behaviours.

4.6.2.5 Timebin Behavioural Analyses

Confirming the expected temporal decline in active behaviours and increase in inactive behaviours across the test session, ANOVA revealed a strong main effect of time for most behavioural measures ($F(11, 99) \geq 2.16$, $p \leq 0.05$). The exceptions being groom duration ($F(11,99) = 1.64$, $p > 0.05$) and groom frequency ($F(11,99) = 1.83$, $p \leq 0.06$), although the latter closely approached significance.

ANOVA revealed only one significant three-way interaction for eat duration (rimonabant x naloxone x time; $F(11,99) = 2.43$, $p < 0.01$). Two significant rimonabant x time interactions were found for eat duration ($F(11,99) = 4.00$, $p < 0.001$) and scratch frequency ($F(11,99) = 7.74$, $p < 0.001$). One naloxone x time interaction was found for rest duration ($F(22,198) = 2.88$, $p < 0.01$). Although the drug x time interactions for other measures failed to reach significance, the F-value for sniff duration ($F(11,99) = 1.85$, $p = 0.056$) closely approached significance. A series of 2-way follow up ANOVAs within each timebin subsequently revealed that rimonabant significantly reduced eat frequency in timebin 2 ($F(1,9) = 10.73$, $p < 0.01$), potentially indicating a rimonabant-induced suppression of the peak feeding response. Furthermore naloxone reduced eat frequency in timebin 10 ($F(1,9) = 6.02$, $p < 0.05$). Similar follow up 2 way (rimonabant x naloxone) ANOVAs for eat duration revealed a rimonabant x naloxone interaction in timebins 4 and 12.

The rimonabant treatment effects on the temporal profiles of eating, scratching and grooming (Figure 4-8) illustrate similar patterns seen in Experiment 1. This is that rimonabant (i) suppressed the peak feeding response, (ii) induced compulsive scratching throughout the session, and (iii) induced compulsive grooming behaviour, particularly over the second half of the session. Unexpectedly, unlike that seen in Experiment 1, naloxone co-treatment displayed relatively weak effects

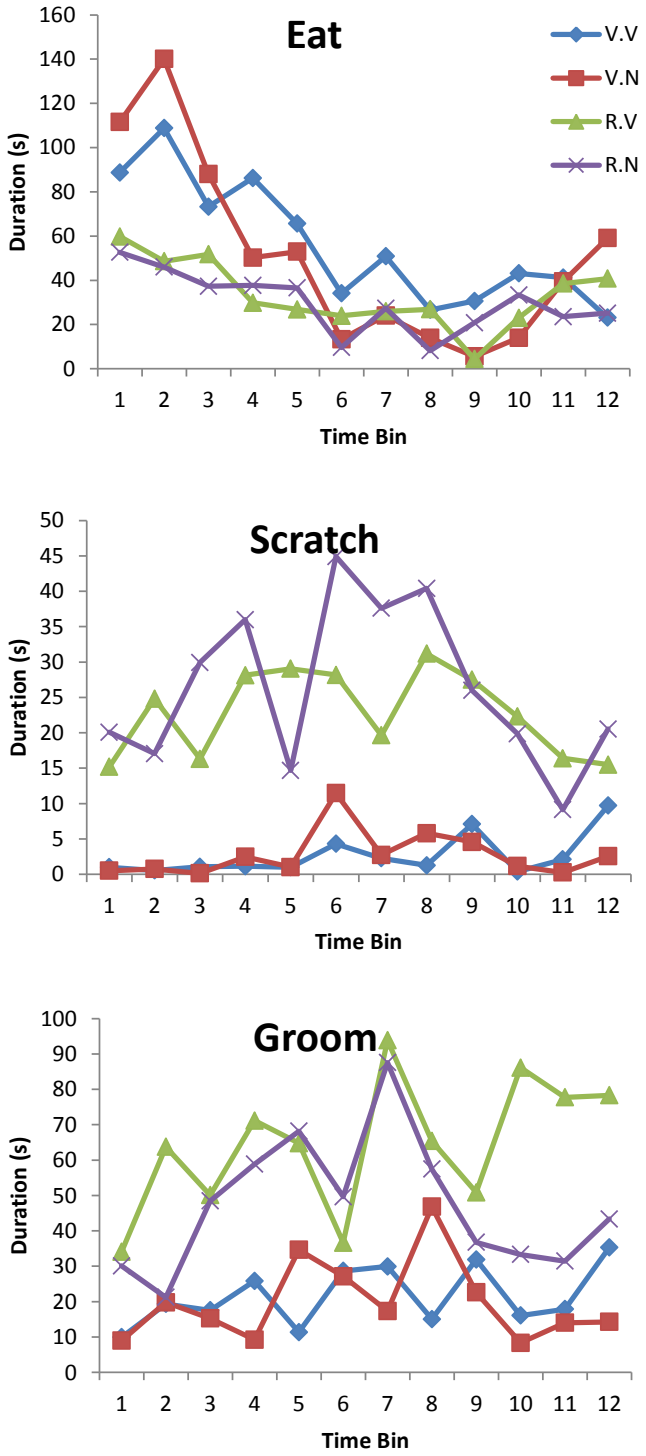


Figure 4-8: Experiment Two. Effects of rimonabant and naloxone, alone and in combination, on the timecourses of eating, scratching and grooming

Data are expressed as the mean duration of each behaviour in the mean duration of each behaviour in 12 x 5-min timebin V = vehicle, R = rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg. See text for further details

on the rimonabant scratching and grooming. The temporal profiles (Figure 4-8) and statistical analysis show little evidence of a naloxone-induced attenuation of rimonabant's scratching and grooming syndrome.

4.6.2.6 Behavioural Satiety Sequence (BSS)

Treatment effects on the behavioural satiety sequence (BSS) are summarised in Figure 4-9. Most unusually, the control condition (VV; top left panel) fails to show a convincing peak feeding response. Normally seen within the first 15mins, the peak should gradually wanes as the test session progresses. Although, the naloxone alone condition (VN; top right panel) does demonstrate this more clearly, the vehicle condition instead demonstrates a gradually waning in duration of what appears to be continuous sampling. Eat-to-rest transitions can be seen within the 7th timebin (30-35 minutes into the session) for both VV and VNL conditions, with resting then becoming the predominant behaviour for the remainder of the test session (more so in VN). Similar to Experiment 1, rimonabant alone (RV; bottom left panel) not only markedly suppressed the peak feeding response, but also disrupted the normal BSS with grooming dominating the profile throughout most the test session. (Eat/Rest cross over at 35mins). Co-administration of naloxone (RN; bottom right panel) fails to restore the peak feeding behaviour. However, it does appear to reduce grooming behaviour towards the end of the session, allowing resting to become the dominant behaviour. An eat-rest transition is still unclear in the RN condition although it does show a groom-rest transition at 40mins.

4.6.2.7 Bodyweight Gain

Data not shown. ANOVA on 7-day absolute bodyweight gain failed to reveal a significant rimonabant x naloxone interaction ($F(2,18) = 0.67$, $p > 0.05$) or indeed significant main effects for either rimonabant ($F(1,9) = 0.32$, $p > 0.05$) or naloxone ($F(2,18) = 0.107$, $p > 0.05$). The lack of treatment effect on weight gain was further confirmed by analyses of percent weight gain. ANOVA failed to reveal a three-way interaction ($F(7,63) = 1.147$, $p > 0.05$), or any two-way interactions ($F(1,9) = 0.122$, $p > 0.05$; rimonabant: $F(1,9) = 0.764$, $p > 0.05$; naloxone: $F(7,63) = 1.36$, $p > 0.05$). Furthermore, there were no main effects of either rimonabant ($F(1,9) = 3.17$, $p > 0.05$) or naloxone ($F(1,9) = 0.085$, $p > 0.05$). The highly significant main effect for day ($F(7,63) = 119.32$, $p < 0.001$) simply reflects the natural growth patterns in all condition.

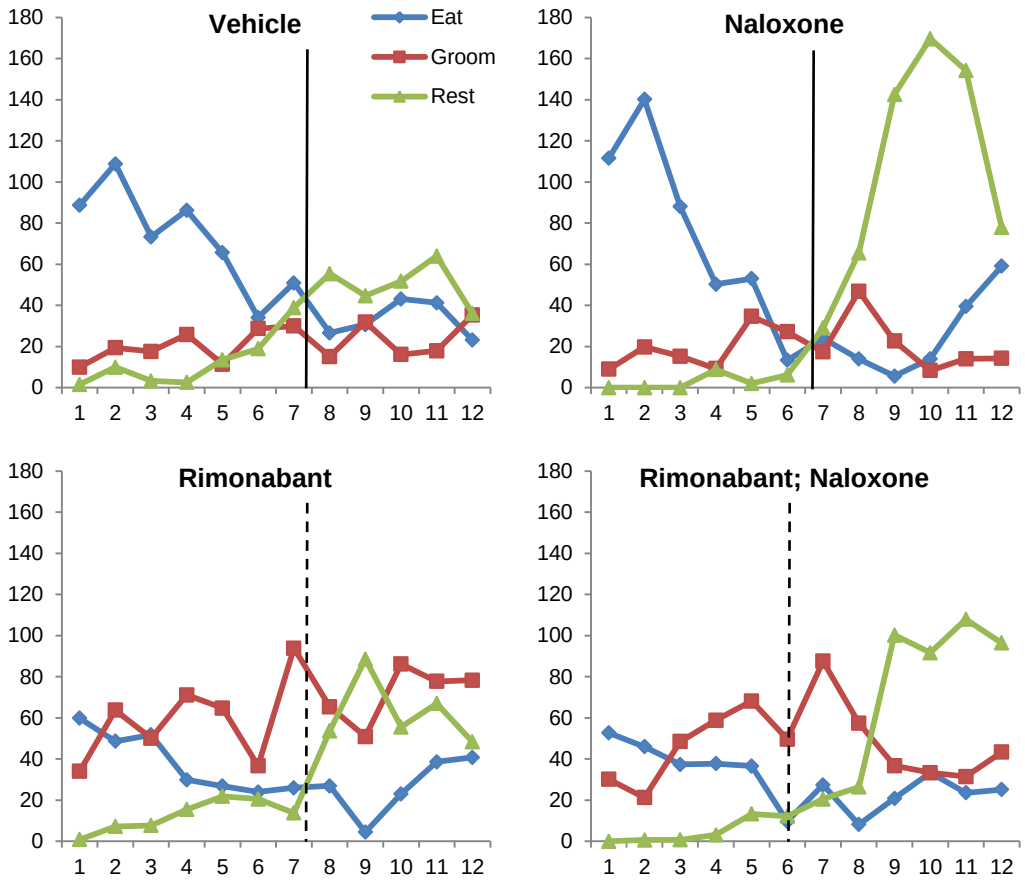


Figure 4-9. Experiment Two. Effects of rimonabant and naloxone, alone and in combination, on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting, this line is dashed for the R treatment conditions in view of the lack of a clear transition; V = vehicle, R = rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg. See text for further details

4.6.3 Summary of Main Findings

The vehicle/control data from this study demonstrated a very unusual BSS profile, somewhat reminiscent of that seen with quinine; i.e. a suppressed peak feeding response, in addition to intermittent food sampling (characterised by an increased number of feed bouts and reduced eating rate), and the virtual absence of resting behaviour (Blundell et al., 1985; Ishii, Blundell, Halford, Rodgers, et al., 2003). This quinine-like behavioural profile indicates appetite suppression as a result of reduced palatability (Ishii, Blundell, Halford, Rodgers, et al., 2003). Furthermore, in this study, naloxone did not have a significant effect on <u>either</u> rimonabant induced anorexia <u>or</u> the scratching/grooming syndrome.

After further investigation, an infestation of *Tribolium confusum* (aka a flour beetle) was discovered within the powered food supply. The suppliers were contacted the

problem quickly resolved (see Appendix 3). However, due to the contamination of the diet, and the unusual behavioural profile of the control animals, the data from this study must be considered suspect. As such, it seemed prudent to repeat the study in the hope of gaining a more reliable control profile with which to compare experimental interventions.

4.7 Experiment Three; Rimonabant (1.5mg/kg) and Naloxone (0.5mg/kg) Interaction

4.7.1 Method

4.7.1.1 Subjects and Design

10 adult male Lister hooded rats (215.94 ± 1.86g on arrival from Charles River, U.K and 479.00 ± 11.03g by the end of the study) were used. A within-subjects design was adopted whereby each subject received all four treatments according to a Latin Square (with a 7 day wash out period): Vehicle + Vehicle (VV); Vehicle + Naloxone 0.05mg/kg (VN); Rimonabant 1.5mg/kg + Vehicle (RV); or Rimonabant 1.5mg/kg + Naloxone 0.05mg/kg (RN).

4.7.1.2 Drugs

Naloxone hydrochloride (Sigma-Aldrich, Poole, UK) was dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. Rimonabant ([N-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide]), purchased from Cambridge Bioscience (Cambridge, UK), was suspended in a small volume of dimethyl sulfoxide (DMSO; Sigma-Aldrich) and subsequently made up to required concentrations in 0.5% methylcellulose (Sigma-Aldrich). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg 30 minutes (rimonabant/vehicle) or 15 minutes (naloxone/vehicle) prior to testing.

4.7.1.3 Procedure

Testing occurred over four weeks, with two test days per week and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square

4.7.1.4 Error

One animal became ill during the first week of the test phase and was removed from the study, with a consequent reduction in sample size to 9

4.7.2 Results

Full statistical details can be found in Appendix 4.

4.7.2.1 Habituation Phase Food Intake

Mash intake again differed over the course of the 5-day habituation period (trial 1: 10.3 ± 1.3g; trial 2: 15.0 ± 1.7g; trial 3: 14.7 ± 0.9g; trial 4: 17.0 ± 1.4g; trial 5: 15.7 ± 1.3g ($F(4,36) = 9.85$, $p < 0.001$). Although food intake on trial 1 was significantly

lower than on trials 3-5 ($p \leq 0.05$), the development of stable intake was confirmed by the lack of a significant difference across trials 2-5 ($p > 0.05$), as well as the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (17.70 ± 0.78g).

4.7.2.2 Test Day Bodyweight

Test-day bodyweights were comparable across treatment conditions (VV: 411.9 ± 15.2g; VN: 418.8 ± 12.3g; RV: 413.5 ± 10.9g; RN: 412.7 ± 14.7g: rimonabant: $F(1,8) = 0.03$, $p > 0.05$; naloxone: $F(1,8) = 0.12$, $p > 0.05$; rimonabant x naloxone: $F(1,8) = 0.08$, $p > 0.05$).

4.7.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.19% throughout the experiment period (range = 0.07-0.29%). Treatment effects on food intake are summarised in Figure 4-10. There was a significant main effect of rimonabant on food intake ($F(1,8) = 115.63$, $p < 0.001$), but no main effect for naloxone ($F(1,8) = 4.46$, $p > 0.05$) or a drug interaction ($F(1,8) = 0.88$, $p > 0.05$).

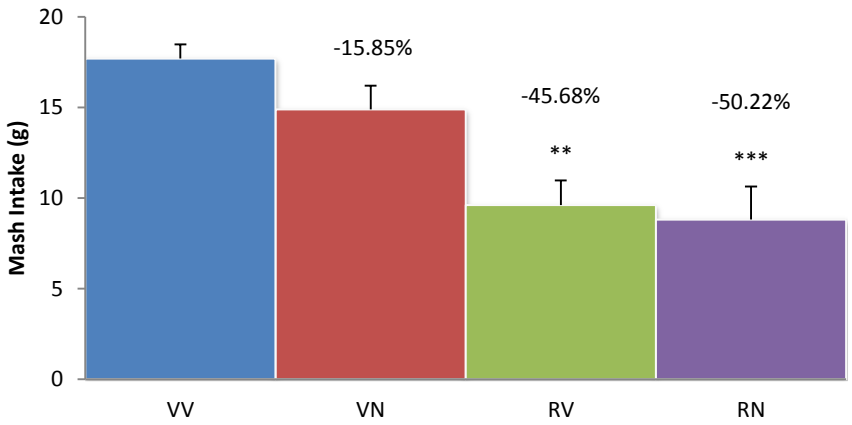


Figure 4-10: Experiment Three. Effects of rimonabant and naloxone, alone and in combination, on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = vehicle, R = Rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg; See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV

Bonferroni comparisons confirmed that, relative to vehicle control (VV), rimonabant both by itself (RV) and in the presence of naloxone (RN) strongly suppressed mash consumption ($p < 0.005$). The inability of the opioid receptor antagonist to alter rimonabant anorexia was confirmed by the lack of significant difference between

the RV and RN treatment conditions. It is important to note that naloxone *per se* (VN) did not significantly influence intake relative to VV control.

4.7.2.4 Total (one-hour) Behavioural Analyses

Treatment effects on behavioural frequencies and durations are illustrated in Figure 4-11, while data for feeding-related parameters are summarised in Table 4-3. Again it should be noted that levels of drinking were extremely low in this study and are not reported.

Significant rimonabant x naloxone interactions were found for the duration both of grooming ($F(1,8) = 15.53$, $p < 0.005$) and scratching ($F(1,8) = 13,26$, $p < 0.01$), while the interaction for groom frequency approached significance ($F(1,8) = 4.40$, $p < 0.07$). There were no other significant interactions ($F(1,8) \leq 3.27$, $p > 0.05$). Significant main effects of rimonabant were found for the frequency and duration of eating, locomotion and rearing ($F(1,8) \geq 7.50$, $p \leq 0.03$) as well as the frequency of scratching ($F(1,8) = 155.21$, $p < 0.001$), duration of sniffing ($F(1,8) = 10.35$, $p < 0.02$), and eat rate ($F(1,8) = 8.36$, $p = 0.02$). Main effects of naloxone were found only for the frequency of eating ($F(1,8) = 12.94$, $p < 0.01$) and duration of resting ($F(1,8) = 6.38$, $p < 0.04$).

Table 4-3: Experiment Three. Acute effects of rimonabant and naloxone, alone and in combination, on eating-related parameters

Data are mean values (± S.E.M). V = vehicle, R = Rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg; See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV

Measure	VV	RV	VN	RN
Latency to locate food (s)	9.31 + 2.33	11.97 + 4.93	6.63 + 2.96	10.98 + 3.48
Latency to eat (s)	9.22 + 2.67	16.22 + 4.86	12.03 + 3.18	13.08 + 7.07
Eat bout duration (s)	10.94 + 1.39	11.19 + 1.79	13.06 + 1.95	10.20 + 1.22
Eating rate (g/min)	1.74 + 0.10	1.40 + 0.13	1.78 + 0.23	1.45 + 0.10

Post-hoc analyses showed that, relative to VV control, neither drug when given alone (RV, VN), or in combination (RN), significantly altered eat rate, the frequency of eating, rearing and locomotion, or the duration of rearing and sniffing. This profile suggests that the significant main effects described above reflect the increased power of main effects analysis. In contrast, whereas naloxone *per se* (VN) was ineffective in altering time spent eating, rimonabant alone (RV, $p < 0.03$) and in combination with naloxone (RN, $p < 0.01$) significantly reduced this measure. The apparent failure of naloxone to block the rimonabant-induced reduction in eat duration was further confirmed by the absence of a significant difference between

RV and RN conditions. A very similar pattern was evident for the duration of locomotion where, in the absence of an intrinsic effect of naloxone (VN), rimonabant both alone (RV; $p < 0.03$) and in combination with naloxone (RN; $p < 0.04$) significantly reduced this behaviour relative to VV control. The lack of significant difference between RV and RN conditions confirmed the inability of naloxone to influence the locomotor suppressant effect of rimonabant.

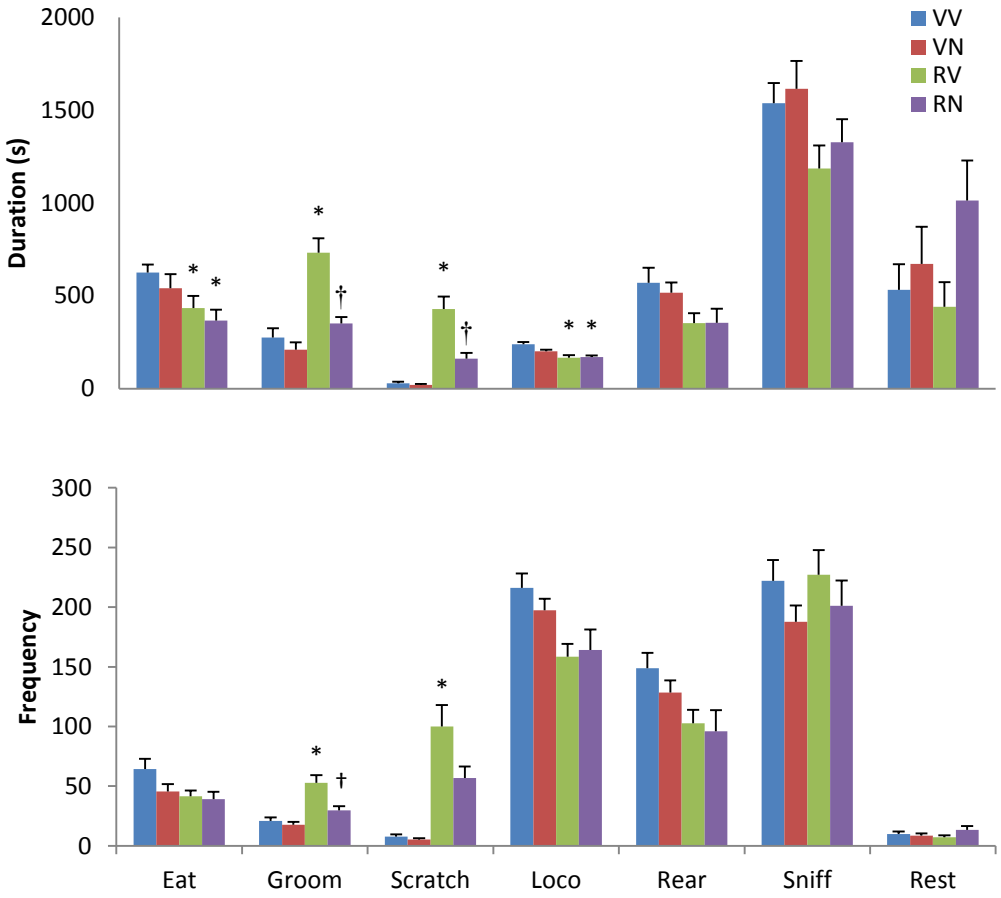


Figure 4-11. Experiment Three. Effects of rimonabant and naloxone, alone and in combination, on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = vehicle, R = rimonabant 1.5 mg/kg; N = naloxone 0.01 mg/kg. See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus VV

Bonferroni comparisons also showed that, relative to VV, rimonabant alone (RV) produced substantial increases in both the frequency and duration of grooming and scratching ($p \leq 0.01$); Figure 4-11. Although naloxone *per se* (VN) was devoid of intrinsic effects on all of these measures, the opioid receptor antagonist strongly attenuated the stimulatory effects of the CB1 receptor antagonist/inverse agonist.

Thus, for all four measures, combined drug treatment (RN) did not differ significantly from VV control. Furthermore, for the frequency and duration of grooming, as well as the duration of scratching, there were significant differences ($p \leq 0.05$) between rimonabant when given alone (RV) and when given in combination with naloxone (RN).

4.7.2.5 Periodic (Timebin) Behavioural Analyses

As confirmed by significant main effects of time for all variables recorded ($F(11,88) \geq 2.04$, $p \leq 0.04$), active behaviours waned and inactive behaviours increased as the session progressed. Although there were no significant three-way (rimonabant x naloxone x time) interactions ($F(11,88) \leq 1.76$, $p > 0.05$) and only two significant naloxone x time interactions (rest duration, sniff duration: $F(11,88) \geq 2.55$, $p \leq 0.01$), ANOVA revealed seven significant rimonabant x time interactions (eat frequency & duration, groom frequency, scratch frequency and duration, rear duration, and sniff duration: $F(11,88) \geq 2.19$, $p \leq 0.03$).

A series of 2-way ANOVAs within each timebin revealed that rimonabant significantly reduced eat duration in timebins 1-3 ($p \leq 0.02$),eat frequency in timebins 1, 3, 5, 6, 11 and 12 ($p \leq 0.054$), rear duration in timebins 4-6 and 8 ($p \leq 0.05$), and sniff duration in timebins 1, 4, 7 and 10 ($p \leq 0.05$). Rimonabant also significantly increased the frequency of grooming in timebins 1-4, 6, 7, 9 and 10 ($p \leq 0.05$) as well as the frequency of scratching in timebins 1-10 and 12 ($p \leq 0.01$) and scratching duration in timebins 1, 2, 5, 10 and 11 ($p \leq 0.02$). Figure 4-12 shows that treatment effects on the timecourses of eating, scratching and grooming were remarkably similar to (but even clearer than) those seen in Experiment 1; namely, that while naloxone failed to impact the anorectic effect of rimonabant, it did markedly attenuate/reverse the effects of the CB1 receptor antagonist/inverse agonist on compulsive scratching and grooming.

4.7.2.6 Behavioural Satiety Sequence (BSS)

Treatment effects on BSS profiles are summarised in Figure 4-13. The control profile (VV; top left panel) confirms that feeding was the predominant response during the first 20-25min. Feeding gradually gave way to resting as the session progressed, with an eat-to-rest transition around 30min. A very similar behavioural pattern was evident under naloxone (VN; top right panel), with some evidence of a slight temporal acceleration (shift to left) in the BSS (see also the effects of naloxone in Experiment 1, Section 4.5).

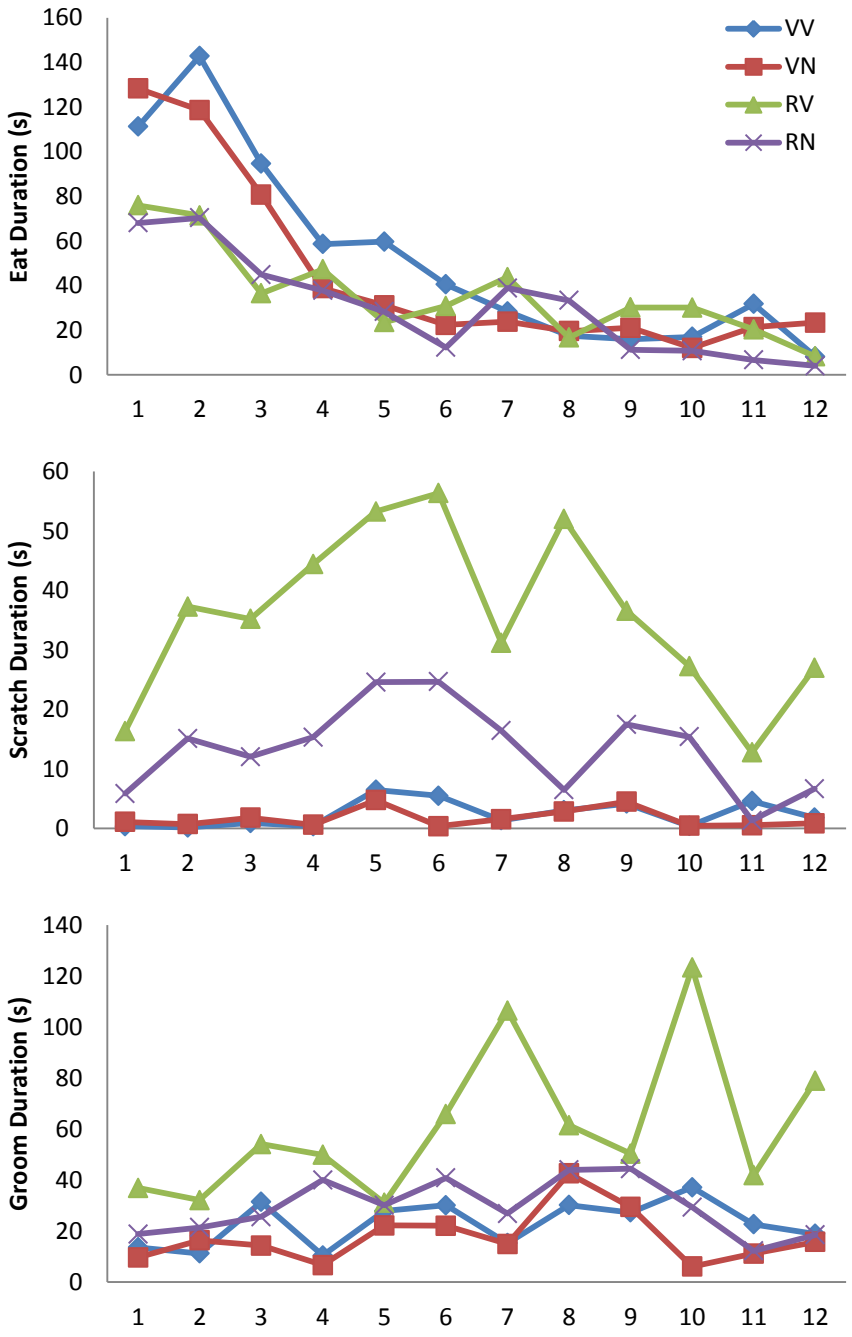


Figure 4-12. Experiment Three. Effects of rimonabant and naloxone, alone and in combination, on the timecourses of eating, scratching and grooming

Data are expressed as the mean duration of each behaviour in 12 x 5-min timebin V = vehicle, R = rimonabant 1.5 mg/kg; NL = naloxone 0.01 mg/kg; NH = naloxone 0.1 mg/kg. See text for further details

When given alone, rimonabant disrupted the BSS (bottom left panel), not only suppressing feeding but also enhancing grooming. Although it is possible to discern an eat-to-rest transition, the behavioural profile under rimonabant is clearly far from normal (compare with VV). Interestingly, combined rimonabant/naloxone treatment (RN; bottom right panel), while doing little to alter the suppressant effect of

rimonabant on peak feeding, once again appeared to 'normalise' the BSS by countering the rimonabant-induced grooming response. Thus, co-treatment produced an acceleration of the BSS relative to RV and VN, and reinstated resting as the predominant behaviour during the second half of the test session.

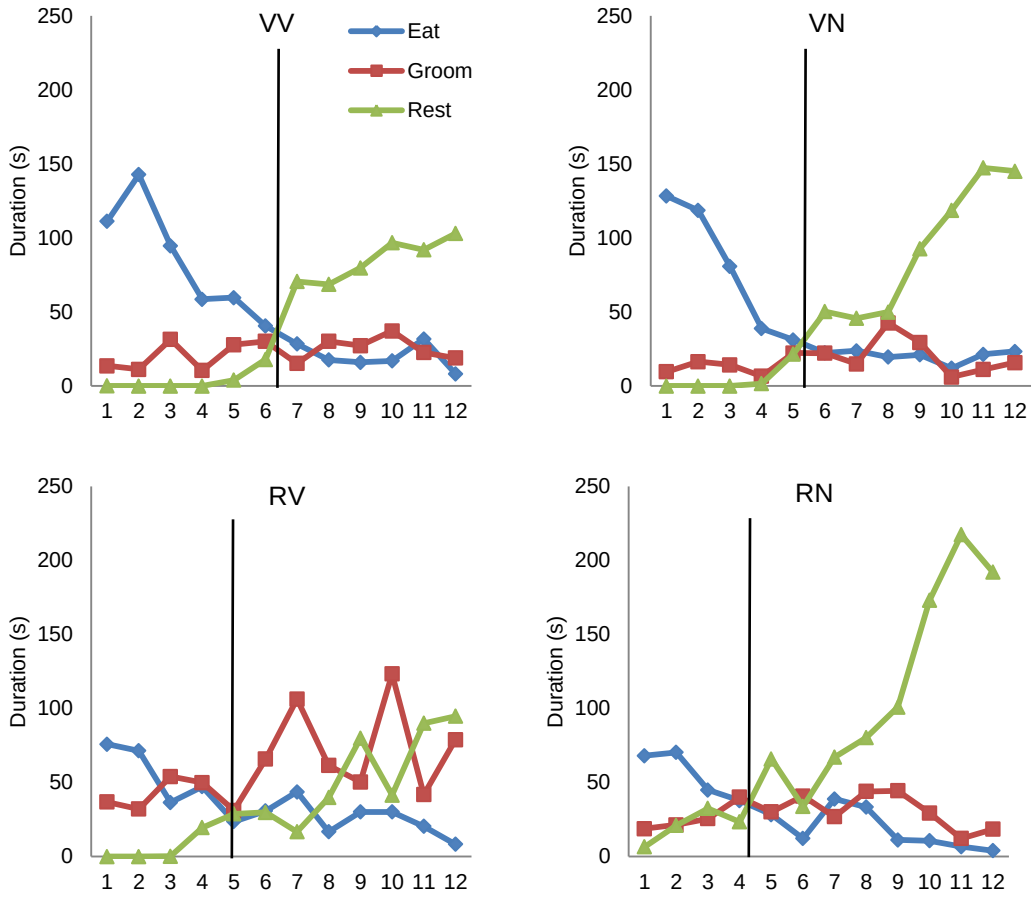


Figure 4-13. Experiment Three. Effects of rimonabant and naloxone, alone and in combination, on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting, this line is dashed for the R treatment conditions in view of the lack of a clear transition; V = vehicle, R = rimonabant 1.5 mg/kg; N = naloxone 0.05 mg/kg. See text for further details

4.7.2.7 Bodyweight Gain

Data not shown. ANOVA on 7-day absolute bodyweight gain failed to reveal a significant rimonabant x naloxone interaction ($F(1,8) = 0.18$, $p > 0.05$) or main effects for either rimonabant ($F(1,8) = 0.02$, $p > 0.05$) or naloxone ($F(1,8) = 0$, $p > 0.05$).

The lack of effect of either drug alone or in combination was further confirmed by analyses of percent weight gain: there was no three-way interaction ($F(6,48) = 0.64$, $p > 0.05$), any two-way interactions (rimonabant x naloxone: $F(1,8) = 0.05$, $p > 0.05$;

rimonabant x day: $F(6,48) = 1.33$, $p > 0.05$; naloxone x day: $F(6,48) = 0.06$, $p > 0.05$), or any main effects for either rimonabant ($F(1,8) = 0.05$, $p > 0.05$) or naloxone ($F(1,8) = 0.003$, $p > 0.05$). The significant main effect for day ($F(6,48) = 259.29$, $p < 0.001$) confirms normal growth patterns over the week following behavioural testing.

4.7.3 Summary of Main Findings

The results of Experiment 3 clearly show that, while markedly attenuating compulsive scratching and grooming, 0.5 mg/kg naloxone failed to have any significant impact on the anorectic response to the CB1 receptor antagonist/inverse agonist. These findings are consistent with those obtained with the higher dose of naloxone in Experiment 1, and suggest that the weak attenuation of rimonabant anorexia by the lower naloxone dose may have been more apparent than real.

4.8 Chapter Four Main Findings

The ‘response competition’ hypothesis holds that the acute anorectic response to conventional CB1 receptor antagonist/inverse agonists (e.g. rimonabant, AM-251) is due primarily (if not exclusively) to the induction of an intense scratching and grooming syndrome that interferes with feeding behaviour during tests of finite duration (Tallett et al., 2007a, 2007b).

- **Experiment 1** showed that sub-anorectic doses of naloxone (0.1 and 1.0mg/kg) dose-dependently attenuate the scratching and grooming syndrome induced by an anorectic dose of rimonabant (1.5mg/kg). Although the higher dose of naloxone (1.0mg/kg) had no effect on the anorectic response to rimonabant, a lower naloxone dose of the opioid receptor antagonist (0.1mg/kg) appeared to slightly attenuate rimonabant anorexia.
- **Experiment 2** showed that an intermediate dose of naloxone (0.5mg/kg) did not significantly affect rimonabant-induced anorexia <u>or</u> the scratching and grooming syndrome. However, due to diet contamination, and an unusual behavioural profile in the control condition, the data from this study was considered questionable.
- **Experiment 3** confirmed that an intermediate dose of naloxone (0.5mg/kg) attenuates the scratching and grooming, but not anorectic, response to rimonabant (1.5mg/kg).

Together with evidence from Hodge and colleagues (Hodge et al., 2008), the present studies imply that the anorectic and pruritic responses to CB1 agents such as rimonabant are largely independent phenomena. As such, the results reported in Chapter 4 failed to support the response competition hypothesis of rimonabant-induced anorexia in rats (Tallett et al., 2007b).

Chapter 5 Monoamine and Opioid System Interactions

5.1 Bupropion

Bupropion (2-tert-butylamino-3-chloropropiophenone-HCl) is a selective catecholamine (dopamine and noradrenaline) presynaptic reuptake inhibitor (Ascher et al., 1995; Ferris & Beaman, 1983). It acts on dopamine and noradrenaline reuptake transporters to decrease reuptake into rat and mouse synaptosomes (pre-synaptic neuronal membranes). The increased synaptic levels of dopamine and noradrenaline subsequently act to inhibit firing via auto-receptor mediated negative feedback mechanisms (Cooper et al., 1994; Stahl., 2004).

In vitro studies have demonstrated that bupropion acts primarily on dopaminergic systems (Ferris et al., 1983; Ferris et al., 1982; Ferris et al., 1981). In vivo studies have supported this, with evidence showing increased dopamine concentrations following bupropion administration (Nomikos et al., 1992). However, doses required to inhibit dopamine transport are far higher than those required to elicit its known therapeutic effects (Golden et al., 1988).

Interestingly, bupropion has minimal effect on indoleamines (serotonin; Golden et al., 1988) and lacks any appreciable affinity for postsynaptic receptors, including histamine, α- and β- adrenergic, acetycholine, serotonin or dopamine (Stahl et al., 2004). Furthermore, despite its known role as an antidepressant, it does not inhibit monoamine oxidase or release catecholamines (Ferris et al., 1978; Soroko et al., 1977).

Bupropion has three metabolites: threo-hydrobupropion (TB), erythro-hydrobupropion (EB) and hydroxybupropion (HB; Ascher et al., 1995). Although the plasma half-life of bupropion is ~10hours, the metabolites take much longer to decay (TB, ~20h; EB, ~27h; HB, ~22h; Laizure et al., 1985). Bupropion's primary metabolite is HB and, in humans and mice, the concentration of HB following bupropion administration is approximately 6-fold higher than bupropion itself. It is thought that this metabolite plays a functional role in bupropion's therapeutic action as an anti-depressant (Ascher et al., 1995; Bondarev et al., 2003; Martin et al., 1990). GlaxoSmithKline even developed this metabolite as a separate drug called radafaxine, but discontinued development in 2006 due to a poor side-effect profile (Halford, 2006). It is pertinent to note that not all of bupropion's metabolites, specifically HB, are produced in significant quantities in rats (Bondarev et al., 2003; Suckow et al., 1986; Welch et al., 1987a).

In the late 1970s, bupropion was identified as a potential target for the treatment of depression (Fabre & McLendon, 1978; Fann et al., 1978; Lineberry et al., 1990; Zung et al., 1983) and, subsequently (1989), an immediate release formulation was approved for the treatment of major depressive disorder (Wellbutrin™). In 1996, a sustained release formulation (Wellbutrin-SR™) was also approved as an atypical antidepressant. In 1997, following reports that bupropion elicited “anti-smoking” effects in the clinical depression trials, bupropion slow release was approved as a smoking cessation aid (Zyban™; Ferry & Johnston, 2003; Fossati et al., 2007; Hurt et al., 1997; Jorenby et al., 1999; Lerman et al., 2004; Tashkin et al., 2001; Tonstad et al., 2003). An extended-release formulation (Wellbutrin-XL™) became available in 2003.

It is now known that bupropion is also an antagonist at nicotinic acetyl choline receptors (Arias, 2009; Miller et al., 2002; Slemmer et al., 2000). Bupropion is found to block nicotine-induced antinociception, hypothermia, hypomotility and seizure activity (Damaj et al., 1999; Slemmer et al., 2000). It is thought to aid smoking cessation via the attenuation of acute nicotine effects, thereby reducing the level of reinforcing effects and craving (Brody et al., 2004; see review: Warner & Shoaib, 2005).

Bupropion has also been used in the treatment of: mania (Shopsin, 1983; Wright et al., 1985); bipolar disorder (Haykal & Akiskal, 1990; Sachs et al., 1994); seasonal affective disorder (Dilsaver et al., 1992; Modell et al., 2005); attention deficit/hyperactivity disorder (Barrickman et al., 1995; Conners et al., 1996; Wender & Reimherr, 1990; Wilens et al., 2005); bulimia (Horne et al., 1988); and obesity (Anderson et al., 2002; Gadde et al., 2001; Greenway, Whitehouse, et al., 2009; Jain et al., 2002).

5.1.1 Bupropion and Appetite Regulation

Early depression trials demonstrated that bupropion did not elicit the weight gain typically seen with other antidepressants (Gardner & Johnston, 1985; for review see: Fava et al., 2005). Furthermore, smoking cessation studies identified that bupropion treatment attenuated post-cessation weight gain (Hurt et al., 1997; Jorenby et al., 1999; Tashkin et al., 2001; Wilcox et al., 2010), an effect lasting up to 2 years (Hays et al., 2001) and persisting beyond the cessation of treatment (Padwal, 2009; Rigotti et al., 2000).

Subsequent preclinical studies showed that bupropion administration reduces food intake in rodents (Billes & Cowley, 2007; Greenway, Whitehouse, et al., 2009; Stairs & Dworkin, 2008; Zarrindast & Hosseininia, 1988), in addition to increasing

energy expenditure (Hasegawa et al., 2005), reducing body temperature (Zarrindast & Abolfathiaraghi, 1992), and increasing thermogenesis (Billes & Cowley, 2007; Liu et al., 2004). Furthermore, human experimental studies showed that bupropion treatment reduced appetite (Chouinard, 1983; Crone & Gabriel, 2004; Musso et al., 1993), and cravings (Jain et al., 2002). Interestingly, however, some studies have failed to demonstrate that bupropion reduces food intake (Hartotruax et al., 1983; Liu et al., 2002; Liu et al., 2004; Miller & Griffith, 1983).

Bupropion-induced appetite suppression is thought to be mediated by effects on dopamine. Evidence demonstrates that drugs that increase dopamine concentrations suppress hunger (Towell et al., 1988), while blockade of dopamine D_1 and D_2 receptors increases food intake and meal size (Clifton et al., 1991). This is consistent with evidence that dopamine levels are suppressed in obese patients (Wang et al., 2001). Furthermore, selective activation of the D_2 receptor reduces NPY mRNA expression in the ARC and increases that of POMC mRNA (see Section 1.2.2), whereas antagonism of the D_2 receptor causes the opposite effect (Pelletier & Simard, 1991; Tong & Pelletier, 1992). Unsurprisingly, therefore, bupropion-induced hypophagia can be blocked by D_2 antagonist, sulpiride (Zarrindast & Abolfathiaraghi, 1992). This supports the role of bupropion as a dopamine reuptake inhibitor, a regulator of appetite, and therefore a potential treatment for obesity.

5.1.2 Bupropion and Non-Appetite Regulatory Behaviours

5.1.2.1 Reward

Catecholamine reuptake inhibitors not only decrease standard chow intake but have also been found to decrease intake of more palatable and rewarding high-fat diets (Billes & Cowley, 2007). In addition, bupropion dose-dependently reduces sucrose- (Rauhut et al., 2003) and food- (Bruijnzeel & Markou, 2003; Stairs & Dworkin, 2008) maintained responding, is self-administered (Bergman et al., 1989; Lamb & Griffiths, 1990; Tella et al., 1997), induces conditioned place preference (CPP; Ortmann, 1985), and substitutes for drugs such as cocaine, amphetamine, methamphetamine, methylphenidate and diethylpropion (see review: Dwoskin et al., 2006). Therefore, bupropion could act to increase the reinforcing effects of food consumption, similar to the perceived mechanism of action for smoking cession.

5.1.2.2 Psychomotor Stimulation

Importantly, bupropion has been found to produce significant psychomotor stimulation in preclinical models. This is characterised by increased levels of

locomotion (Billes & Cowley, 2007; Bourin et al., 1998; Carrasco et al., 2004; Cooper, 1980; Gomez et al., 2008; Howard et al., 1978; Martin et al., 1990; Nielsen et al., 1986; Paterson et al., 2010; Redolat, Gomez, et al., 2005; Redolat, Vidal, et al., 2005; Santamaria & Arias, 2010; Sidhpura et al., 2007; Soroko et al., 1977) and sniffing behaviour (Billes & Cowley, 2007; Muley et al., 1984; Zarrindast et al., 1996; Zarrindast & Hosseininia, 1988). Additionally, bupropion has been shown to reverse tetrabenazine-induced sedation (Cooper et al., 1994). Tetrabenazine is commonly used for the treatment of hyperkinetic disorders, and is thought to act via the degradation of monoamines such as dopamine. Thus, bupropion-induced locomotion is thought to be mediated by increases in dopamine function (Santamaria & Arias, 2010; Sidhpura et al., 2007; Zarrindast & Minaian, 1991).

It has been argued that the anorectic and psychomotor responses to bupropion are independent phenomena (Billes & Cowley, 2007; Cooper, 1980; Zarrindast & Minaian, 1991). For example, Billes & Cowley (2007) found that incremental increases in bupropion dose produce independent changes in locomotion activity and intake (Billes & Cowley, 2007). Despite this evidence, however, both effects are normally seen at similar dose levels.

5.1.3 Bupropion and Therapeutic Potential for Treatment of Obesity

Bupropion's effects on appetite and weight gain have since been supported by randomised, double-blind, placebo-controlled studies (Anderson et al., 2002; Gadde et al., 2001; Jain et al., 2002). A meta-analysis revealed a mean weight loss of 2.66 kg (Li et al., 2005), an effect maintained for up to one year (Croft et al., 2002).

In the initial trials, overweight and obese patients taking bupropion (~350mg/day) lost 4.9% baseline bodyweight, producing significantly higher weight loss than that seen in the placebo group (-1.3 %) after 8 weeks and, after 24 weeks, completers had lost 12.9% of baseline bodyweight (Gadde et al., 2001). A similar, 24 week trial (Anderson et al., 2002), found that patients who completed the bupropion treatment (400mg/day) lost 10.1% of baseline bodyweight, significantly higher than placebo (-5.0%). At 56 weeks (Croft et al., 2002), patients who completed the bupropion treatment (300mg/day) had lost 1.5kg, an effect significantly higher than placebo (-0.02kg).

In line with earlier evidence from depressed patients (Croft et al., 2000; Settle et al., 1999), a trial with obese patients exhibiting depressive symptoms (as indicated by BDI-II scores between 10 and 30; Jain et al., 2002) found that completers in the

bupropion treatment group (sustained release; 400mg/day) produced weight loss of -5.8kg (~6.0% baseline) over a 6 month period (Placebo, -2.8kg). Furthermore, those treated with bupropion reported significantly greater reductions in craving and hunger than did those treated with placebo. Evidence from a more recent study (White & Grilo, 2013) with binge eating disorder patients also found bupropion treatment (300mg/day) to significantly increase net weight loss (-1.68kg) compared to placebo during an 8 week trial.

Despite evidence that bupropion can produce mild weight loss (average 2.8 kg over 24-52 weeks), equivalent to that seen with sibutramine and orlistat (Anderson et al., 2002; Gadde et al., 2001; Jain et al., 2002), it falls short of the current regulatory criterion (a minimum 5 kg weight loss) for marketing approval (Heal et al., 2009; Kennett & Clifton, 2010).

5.2 The combination of Bupropion and Naltrexone

5.2.1 Naltrexone vs. Naloxone

As discussed in Chapter 4, naltrexone is an opioid receptor antagonist primarily used for the treatment of opioid addiction (Herz, 1997; Minozzi et al., 2011) and alcohol dependence (Garbutt, 2010; Garbutt et al., 1999), but which is also associated with appetite regulation (Lee & Fujioka, 2009).

There are known pharmacokinetic and pharmacodynamic differences between naloxone and naltrexone (Goldstein & Naidu, 1989; Magnan et al., 1982; Raynor et al., 1994; Tepperman et al., 1983). Although receptor selectivity is the same between the two opioid antagonists ($\mu \geq \kappa \geq \delta$; Goldstein & Naidu, 1989; Magnan et al., 1982; Raynor et al., 1994), the primary difference is the half-life. Naloxone has a half-life of ~1.5hours, whereas naltrexone has a half-life of up to 4 hours (Berkowitz et al., 1976). This makes it a more viable option for clinical treatment.

In contrast to the developments with naloxone (see Chapter 4), comparatively little is known about the behavioural selectivity of the anorectic response to naltrexone. It is known that, similar to naloxone, the longer-action opioid antagonist naltrexone reduces motivation to eat post-ingestion (Kirkham & Blundell, 1986), an effect not explained by locomotor impairment (Cooper & Turkish, 1989). Despite this, and evidence that it acutely suppresses appetite in humans, naltrexone has not by itself proven clinically useful in the management of obesity (Yeomans & Gray, 2002).

5.2.2 The Combination; Contrave™

Previous evidence had implicated naltrexone in the reduction of post-cessation weight gain in smokers (King et al., 2006; King et al., 2013; Krishnan-Sarin et al., 2003; O'Malley et al., 2002; Omalley et al., 1992). Therefore, it seemed logical to assess the combination of bupropion and naltrexone for the treatment of weight concerned smokers. Disappointingly however, these studies found no significant differences in weight gain (Toll et al., 2008; Wilcox et al., 2010).

The combination was then considered within the domain of obesity research when Greenway (2009) proposed that bupropion's influence on food intake and weight gain may be self-limiting due to an opioid-mediated (β-endorphin) negative feedback effect on POMC neurons in the ARC (Cowley et al., 2001; Greenway, Whitehouse, et al., 2009). The co-administration of an opioid antagonist, such as naltrexone, should inhibit this negative feedback, augmenting the stimulation of POMC neurons, with the aim of overcoming the plateau demonstrated in previous studies using bupropion alone (Anderson et al., 2002). Electrophysiological recordings of POMC neurons successfully demonstrated a positive effect of naltrexone on bupropion-induced POMC firing rates (Greenway, Whitehouse, et al., 2009).

The above hypothesis received support from initial animal and human studies (Greenway, Dunayevich, et al., 2009; Greenway, Whitehouse, et al., 2009) and the combination, now labelled Contrave™, underwent development with Orexigen Therapeutics Inc as a potential treatment for obesity. The combination demonstrated an additive inhibition of intake in obese (but not lean) mice. One interpretation of this finding is that basal activity of POMC neurons is lower in obese mice, possibly due to obesity-induced leptin resistance in POMC neurons (Cowley et al., 2001). The clinical proof-of-concept study (16 weeks) demonstrated greater weight loss with the combination of 50mg/day naltrexone plus 300mg/day bupropion (-3.7%) than with placebo (-0.6%) or either drug treatment alone (-1.7% naltrexone; -3.2% bupropion). A longer 24 week trial (Greenway, Dunayevich, et al., 2009) demonstrated stronger results, with the combination of naltrexone (16mg/day or 32mg/day) plus bupropion (400mg/day) producing significant reductions in baseline weight (-5.4%) compared to the placebo group (-0.8%) or either drug treatments alone (-1.2% naltrexone 48mg/kg; -2.7% bupropion).

The Orexigen Therapeutics Contrave™ Obesity Research (COR) Programme consists of four phase III, 56-week trials (COR-I, COR-II, COR-BMOD and COR-Diabetes; see review: (Katsiki et al., 2011). The first (COR-I; Greenway et al. 2010)

employed 1482 women and 260 men. Weight loss started at week 4 and continued to be significantly higher than placebo up to week 56. The 870 completers lost significantly more baseline bodyweight (8.1%, 32mg naltrexone plus bupropion; -6.7%, 16mg naltrexone plus bupropion) compared to placebo (-1.8%). The study found significant improvements in a range of parameters including; waist circumference, insulin resistance, cholesterol, triglycerides, physical function, self-esteem, sexual life, public distress and measures of food craving and eating control. The naltrexone plus bupropion treatment also reduced blood pressure although, interestingly, failed to reduce heart rate, despite a drop seen in the placebo group.

The COR-II study (Apovian et al., 2013; Rubino et al., 2010) used 1496 patients, and produced similar findings to the first phase III trial. The patients taking the combination treatment lost significantly more baseline bodyweight (6.4%) compared to placebo (-1.2%). 56.3% of patients receiving the combination treatment lost a clinical meaningful amount of weight (≥5% of baseline bodyweight; placebo, 17.1%).

The COR-BMOD study (Wadden et al., 2011) assessed naltrexone (32mg/day) plus bupropion (360mg/day) as an adjunct to intensive behaviour modification. Completer data showed that those in the combination plus behaviour modification group produced a greater percentage baseline weight loss (-11.5%) than those in the placebo plus behaviour modification group (-7.3%). Again there were significant improvements, compared to placebo, in measures of waist circumference, plasma triglycerides, insulin resistance, physical function, and self-esteem.

The COR-Diabetes study (Hollander et al., 2010) assessed the combination in overweight/obese subjects with type 2 diabetes. Week 56 saw the combination produce reductions in baseline bodyweight (-5.0%) significantly higher than seen with placebo (-1.8%), alongside significant improvements (vs. placebo) in waist circumference, plasma triglycerides and insulin resistance.

Overall, the results of the COR programme results demonstrate that Contrave™ is an effective combination for weight loss (see review: Padwal, 2009; Plodkowski et al., 2009). It also shows improvements in some cardiovascular and mood measures. Despite this, there are still some concerns regarding elevated systolic and diastolic blood pressure and pulse rate (Katsiki et al., 2011). As it was denied approval by the FDA in 2011, Contrave™ is now being evaluated in the Light Study, a multicentre, randomized, double-blind, placebo-controlled long-term research trial designed to further assess the cardiovascular health outcomes of Contrave™.

5.3 Rationale: Chapter Four

Although co-treatment with bupropion and naltrexone has been reported (in rodents & obese humans) to produce significantly greater reductions in intake and bodyweight than either drug given alone (Contrave™; Greenway, Dunayevich, et al., 2009; Greenway et al., 2010; Greenway, Whitehouse, et al., 2009; Wadden et al., 2011; Wadden et al., 2009), comparatively little is known about the effects of these agents, either alone or together, on behaviour within the feeding context.

In view of this gap in the literature, the aims of the studies reported in Chapter 5 are to employ BSS methodology (review: Rodgers et al., 2010) to systematically and comprehensively profile the effects of bupropion and naltrexone, alone and in combination, on ingestive and non-ingestive behaviours in non-deprived male rats exposed to palatable mash.

5.4 Experiment Four; Bupropion Dose-Response

5.4.1 Method

For the main methodological details, refer to General Methods (Chapter 3).

5.4.1.1 Subjects and Design

10 adult male Lister hooded rats (209.37 ± 1.91g on arrival from Charles River, U.K and 397.02 ± 9.46g by the end of the study) were employed for this study. A within-subjects design was adopted whereby each subject received all four experimental conditions according to a Latin Square (with a 3-day wash out period): Vehicle (V); Bupropion 10mg/kg (B10); Bupropion 20mg/kg (B20); and Bupropion 40mg/kg (B40).

As bupropion is eliminated rapidly in the rat (Suckow et al., 1986), with an elimination half-life between 11-14hours (Bryant et al., 1983; Welch et al., 1987b), a 3-day wash out period was considered acceptable.

5.4.1.2 Drugs

Bupropion hydrochloride (Sigma-Aldrich, Poole, UK) was dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. Doses of bupropion (10, 20 and 40 mg/kg) were selected based on previous research on food intake to span the full range from ineffective to sub-maximal (Billes & Cowley, 2007; Greenway, Whitehouse, et al., 2009; Stairs & Dworkin, 2008; Zarrindast & Hosseininia, 1988). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg 30 minutes prior to testing

5.4.1.3 Procedure

Testing occurred over two weeks, with four test days per week, 5 animals tested per day, and a 3 day wash-out period. Treatment order was counterbalanced both within and between test days according to the Latin Square.

5.4.2 Results

Full statistical details can be found in Appendix 5. It should be noted that, as animals did not show appreciable amounts of scratching during Experiment 4, data for these variables are not reported.

5.4.2.1 Habituation Phase Food Intake

Mash consumption differed significantly over the course of habituation ($F(4,36) = 18.86$, $p < 0.001$), with intake on the first two trials predictably lower ($p \leq 0.01$) than

on the remaining trials (trial 1: 9.86 ± 0.80g; trial 2: 12.42 ± 0.59g; trial 3: 17.15 ± 0.77g; trial 4: 16.90 ± 1.97g; trial 5: 19.86 ± 1.17g). Although intake on the first trial differed significantly from trials 3, 4 and 5 ($p \leq 0.01$), and intake on trial 2 from trials 3 and 5 ($p \leq 0.01$), the development of a stable intake pattern was confirmed by the lack of difference across trials 3-5. Further emphasised by the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (17.89 ± 1.08g).

5.4.2.2 Test Day Bodyweight

Test-day bodyweights were comparable across the various treatment conditions (V: 366.3 ± 10.9g; B10: 369.2 ± 7.4g; B20: 370.4 ± 8.4g; B40: 370.4 ± 9.0g ($F(3,27) = 0.17$, $p > 0.05$).

5.4.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.17% throughout the experiment (range = 0.15 - 0.21%). Treatment effects on food intake are summarised in Figure 5-1. There was a significant main effect of bupropion on food intake ($F(3,27) = 7.03$, $p = 0.001$). Bonferroni comparisons showed a significant suppression (21% reduction relative to vehicle control, $p < 0.03$) only at the highest dose tested (40 mg/kg).

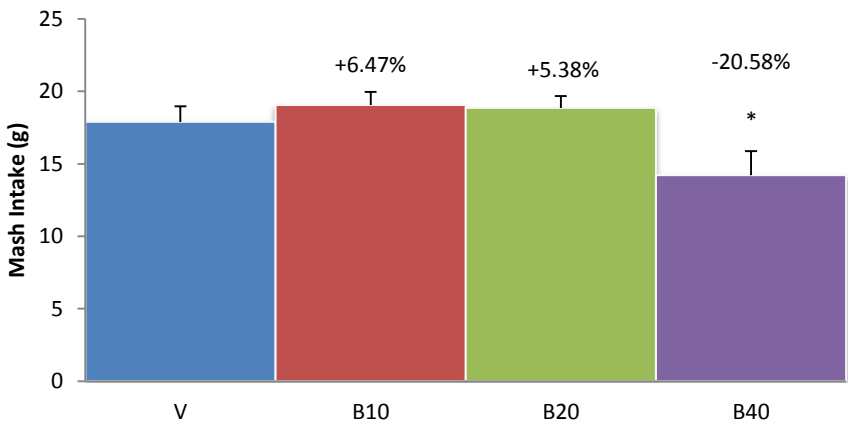


Figure 5-1: Experiment Four. Effects of acute bupropion on mash intake by Non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = Vehicle, B10 = Bupropion 10 mg/kg; B20 = Bupropion 20 mg/kg; B40 = Bupropion 40 mg/kg. See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus V

5.4.2.4 Total (one-hour) Behavioural Analyses

Treatment effects on total 1-h frequency and duration scores for ingestive and non-ingestive behaviours are illustrated in Figure 5-2, while data for eating-related parameters are summarised in Table 5-1.

ANOVA revealed significant treatment effects on the average duration of eating bouts ($F(3,27) = 7.19$, $p < 0.001$), the frequency and duration of eating ($F(3,27) \geq 11.59$, $p \leq 0.001$), resting ($F(3,27) \geq 4.58$, $p \leq 0.01$), locomotion ($F(3,27) \geq 13.71$, $p \leq 0.002$) and sniffing ($F(3,27) \geq 11.72$, $p \leq 0.001$), and both the duration of grooming ($F(3,27) = 10.46$, $p < 0.001$) and the frequency of rearing ($F(3,27) = 18.15$, $p < 0.001$). Treatment did not significantly influence the frequency of grooming or the duration of rearing ($F(3,27) \leq 2.41$, $p \geq 0.05$), nor did it affect the latency of locate the food source, the latency to commence eating, or the average rate of eating ($F(3,27) \leq 1.40$, $p \geq 0.05$).

Table 5-1: Experiment Four. Acute effects of Bupropion on eating-related parameters (Mean ± SE). See text for further details. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 versus VV

Measure	Vehicle	Bupropion 10mg/kg	Bupropion 20mg/kg	Bupropion 40mg/kg
Latency to locate food (s)	7.60 ± 1.20	12.39 ± 3.15	9.02 ± 2.02	8.62 ± 2.97
Latency to eat (s)	34.13 ± 4.68	25.27 ± 6.29	18.83 ± 4.32	25.36 ± 7.18
Eat bout (s)	15.43 ± 2.45	17.81 ± 5.04	15.79 ± 4.24	6.57 ± 1.59**
Eat rate (g/min)	1.31 ± 0.05	1.41 ± 0.06	1.30 ± 0.06	1.49 ± 0.11

As shown in Table 5-1 and Figure 5-2, Bonferroni comparisons confirmed that behaviour was primarily affected at the highest dose of bupropion (40 mg/kg), which significantly reduced time spent eating ($p < 0.05$), the average duration of eating bouts ($p < 0.01$) and grooming duration ($p = 0.052$), while significantly increasing the frequency of eating and rearing ($p \leq 0.01$), and both the frequency and duration of locomotion and sniffing ($p \leq 0.03$). An increase in locomotion frequency was also observed at 20 mg/kg ($p = 0.021$)

Despite significant treatment effects on rest frequency and duration, and the apparent elimination of resting from the behavioural profile at the highest dose of bupropion (Figure 5-2), post-hoc tests revealed only a significant difference between drug doses (10 vs 40 mg/kg) and not between drug and vehicle control.

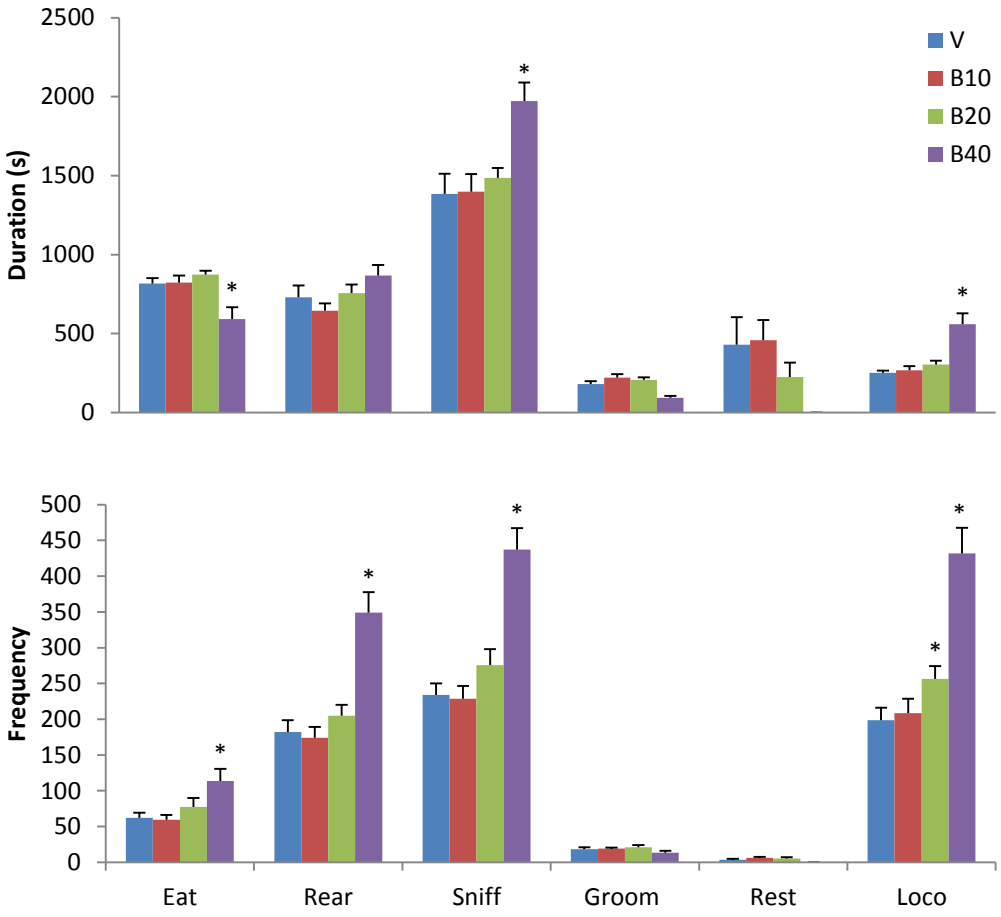


Figure 5-2: Experiment Four. Effects of acute bupropion on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = Vehicle, B10 = Bupropion 10 mg/kg; B20 = Bupropion 20 mg/kg; B40 = Bupropion 40 mg/kg. See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus V

However, as resting is normally absent during the first half of the test session (Figure 5-3), data were re-analysed focusing purely on the frequency and duration of resting during the second half of the session (timebins 7-12 inclusive). While significant treatment effects were confirmed (frequency: $F(3,27) = 5.18$, $p \leq 0.01$; duration: $F(3,27) = 4.58$, $p \leq 0.01$), the higher variability in these datasets once again precluded detection of a significant drug effect versus vehicle control.

5.4.2.5 Periodic (Timebin) Behavioural Analyses

Timebin analyses confirmed the normal temporal pattern of behaviour during the 1h test; a gradual reduction in most active behaviours and increase in resting as the session progressed (e.g. Ishii et al. 2003; Tallett et al. 2008a&b, 2009a&b). Thus, with the exception of groom frequency ($F(11,99) = 1.27$, $p > 0.05$), significant main

effects of time were found for the frequency ($F(11,99) \geq 7.18$, $p \leq 0.001$) and duration ($F(11,99) \geq 2.70$, $p \leq 0.005$) of all behavioural measures.

Significant treatment x time interactions were found for the duration of eating ($F(33,297) = 3.26$, $p < 0.001$), the frequency and duration of resting ($F(33,297) \geq 1.62$, $p \leq 0.02$); and the frequency of locomotion ($F(33,297) = 1.49$, $p = 0.05$).

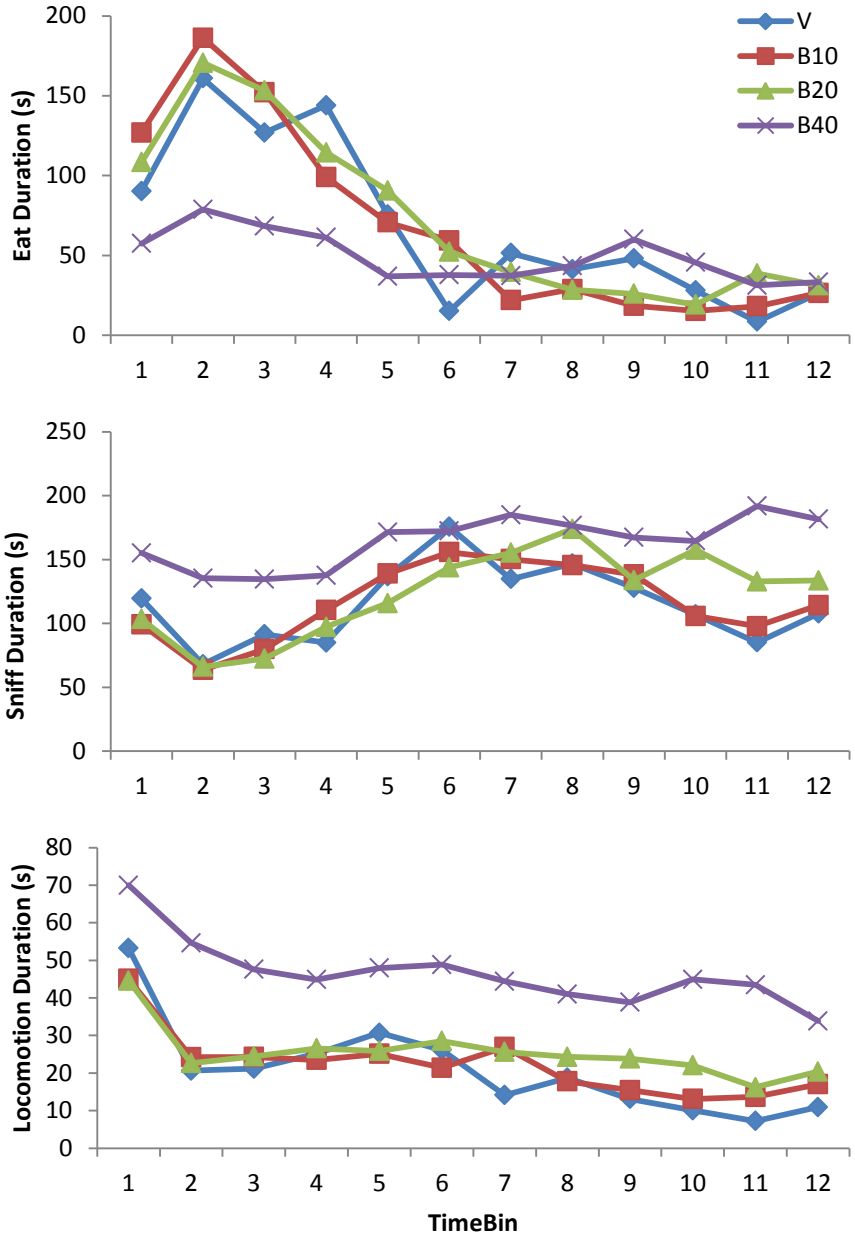


Figure 5-3: Experiment Four. Effects of acute bupropion on the timecourses of eating, sniffing and locomotion in male rats during a 1-h test with palatable mash

Data are expressed as the mean duration of each behaviour in 12 x 5-min timebin. V = Vehicle, B10 = Bupropion 10 mg/kg; B20 = Bupropion 20 mg/kg; B40 = Bupropion 40 mg/kg. See text for further details

A series of one-way ANOVAs within each timebin indicated that B40 significantly reduced time spent eating at several timepoints during the first half of the test session ($F(3,27) \geq 4.05$, $p \leq 0.02$), and increased both the frequency and duration of locomotion throughout the entire test (timebins 1-12; $F(3,27) \geq 4.03$, $p \leq 0.02$; see Figure 5-3).

Similar follow-up analyses of the treatment x time interactions for resting failed to reveal a significant effect of bupropion on rest frequency or duration at any individual timepoint. This outcome is consistent with that of the analyses for total resting scores (Figure 5-2).

5.4.2.6 Behavioural Satiety Sequence (BSS)

Treatment effects on the BSS are shown in Figure 5-4. Consistent with previous research (Ishii et al; 2003; Tallett et al. 2008a&b, 2009a&b), and work within the current thesis, the profile for the vehicle control (V; top left panel) shows a typical peak feeding response during the first half of the test. As resting gradually replaces eating as the predominant behaviour, a clear eat-to-rest transition can be seen around timebin 8. A similar pattern of behaviour is seen in the B10 or B20 conditions. However, at 40 mg/kg, behaviour is completely disrupted with the typical BSS replaced by high levels of locomotor activity, low levels of grooming, and a virtual absence of resting behaviour.

5.4.2.7 Bodyweight Gain

Data not shown. ANOVA failed to reveal a significant effect of treatment on 3-day absolute weight gain ($F(3,27) = 0.2$, $p > 0.05$) or daily percent bodyweight change ($F(3,27) = 0.44$, $p > 0.05$). As is typical, a main effect of day ($F(2,18) = 84.16$, $p < 0.001$) confirms treatment-independent general increase in bodyweight.

5.4.3 Summary of Main Findings

Experiment 4 confirmed that acute bupropion (40 mg/kg) suppresses food intake in rodents, a finding consistent with previous research (Billes & Cowley, 2007; Greenway, Whitehouse, et al., 2009; Stairs & Dworkin, 2008; Zarrindast & Hosseininia, 1988). However, at the same dose level (but not 10-20 mg/kg), the compound significantly suppressed grooming while markedly stimulating rearing, sniffing and locomotion. Interestingly, this behavioural activation even extended to the frequency of eating even though actual time spent eating was significantly reduced.

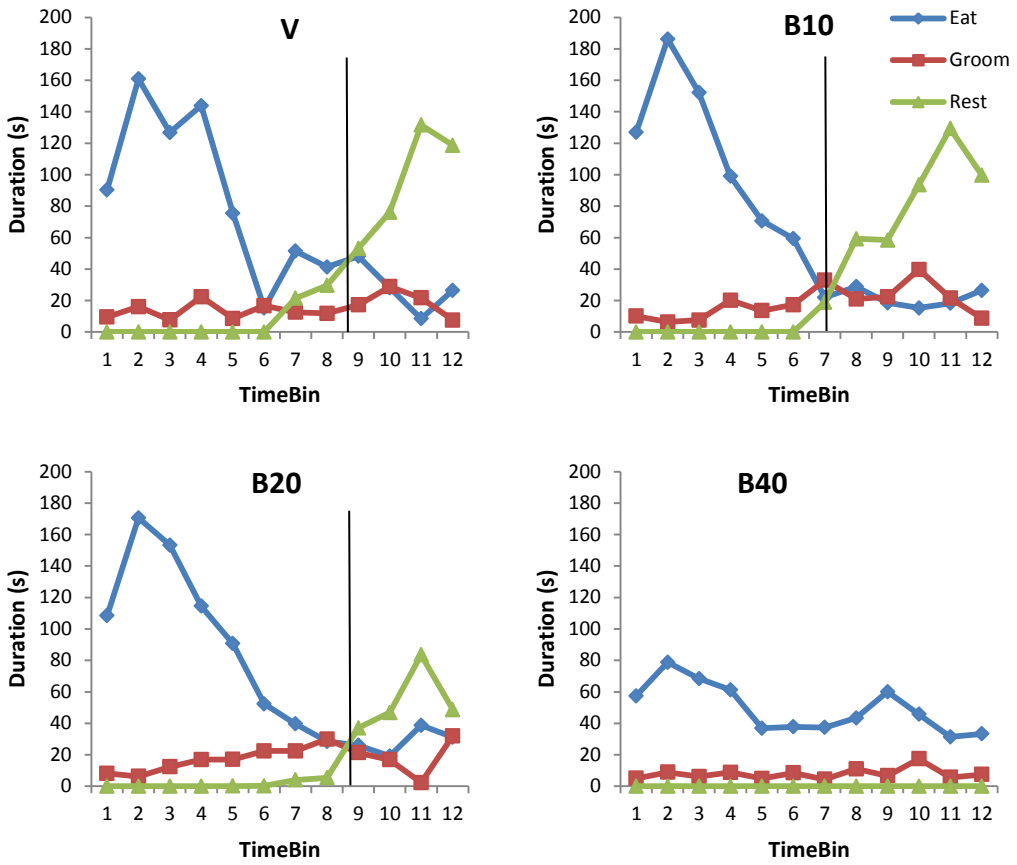


Figure 5-4: Experiment Four. Effects of acute bupropion on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. V = Vehicle, B10 = Bupropion 10 mg/kg; B20 = Bupropion 20 mg/kg; B40 = Bupropion 40 mg/kg. See text for further details

Although locomotor stimulation does not always lead to a suppression of food intake (e.g. Cooper & Vanderhoek, 1993; Vanrossum & Simons, 1969), it would seem parsimonious to argue that the acute anorectic effect of bupropion (40 mg/kg) in Experiment 4 may have been secondary to the marked increase in non-ingestive behaviours (however, see: Billes & Cowley, 2007).

5.5 Experiment Five; Naltrexone Dose-Response

5.5.1 Method

5.5.1.1 Subjects and Design

10 adult male Lister hooded rats (204.83 + 1.79g on arrival from Charles River, U.K and 418.07 + 8.23g by the end of the study) were employed for this study. A within-subjects design was adopted whereby each subject received all four experimental conditions according to a Latin Square (with a 7 day wash out period): Vehicle (V); Naltrexone 0.1mg/kg (Ntx0.1); Naltrexone 1.0mg/kg (Ntx1.0); and Naltrexone 3.0mg/kg (Ntx3.0).

5.5.1.2 Drugs

Naltrexone hydrochloride (Sigma-Aldrich, Poole, UK) was dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. Doses of naltrexone (0.1, 1.0 and 3.0 mg/kg) were selected based on previous research on food intake to span the full range from ineffective to sub-maximal (Apfelbaum & Mandenoff, 1981; Cooper & Turkish, 1989; Hadjimarkou et al., 2004; Jackson & Sewell, 1985a, 1985b; Kirkham & Blundell, 1986, 1987; Markskaufman & Balmagiya, 1985; Sanger & McCarthy, 1982). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg 15 minutes prior to testing

5.5.1.3 Procedure

Testing occurred over four weeks, with two test days per week and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square.

5.5.1.4 Error

Although apparently quite healthy, one rat failed to consume any significant quantity of mash during habituation week and was therefore excluded from the study.

5.5.2 Results

Full statistical details can be found in Appendix 6. It should be noted that, as animals did not show appreciable amounts of scratching during Experiment 5, data for these variables are not reported.

5.5.2.1 Habituation Phase Food Intake

Mash consumption differed significantly over the course of habituation ($F(4,32) = 19.23$, $p < 0.001$), with intake on trial 1 (10.28 + 0.77g) significantly lower ($p \leq$

0.005) than on trials 2-5 (trial 2: 15.35 ± 1.23g; trial 3: 17.56 ± 0.90g; trial 4: 17.82 ± 0.86g; trial 5: 16.52 ± 0.93g). The development of a stable intake pattern was confirmed by the lack of significant difference across trials 2-5, as well as the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (14.91 + 0.90g).

5.5.2.2 Test Day Bodyweight

Test-day bodyweights did not vary significantly across treatment conditions (vehicle: 370.7 ± 8.6g; Ntx0.1: 377.0 ± 8.5g; Ntx1.0: 382.6 ± 8.6g; Ntx3.0: 378.4 ± 7.5g: $F(3,24) = 0.77$, $p > 0.05$).

5.5.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.19% throughout the experiment (range = 0.15 – 0.33%). Treatment effects on food intake are summarised in Figure 5-5. Naltrexone dose-dependently suppressed 1h mash intake ($F(3,24) = 18.13$, $p < 0.001$). Bonferroni post-hoc analysis confirmed that this effect was statistically significant only at the highest dose tested (3.0 mg/kg; 56 % reduction relative to vehicle control, $p = 0.001$).

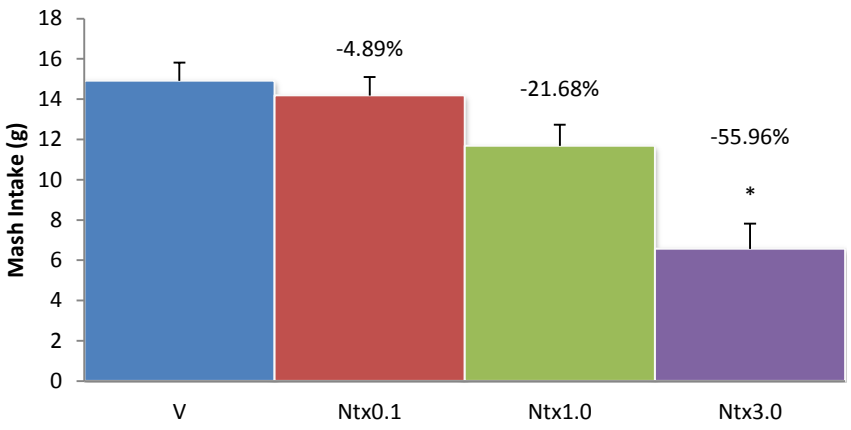


Figure 5-5: Experiment Five. Effects of acute naltrexone on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = vehicle, Ntx0.1 = naltrexone 0.1 mg/kg; Ntx1.0 = naltrexone 1.0 mg/kg; Ntx3.0 = naltrexone 3.0 mg/kg. See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus V

5.5.2.4 Total (one-hour) Behavioural Analyses

Treatment effects on total 1-h frequency and duration scores for ingestive and non-ingestive behaviours are illustrated in Figure 5-6, while data for eating-related parameters are summarised in Table 5-2.

Naltrexone did not significantly alter latencies to locate the food source or to commence eating ($F(3,24) \leq 1.60$, $p \geq 0.05$), nor did it significantly affect the duration of eating bouts or the rate of eating ($F(3,24) \leq 2.49$, $p \geq 0.05$). Nevertheless, it is interesting to note the trend towards a dose-dependent reduction in the rate of eating with naltrexone (Table 5-2).

Table 5-2: Experiment Five. Acute effects of naltrexone on eating-related parameters

(Mean ± SE). See text for further details. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus V

Measure	Vehicle	Bupropion 10mg/kg	Bupropion 20mg/kg	Bupropion 40mg/kg
Latency to locate food (s)	5.70 ± 2.25	5.29 ± 1.84	4.93 ± 1.95	6.65 ± 2.84
Latency to eat (s)	27.67 ± 6.57	57.84 ± 12.48	37.50 ± 11.43	41.28 ± 12.08
Eat bout (s)	10.24 + 1.15	11.12 ± 1.08	12.29 ± 1.13	13.25 ± 1.57
Eat rate (g/min)	1.42 ± 0.10	1.40 ± 0.08	1.25 ± 0.08	1.10 ± 0.12

However, ANOVA did reveal significant treatment effects of frequency and duration of eating ($F(3,24) \geq 9.99$, $p \leq 0.001$). ANOVA also, revealed significant treatment effects for the frequency and duration of: locomotion ($F(3,24) \geq 3.16$, $p \leq 0.05$), and rearing ($F(3,24) \geq 5.04$, $p \leq 0.008$), as well as the duration of resting ($F(3,24) = 6.63$, $p = 0.012$) and the frequency of sniffing ($F(3,24) = 6.53$, $p = 0.002$). Treatment did not significantly influence any other behavioural measure ($F(3,24) \leq 2.46$, $p \geq 0.05$).

Bonferroni post-hoc analysis demonstrated that naltrexone was behaviourally most effective at the highest dose tested (3.0 mg/kg) with significant reductions in both the frequency and duration of eating ($p \leq 0.01$) and the frequency of locomotion and sniffing ($p \leq 0.05$), as well as significant increases in the frequency and duration of resting ($p \leq 0.02$) (see Figure 5-6). The lower doses of the opioid receptor antagonist suppressed ($p \leq 0.03$) eat frequency at 1.0 mg/kg and sniff frequency at 0.1 mg/kg. Despite significant ANOVA main effects for the duration of locomotion, as well as the frequency and duration of rearing, post-hoc tests failed to identify any significant drug-vehicle differences for these measures.

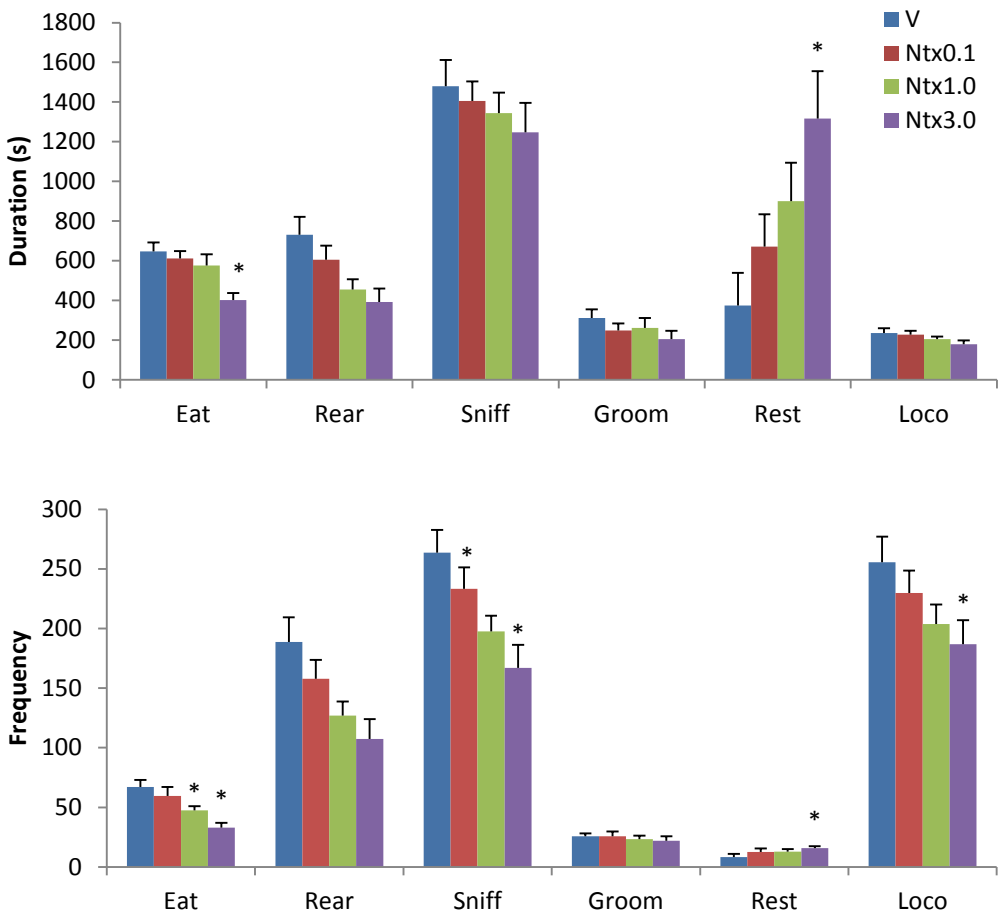


Figure 5-6: Experiment Five. Effects of acute naltrexone on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = vehicle, Ntx0.1 = naltrexone 0.1 mg/kg; Ntx1.0 = naltrexone 1.0 mg/kg; Ntx3.0 = naltrexone 3.0 mg/kg. * $p < 0.05$ versus V. See text for further details.

5.5.2.5 Periodic (Timebin) Behavioural Analyses

Timebin analyses confirmed the gradual reduction in most active behaviours and an increase in resting over the 1h test session (e.g. Ishii et al., 2003; Tallett et al., 2008a&b; 2009a&b). As expected, significant main effects of time were found for the frequency ($F(11,88) \geq 10.98$, $p \leq 0.001$) and duration ($F(11,88) \geq 7.41$, $p \leq 0.001$) of all behavioural measures, with the exception of the frequency and duration of grooming and scratching ($F(11,88) \leq 1.81$, $p > 0.05$).

Significant treatment x time interactions were found for the frequency and duration of eating ($F(33,264) \geq 1.60$, $p \leq 0.03$), rearing ($F(33,264) \geq 1.91$, $p \leq 0.01$) and sniffing ($F(33,264) \geq 1.54$, $p \leq 0.05$), as well as for the duration of resting ($F(33,264) = 1.60$, $p = 0.024$) and the frequency of locomotion ($F(33,264) = 1.58$, $p < 0.03$).

5.5.2.5.1 Treatment x Time Interaction Analysis

These significant drug x time interactions were further explored by a series of one-way ANOVAs within each timebin.

5.5.2.5.1.1 Eating

Significant treatment effects were found for eat frequency in timebins 1-3 ($F(3,24) \geq 3.85$, $p \leq 0.03$) and for eat duration in timebins 1-4 ($F(3,24) \geq 4.50$, $p \leq 0.02$). Somewhat unexpectedly, the lowest dose of naltrexone (0.1 mg/kg) significantly increased time spent eating in timebin 1 ($p \leq 0.05$) but reduced the frequency of eating in timebin 4 ($p < 0.01$), whereas the highest dose of naltrexone (3.0 mg/kg) reduced eat frequency in both timebins 1 and 4 ($p \leq 0.05$).

5.5.2.5.1.2 Resting

Naltrexone significantly affected rest duration in timebin 8 and timebin 9 ($F(3,24) \geq 5.00$, $p \leq 0.01$), with Bonferroni comparisons confirming significant enhancement of this measure by the highest dose of the compound ($p \leq 0.02$).

5.5.2.5.1.3 Locomotion

Significant effects of drug treatment were found for locomotion frequency in timebin 1 and timebin 8-9 inclusive ($F(3,24) \geq 3.40$, $p \leq 0.04$), with significant suppression evident at the lowest dose in timebin1 ($p < 0.03$), and at the highest dose in timebins 8 and 9 ($p \leq 0.03$).

5.5.2.5.1.4 Rearing

Significant effects of naltrexone were found for rear frequency in T3-9 inclusive ($F(3,24) \geq 3.48$, $p \leq 0.04$) and for rear duration in timebin 1 and timebin 5-9 inclusive ($F(3,24) \geq 3.24$, $p \leq 0.04$). Post-hoc tests showed that rear frequency was suppressed by naltrexone 1.0 mg/kg in T6 ($p < 0.02$) and by the highest dose in timebin 5, 8 and 9 ($p \leq 0.05$), while rear duration was suppressed by the lowest dose in timebin 1 ($p < 0.01$) and by the highest dose in timebin 5 ($p < 0.005$).

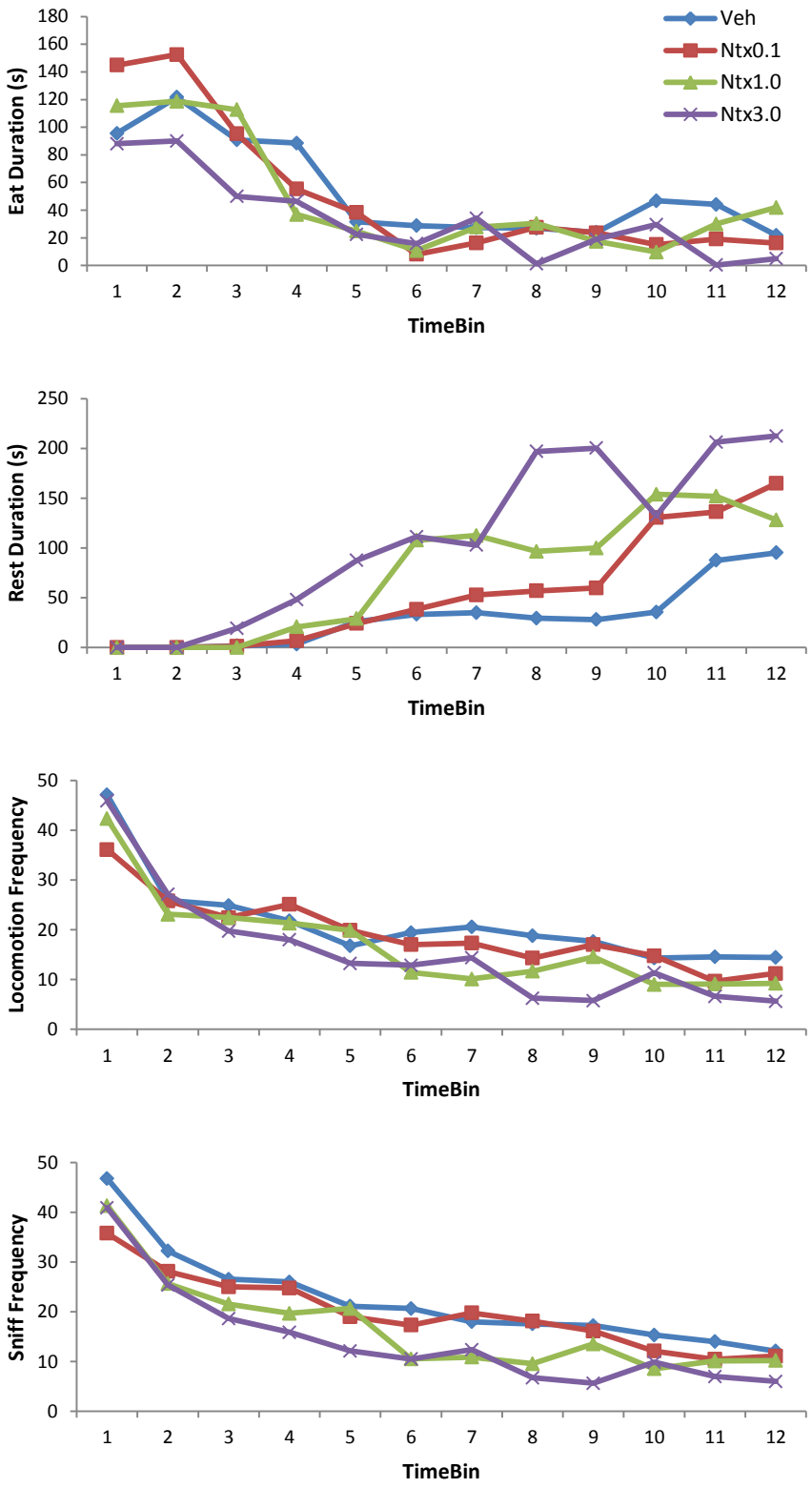


Figure 5-7: Experiment Five. Effects of acute naltrexone on the timecourses of eating, resting, locomotion and sniffing in male rats during a 1-h test with palatable mash

Data are expressed as the mean duration of each behaviour in 12 x 5-min timebin. V = vehicle, Ntx0.1 = naltrexone 0.1 mg/kg; Ntx1.0 = naltrexone 1.0 mg/kg; Ntx3.0 = naltrexone 3.0 mg/kg. See text for further details.

5.5.2.5.1.5 Sniffing

Significant treatment effects were found for sniff frequency in timebin 1 and timebins 3-9 inclusive ($F \geq 3.37$, $p \leq 0.04$) and for sniff duration in timebin 1, 9 and 12 ($F(3,24) \geq 3.62$, $p \leq 0.03$). Sniff frequency was significantly reduced by naltrexone 0.1 mg/kg in T1 ($p < 0.001$), by naltrexone 1.0 mg/kg in timebin 6 ($p < 0.01$), and by naltrexone 3.0 mg/kg in timebins 8 and 9 ($p \leq 0.02$). In addition, sniff duration was suppressed by naltrexone 3.0 mg.kg but only in timebin 9 ($p < 0.04$).

Overall, these temporal analyses indicate that, while naltrexone suppressed eating in the early part of the session, resting was increased and other behaviours inhibited somewhat later in proceedings – a profile consistent with an increase in and earlier onset of postprandial resting. Figure 5-7 illustrates the timecourse patterns for time spent eating and resting, as well as the frequency of sniffing, locomotion and rearing.

5.5.2.6 Behavioural Satiety Sequence (BSS)

The effects of naltrexone (0.1-3.0 mg/kg) on the BSS are summarised in Figure 5-8. As seen in previous studies, the vehicle profile (left top panel) shows a peak feeding response during the first 20 min of the test. Over time, resting gradually begins to replace eating as the predominant behaviour with the first clear eat-rest transition occurring circa timebin 6. Although the structural integrity of the BSS is fully maintained under all doses of naltrexone, Figure 5-8 shows a dose-dependent acceleration (shift to left) in the entire behavioural pattern, an effect most evident at the highest dose tested (3.0 mg/kg; timebin 4). It is particularly important to note that increased resting seen in response to naltrexone occurred after (and not before) the ingestion of food.

5.5.2.7 Bodyweight Gain

Data not shown. ANOVA failed to reveal a significant effect of naltrexone on 3-day absolute weight gain (vehicle: 8.63 + 1.77g; Ntx0.1: 8.26 + 1.14g; Ntx1.0: 8.93 + 0.94g; Ntx3.0: 7.46 + 0.69g; $F(3,24) = 0.32$, $p > 0.05$). Similarly, while the analysis of percent bodyweight change over days post-treatment confirmed a general increase in bodyweight over time (main effect DAY: $F(2,16) = 127.49$, $p < 0.001$), it too failed to support a significant main effect of treatment ($F(3,24) = 1.49$ $p > 0.05$) or a treatment x day interaction ($F(6,48) = 0.94$, $p > 0.05$).

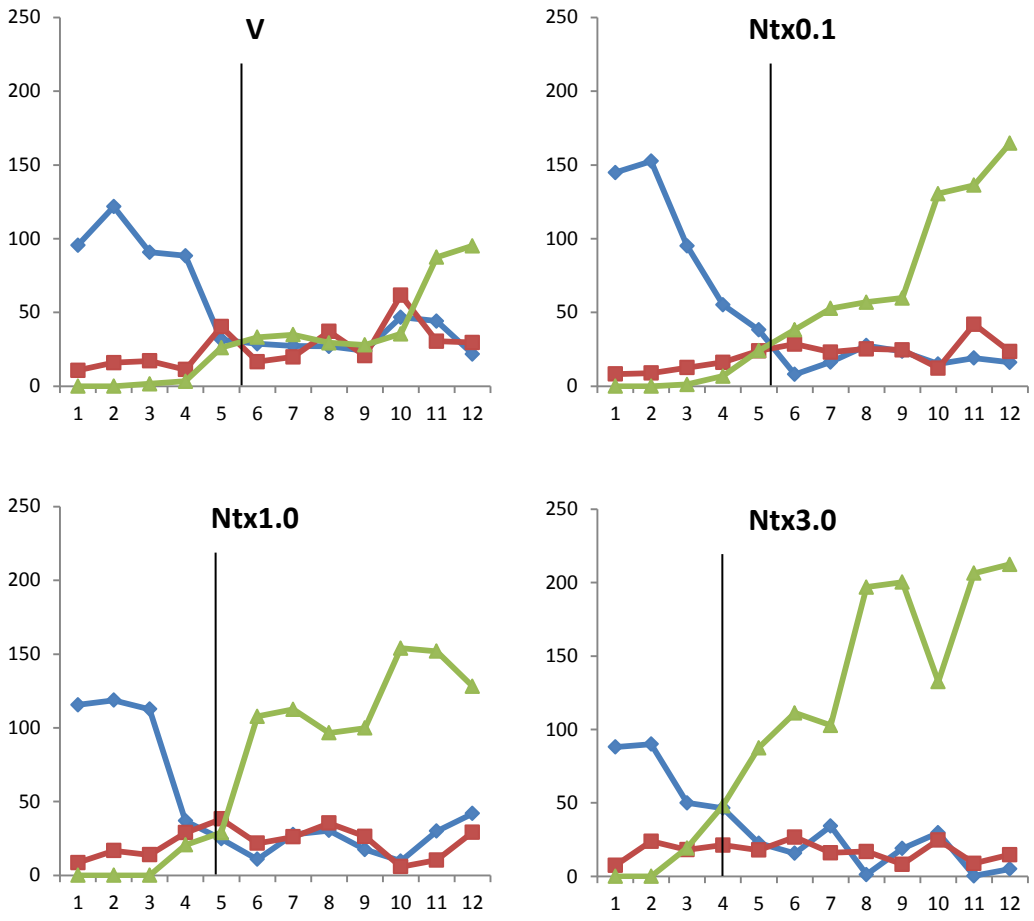


Figure 5-8: Experiment Five. Effects of acute naltrexone on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. V = Vehicle, Ntx0.1 = naltrexone 0.1 mg/kg; Ntx1.0 = naltrexone 1.0 mg/kg; Ntx3.0 = naltrexone 3.0 mg/kg. See text for further details

5.5.3 Summary of Main Findings

The results of Experiment 5 confirm that naltrexone dose-dependently reduced mash consumption (Apfelbaum & Mandenoff, 1981; Cooper & Turkish, 1989; Hadjimarkou et al., 2004; Jackson & Sewell, 1985a, 1985b; Kirkham & Blundell, 1986, 1987; Markskaufman & Balmagiya, 1985; Sanger & McCarthy, 1982), in addition to the frequency and duration of feeding behaviour (3.0mg/kg; Cooper & Turkish, 1989; Kirkham & Blundell, 1987; Tallett et al., 2008a). Consistent with previously reported effects for broad spectrum opioid antagonists (naloxone; Tallett et al., 2008a), naltrexone did not significantly alter the time taken to locate the food or to commence eating, nor did it affect the average duration of eating bouts or eating rate.

Naltrexone treatment also led to a modest acceleration (gradual shift to the left in the eat-to-rest transition) in the satiety sequence, without compromising behavioural structure. This profile for naltrexone is quite similar to that previously obtained with naloxone (Tallett et al. 2008). Somewhat unexpectedly, naltrexone was less potent than naloxone in terms of the minimum effective anorectic dose (3.0 mg/kg vs 1.0 mg/kg) but had a wider range of behavioural activity.

5.5.4 Design of Experiment Six

Based on the data from the bupropion dose response study (Experiment 4), a sub-anorectic dose of bupropion was selected. Experiment 4 confirmed that acute bupropion 20mg/kg did not significantly suppress food intake. Nor did it produce behavioural stimulation, as characterised by a significant increase in rearing, sniffing or locomotion. Therefore, a bupropion dose of 20mg/kg was selected for the interaction study.

Based on the data from the naltrexone dose response study (Experiment 5), a sub-anorectic dose and a threshold dose of naltrexone were selected. Experiment 5 confirmed that naltrexone 0.1 and 1.0mg/kg did not significantly supress food intake. While, these doses did lead to a small acceleration in the BSS, there was no evidence of behavioural disruption. Therefore, 0.1 and 1.0 mg/kg of naltrexone were selected for the interaction study.

5.6 Experiment Six; Bupropion (20mg/kg) and Naltrexone (1.0 and 0.1mg/kg) Interaction

5.6.1 Method

5.6.1.1 Subjects and Design

10 adult male Lister hooded rats (205.13 + 2.25g on arrival from Charles River, U.K and 444.32 + 9.51g by the end of the study) were employed for this study. A within-subjects design was adopted whereby each subject received all six experimental conditions according to a Latin Square (with a 7-day wash out period: Vehicle + Vehicle (Saline; VV); Vehicle + Naltrexone (0.1mg/kg; VNL); Vehicle + Naltrexone (1.0mg/kg; VNH); Bupropion (20mg/kg) + Vehicle (BV); Bupropion (20mg/kg) + Naltrexone (0.1mg/kg; BNL); and Bupropion (20mg/kg) + Naltrexone (1.0mg/kg; BNH).

5.6.1.2 Drugs

Bupropion hydrochloride and naltrexone hydrochloride (both sourced from Sigma-Aldrich, Poole, UK) were both dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. Doses of bupropion (20 mg/kg) and naltrexone (0.1, and 1.0 mg/kg) were selected based on data obtained in Experiments 4 and 5. All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml//kg either 30 minutes (bupropion or vehicle) or 15 minutes (naltrexone or vehicle) prior to testing.

5.6.1.3 Procedure

Testing occurred over four weeks, with two test days per week, and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square.

5.6.2 Results

Full statistical details can be found in Appendix 5. It should be noted that, as animals did not show appreciable amounts of scratching during Experiment 6, data for these variables are not reported.

5.6.2.1 Habituation Phase Food Intake

As expected, intake differed significantly over the course of habituation ($F(4,36) = 8.82$, $p < 0.001$), with intake on trial 1 (13.83 + 0.85g) significantly lower ($p \leq 0.05$) than on trials 2, 3 and 5 (trial 2: 17.58 ± 0.94g; trial 3: 19.00 ± 1.27g; trial 4: 19.29 ±

1.98g; trial 5: 20.36 ± 1.29g). The lack of significant difference across trials 2-5 confirmed the development of a stable intake pattern, as well as the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (19.30 ± 1.24g).

5.6.2.2 Test Day Bodyweight

Test-day bodyweights did not differ significantly across treatment conditions: VV: 399.2 ± 14.3g; VNL: 399.0 ± 12.4g; VNH: 401.1 ± 11.3g; BV: 403.9 ± 7.2g: BNL: 408.0 + 10.8g; BNH: 403.4 + 12.3g (main effect bupropion, $F(1,9) = 0.30$, $p \geq 0.05$; main effect naltrexone, $F(2,18) = 0.08$, $p \geq 0.05$; interaction: $F(2,18) = 0.05$, $p \geq 0.05$).

5.6.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.18% throughout the experiment (range = 0.09 - 0.35%). Treatment effects on food intake are summarised in Figure 5-9. ANOVA confirmed significant main effects for bupropion ($F(1,9) = 33.74$, $p < 0.001$) and naltrexone ($F(2,18) = 10.71$, $p = 0.005$), but no significant interaction ($F(2,18) = 0.64$, $p > 0.05$).

Post-hoc comparisons revealed that mash intake was significantly suppressed both by bupropion and the higher dose naltrexone (1.0mg/kg) when given alone ($p \leq 0.01$ vs VV), and by bupropion in combination with each dose of naltrexone ($p \leq 0.001$ vs VV). The apparently greater suppressant effect of co-administration is supported by significant differences between (i) the lower dose of naltrexone (0.1mg/kg) in the presence versus absence of bupropion (BNL vs VNL; $p < 0.01$), and (ii) bupropion in the presence versus absence of the higher dose of naltrexone (1.0mg/kg; BNH vs BV; $p < 0.05$). An additive anorectic effect of co-treatment is further supported by comparisons between the actual percentage reductions in intake (relative to VV control) for the treatment combinations and those predicted by simply adding the effects of the individual treatments: VNL (14.3%); VNH (26%); BV (23.1%); BNL (37.7% actual, vs 37.4% calculated); BNH (40.8% actual, vs 49.1% calculated).

5.6.2.4 Total (one-hour) Behavioural Analyses

Data for feeding-related measures are summarised in Table 5-3, while treatment effects on the total frequency and duration of ingestive and non-ingestive elements are shown in Figure 5-10.

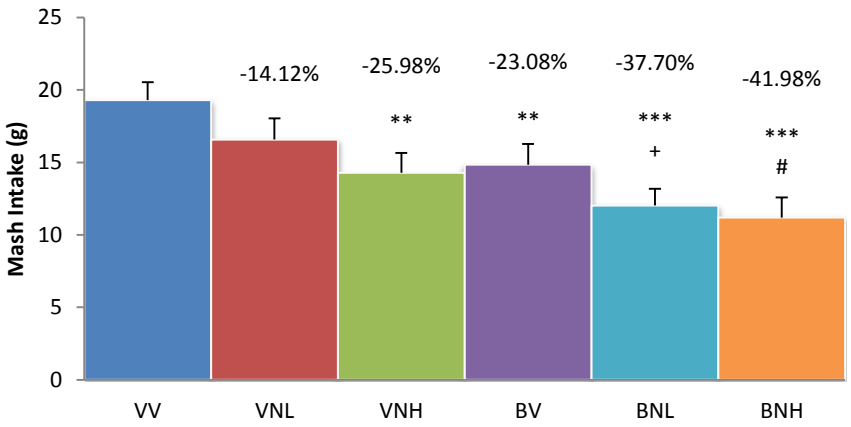


Figure 5-9: Experiment Six. Effects of bupropion and naltrexone, alone and in combination, on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = vehicle, B = bupropion 20 mg/kg NL = naltrexone 0.1 mg/kg; NH = naltrexone 1.0 mg/kg; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs VV; **+** $p < 0.01$ vs VNL; # $p < 0.05$ v BV. See text for full details.

Significant bupropion x naltrexone interactions were found for the frequency of rearing and sniffing and for time spent grooming ($F(2,18) \geq 10.42$, $p \leq 0.01$), while significant main effects of bupropion were found for the frequency of eating ($F(1,9) = 12.44$, $p \leq 0.01$), eat bout duration ($F(1,9) = 14.01$, $p = 0.005$), eating rate ($F(1,9) = 13.64$, $p = 0.005$), the duration of sniffing ($F(1,9) = 50.57$, $p < 0.001$), and both the frequency and duration of locomotion ($F(1,9) \geq 29.61$, $p \leq 0.001$). In addition, significant main effects of naltrexone were found for eating rate ($F(2,18) = 8.37$, $p = 0.003$), rear duration ($F(2,18) = 8.04$, $p < 0.005$), and both the frequency and duration of resting ($F(2,18) \geq 4.49$, $p \leq 0.05$). No other interactions or main effects were significant.

As summarised in Figure 5-10, bupropion increased the frequency of rearing and sniffing ($p \leq 0.01$, BV vs VV), effects that were blocked by the intrinsically-inactive higher dose of naltrexone ($p \leq 0.01$; BNH vs BV). Relative to VV control, BV also increased the frequency ($p < 0.01$) and duration ($p < 0.05$) of locomotion, effects that were non-significantly attenuated by co-administration of the higher dose of naltrexone. No significant pairwise contrasts were found in post-hoc followups to the reported main effects of bupropion on eat frequency, eat bout duration or sniff duration, or of naltrexone on rest frequency and rear duration. Although both bupropion and naltrexone tended to individually reduce the rate of eating relative to VV control, the largest effects were seen in animals receiving combined treatment (Table 5-3), with post-hoc comparisons confirming a significant reduction in eat rate

only for the combination of bupropion and the higher dose of naltrexone (BNL vs VV, $p < 0.05$). It is also worth noting that, the difference in eating rate between the latter condition and bupropion given alone (BV) approached statistical significance ($p = 0.072$). Finally, relative to VV control, the higher dose of naltrexone reduced the duration of grooming and increased the duration of resting ($p < 0.05$). Despite the observation that the latter effect of the opioid receptor antagonist was significantly attenuated by co-administration of bupropion (BNL vs VNL; $p < 0.05$), it should be noted that bupropion by itself (albeit non-significantly) reduced time spent resting and that a simple cancellation effect most probably occurred (Figure 5-10).

Table 5-3: Experiment Six. Effects of bupropion and naltrexone, alone and in combination, on mash intake and feeding-related parameters in male rats exposed for 1h to palatable mash

Data are presented as mean values (± SEM). s = seconds, g = grams. V = vehicle, B = bupropion 20 mg/kg NL = naltrexone 0.1 mg/kg; NH = naltrexone 1.0 mg/kg; * $p < 0.05$ vs VV. See text for full details.

Measure	VV	VNL	VNH	BV	BNL	BNH
Latency to locate food (s)	7.14 ± 1.74	7.90 ± 1.79	7.03 ± 2.19	11.95 ± 3.11	5.93 ± 1.93	7.87 ± 3.69
Latency to eat (s)	20.65 ± 3.87	28.33 ± 4.66	30.18 ± 7.35	32.10 ± 9.41	22.57 ± 4.06	18.90 ± 5.00
Eat bout (s)	11.12 ± 1.63	10.42 ± 1.36	9.97 ± 1.55	6.97 ± 1.12	9.26 ± 1.97	9.19 ± 1.80
Eat rate (g/min)	1.70 ± 0.07	1.66 ± 0.14	1.63 ± 0.13	1.66 ± 0.13	1.36 ± 0.15	1.15 ± 0.11*

5.6.2.5 Periodic (Timebin) Behavioural Analyses

As found in Experiment 5, significant main effects of time were found for the frequency ($F(11,99) \geq 8.03$, $p \leq 0.001$) and duration ($F(11,99) \geq 4.08$, $p \leq 0.001$) of all behavioural measures, with the exception of groom frequency ($F(11,99) < 1.13$, $p > 0.05$). Few parameters showed significant drug interactions involving time, the exceptions being 3-way (bupropion x naltrexone x time) interactions for the frequency of rearing and sniffing ($F(22,198) \geq 1.58$, $p \leq 0.05$), and a 2-way (bupropion x time) interaction for rest duration ($F(11,99)= 2.40$, $p = 0.01$). The naltrexone x time interaction for rest frequency also closely approached statistical significance ($F(22, 198) = 1.57$, $p < 0.06$).

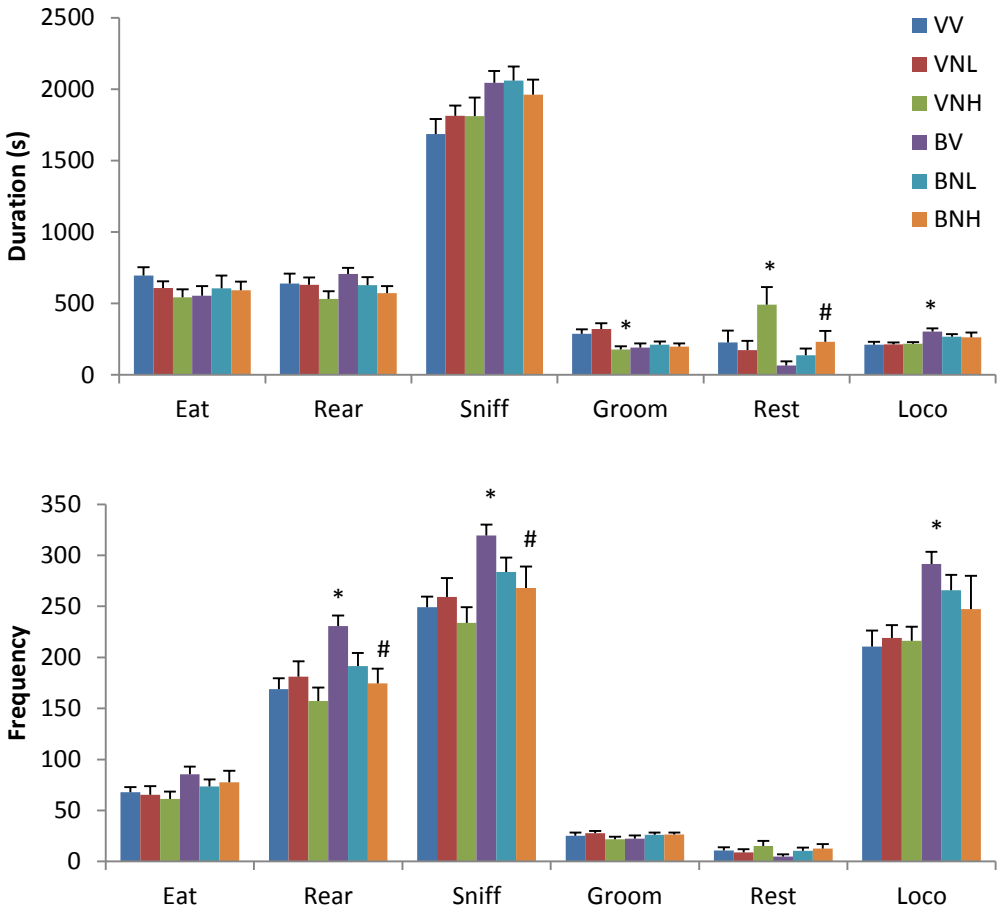


Figure 5-10: Experiment Six. Effects of bupropion and naltrexone, alone and in combination, on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = vehicle, B = bupropion 20 mg/kg NL = naltrexone 0.1 mg/kg; NH = naltrexone 1.0 mg/kg; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs VV; **+** $p < 0.01$ vs VNL; # $p < 0.05$ v BV. See text for full details.

Significant interactions were further explored by a series of two-way ANOVAs (& post-hoc tests) within each timebin. These analyses showed that bupropion alone significantly increased rear frequency in timebins 2, 3 and 9 (BV vs VV; $p \leq 0.03$), effects that were significantly blocked by the co-administration of the higher dose of naltrexone (BNH vs BV; $p \leq 0.05$). Similarly, sniff frequency was significantly increased by bupropion alone (BV vs VV) in timebins 1 and 2 ($p \leq 0.04$), with additional increases in timebins 3 and 8 closely approaching significance ($p \leq 0.07$). Given the pattern of results for rearing, it is interesting to note that the bupropion-induced increases in sniff frequency in timebins 1 and 3 were almost significantly attenuated by co-administration of the higher dose of naltrexone (BNH vs BV, $p \leq 0.08$). Despite the significant main effects and/or interactions for resting parameters (indicating overall increases under naltrexone & decreases under bupropion), high

within-timebin variance largely precluded detection of meaningful significant pairwise contrasts.

5.6.2.6 Behavioural Satiety Sequence (BSS)

Figure 5-11 illustrates the BSS profiles for each of the 6 treatment conditions. Although the absolute level of resting in the second half of the session was not as great as seen in earlier experiments, the control BSS profile (VV; top left panel) nevertheless shows the typical peak feeding response in the first 15-20 min of the test. Feeding gradually gives way to grooming and resting as time progressed, with an eat-to-rest transition occurring around 35min into the test. Although neither dose of naltrexone when given alone interfered with normal behavioural structure (centre & bottom left panels), VNH modestly accelerated the sequence (shift to the left) by suppressing the peak feeding response and producing an earlier transition to, as well as higher levels of, resting. Given alone, BV also suppressed the peak feeding response but virtually eliminated resting behaviour, a pattern consistent with behavioural disruption (top right panel). Interestingly, while still displaying a reduction in the peak feeding response, co-administration of either dose of naltrexone with bupropion (centre & bottom right panels) appeared to reinstate a more normal behavioural structure with eat-to-rest transitions once again discernible around 35-40min.

5.6.2.7 Bodyweight Gain

Data not shown. As with the previous dose-response experiments, ANOVA failed to reveal any significant main effects or interactions for 3-day absolute weight gain (main effect bupropion: $F(1,9) = 0.96$, $p > 0.05$; main effect naltrexone: $F(2,18) = 0.07$, $p > 0.05$; interaction: $F(2,18) = 0.24$, $p > 0.05$). Again the analysis of percent bodyweight c6hange over days post-treatment confirmed normal growth patterns (main effect day: $F(2,18) = 71.36$, $p < 0.001$), and it too failed to reveal any significant drug main effects or interactions (main effect bupropion: $F(1,9) = 2.28$, $p > 0.05$; main effect naltrexone: $F(2,18) = 0.31$, $p > 0.05$; interaction: $F(2,18) = 0.24$, $p > 0.05$).

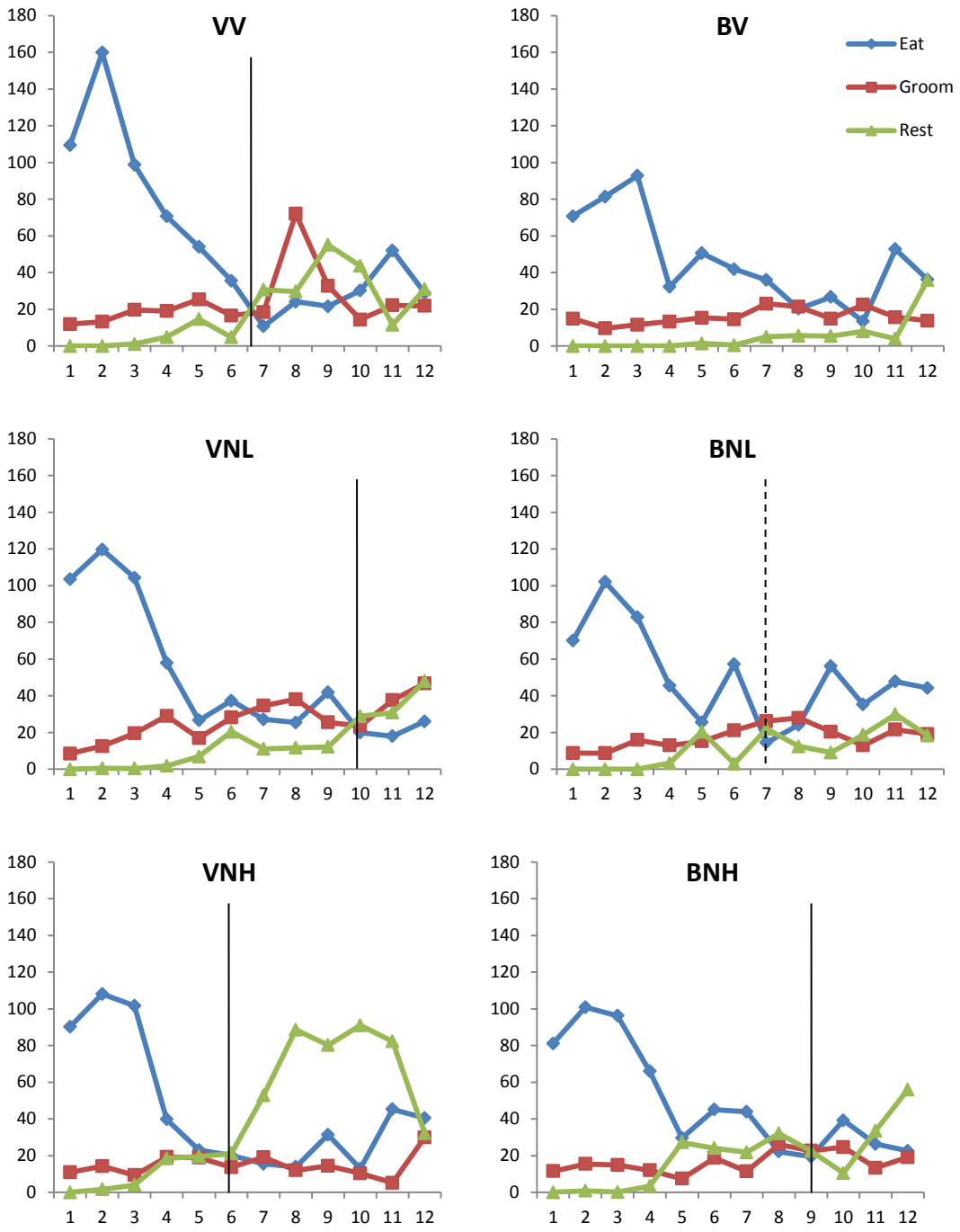


Figure 5-11: Experiment Six. Effects of bupropion and naltrexone, alone and in combination, on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. V = vehicle, B = bupropion 20 mg/kg NL = naltrexone 0.1 mg/kg; NH = naltrexone 1.0 mg/kg; See text for full details.

5.6.3 Summary of Main Findings

In Experiment 6, bupropion (20mg/kg) somewhat unexpectedly exerted significant anorectic activity by itself and produced evidence consistent with psychomotor stimulation (e.g. increased locomotion, rearing & sniffing). Furthermore, the higher naltrexone dose (1.0 mg/kg) produced a statistically significant reduction of intake. However, it is pertinent to note that the actual % suppression was very similar to Experiment 5 (22%, Experiment 5 vs. 26%, Experiment 6). Despite these profiles, the original aim of the study remained intact; to study the behavioural effects of these two drugs when co-administered at sub-maximal dose levels.

The results showed that the combinations produced significantly greater reductions in mash intake (37-41%) than those produced by either agent alone (14-26%). However, unlike the theoretically-predicted synergistic interaction for the bupropion and naltrexone combination (Greenway, Whitehouse, et al., 2009), our findings are indicative of only an additive interaction. The reductions seen in response to combined treatment closely matched those simply calculated by the addition of reductions in response to the constituent agents.

Interestingly, and unexpectedly, the higher dose of naltrexone (1.0 mg/kg) also counteracted the stimulant effects of bupropion (20 mg/kg) on locomotion, rearing and sniffing, an effect statistically significant for the latter two measures.

5.7 Chapter Five Main Findings

As comparatively little is known about the combination Contrave™, Chapter 5 aimed to comprehensively profile the effects of bupropion and naltrexone, alone and in combination, on ingestive and non-ingestive behaviours using BSS methodology.

- **Experiment 4** showed that the highest dose of bupropion (40mg/kg) suppressed intake and feeding behaviour. However, the same dose level (but not 10 or 20mg/kg) produced locomotor stimulation, characterised by marked stimulation of rearing, sniffing and locomotion behaviour.
- **Experiment 5** showed that naltrexone dose-dependently reduces food intake, and significantly suppressed the frequency and duration of feeding behaviour at the highest dose tested (3.0mg/kg). Naltrexone also produced a dose-dependent acceleration in the BSS, without evidence of behavioural disruption.
- **Experiment 6** showed that bupropion (20mg/kg) and the higher dose of naltrexone (1.0mg/kg), alone produced anorectic effects. The combination of bupropion and naltrexone produces a reduction in intake indicative of an additive interaction. Similar to the findings of Chapter 4, it was found the addition of the opioid receptor antagonist counteracted any undesirable side-effects; in this case the stimulant effects of bupropion. Interestingly, the latter finding indicates that the anorectic response to acute bupropion is not explained by its psychomotor stimulant property.

The work reported in Chapter 5 confirms the additive effect of naltrexone and bupropion combination Contrave™ (Greenway, Whitehouse, et al., 2009). It also shows that the addition of the opioid receptor antagonists counters the psychomotor stimulant effects of bupropion.

Chapter 6 Serotonergic and Opioid System Interactions

6.1 The Serotonergic System

The discovery of serotonin (5-hydroxytryptamine; 5-HT) was reported almost simultaneously by two separate research groups. In the 1930s, Page et al., identified the molecule “serotonin”, named after its origin in blood ‘serum’ and its effect on vascular muscle ‘tone’ (Rapport et al., 1948). Meanwhile, Erspamer discovered “enteramine”, a molecule secreted by the enterochromaffin cells of the GIT and found to contract vascular muscle (Erspamer, 1940; cited in Feldman et al., 1997). It wasn’t until the 1950s that it was confirmed that these molecules were one and the same (Erspamer & Asero, 1952).

Although widely distributed (Twarog et al., 1953), the location of 9 primary serotonergic cell bodies and pathways were identified using histofluorescence techniques by Dahlstrom and Fuxe in the 1960s (Dahlstrom & Fuxe, 1964), named B1 – B9, as can be seen in Figure 6-2. The serotonergic cells were located primarily within the dorsal raphe nucleus (Tork, 1990) and the reticular formation of the lower brain stem. The neurons are clustered into two groups: the caudal group (B1-4), projecting to the brainstem and spinal cord; and the rostral group (B5-9), projecting to the forebrain (Hornung, 2003).

Serotonin is an indoleamine neurotransmitter synthesised from tryptophan into 5-hyroxytryptophan (5-HTP) that is then decarboxylated at the terminal to produce serotonin. Serotonin is stored primarily in presynaptic vesicles and, on release, stimulates both pre- and post-synaptic receptors until it is reabsorbed or broken down to 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase (MAO; Halford et al., 2007). There are seven serotonin receptor families 5-$HT_{1–7}$ and, to the authors knowledge, currently 14 identified receptor subtypes (see reviews: Barnes & Sharp, 1999; Boess & Martin, 1994; Hoyer et al., 2002; Nichols & Nichols, 2008; see Table 6-1).

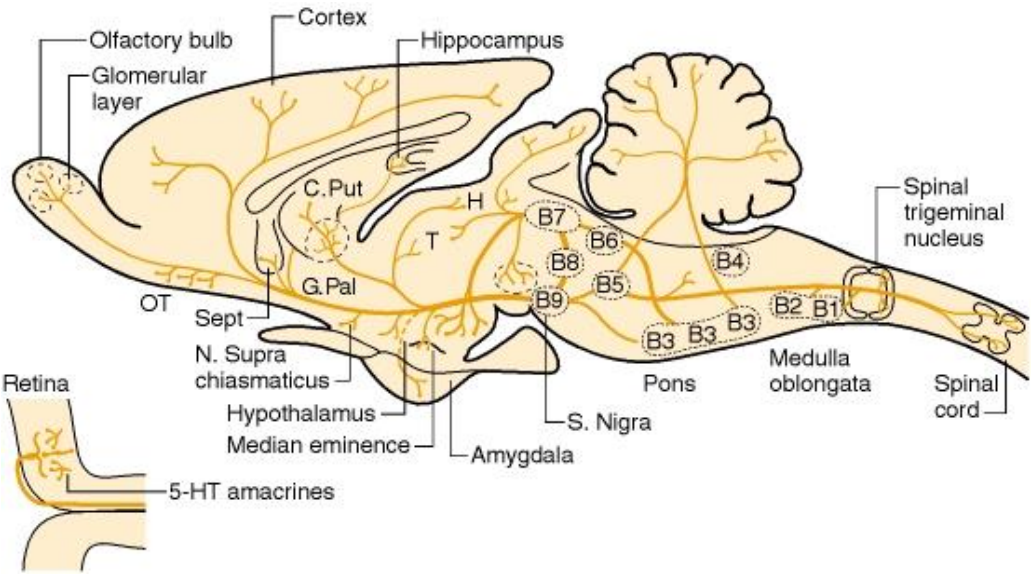


Figure 6-1: Schematic drawing depicting the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections.

OT, olfactory tuberculum; Sept, septum; C. Put, nucleus caudate-putamen; G. Pal, globus pallidus; T,thalamus; H, habenula; S. Nigra, substantia nigra. Source: Siegel et al., (1999)

6.1.1 Serotonin and Appetite Regulation

Serotonin was linked to the control of food intake and feeding behaviour 30 years ago, and now there are more data on the effects of serotonin on human appetite and rodent eating behaviour than for any other peripheral or central target (Halford et al., 2007).

As serotonin is a key factor in appetite regulation, its levels and function should reflect the organism's nutritional status, fed or fasted. For example, a nutritional deficit reduces endogenous serotonin and increases 5-HT receptor sensitivity. Evidence shows that malnourished animals have low serotonin levels (Barragan-Mejia et al., 2002) and the sensitivity of 5-HT_{2C} receptors appears to be increased following dieting (Cowen et al., 1996). Similarly, in humans, tryptophan levels are reduced after a 4-week hypocaloric diet (Wolfe et al., 1997) and 5-HIAA levels are reduced in anorexic patients (Kaye et al., 1988). Additionally, obese models demonstrate abnormal serotonin levels. For example, obese animals (Hassanain & Levin, 2002; Meguid et al., 2000; Svec et al., 2002) and humans (Breum et al., 2003) display lower levels of serotonin and its metabolites than their lean counterparts.

In 1977, Blundell proposed that the serotonergic system not only has an inhibitory role in feeding but a more significant role as a satiety factor (Blundell, 1977).

Numerous studies employing a BSS methodology have since supported the role of increased serotonin levels in the selective acceleration of the BSS (see (Rodgers et al., 2010).

6.1.2 Serotonin Mechanism of Action on Appetite

Serotonin-induced anorexia is thought to be mediated in the PVN, via its impact on orexigenic and anorexgenic neuropeptides such as NPY, POMC and their associated derivatives (Halford et al., 2003).

The administration of serotonin releasers, such as fenfluramine and dex-fenfluramine (d-FEN; see Section 6.1.3.1), decreases levels of NPY (Choi et al., 2006; Dryden, Frankish, et al., 1996), whereas 5-HT antagonists have been shown to increase NPY in the hypothalamus (Dryden et al., 1995). Furthermore, reduced serotonin availability is reported to decrease the density of NPY neurons in the hypothalamus (Compan et al., 1996). Similarly, administration of 5-HT receptor agonists has been found to attenuate NPY-induced hyperphagia (Grignaschi et al., 1995; Rogers et al., 1991; however see: Brown & Coscina, 1995).

More specifically, the PVN has been implicated in the NPY-serotonin relationship, although, currently, only 5-HT_{2C} receptor agonists/antagonists administered into the PVN have been shown to impact NPY-mediated feeding responses (Currie, 2003). Moreover, NPY neurons within the PVN are hyperpolarised by 5-HT_{1B} receptor agonist administration (Heisler et al., 2006; see Section 6.1.4).

Additionally, the anorectic action of serotonin can be attenuated by pharmacological and genetic inactivation of melanocortin receptors (Heisler et al., 2003; Heisler et al., 2002; Heisler et al., 2006; Lam et al., 2008). Evidence shows that administration of d-FEN or *m*CPP (*meta*-Chlorophenylpiperazine; see Section 6.1.4.1) activates POMC neurons in the ARC (Heisler et al., 2002; Lam et al., 2008), causing the release of α-MSH which then acts at MC3 and MC4 receptors. Furthermore, pharmacological or genetic blockade of these receptors attenuates the anorectic effect of serotonin (Heisler et al., 2003). In fact, specific 5-HT_{2C} and 5-HT_{1B} receptor activation (see Section 6.1.4) promotes the release of α-MSH, whilst simultaneously inhibiting the release of AgRP (Heisler et al., 2006). The inhibition of AgRP decreases the inhibitory effect on POMC neurons, further promoting the release of α-MSH. Evidence demonstrates that d-FEN-induced anorexia is attenuated in mice with ectopic expression AgRP (Heisler et al., 2006).

Serotonin has also been thought to elicit its appetite effects via interaction with: orexin (Muraki et al., 2004; see Section 1.4.3), oxytocin (Jorgensen et al., 2003; see

Section 1.5.2.3) and noradrenaline (James et al., 2000; see Chapter 5); and CRH (see review: Lam et al., 2010; see Section 1.5.2.4).

Interestingly, the systemic administration of 5-HT (with poor BBB penetration) has also been found to reduce food intake (Edwards, 1991; Fletcher & Burton, 1984; Pollock & Rowland, 1981). Furthermore, serotonin is the main transmitter in the gastrointestinal nervous system that regulates peripheral hormones (Feldberg & Toh, 1953). Together with evidence of a prolonged effect of bodyweight loss beyond that of intake suppression (Connoley et al., 1995; Day & Bailey, 1998; Vickers et al., 2000), these findings suggest an additional peripheral action of serotonin.

6.1.3 Therapeutic Potential of Serotonergic Drugs

Administration of the 5-HT precursor, 5-HTP, reduces self-reported food intake and induces weight loss in obese patients for up to 12 weeks (Cangiano et al., 1992). In contrast, neurotoxic lesioning of serotonin neurons via 5,7-dihydroxytryptamine (5,7-DHT), blocks serotonin-induced hypophagia and increases food intake (Mackenzie et al., 1979). Similarly, administration of *para*-chlorophenlyanine (*p*CPA), an inhibitor of tryptophan hydroxylase, and therefore of serotonin synthesis, also blocks serotonin induced hypophagia and increases intake (Breisch et al., 1976; Mackenzie et al., 1979).

The above evidence clearly demonstrates that increased serotonin levels enhance satiation and meal termination (Blundell & Halford, 1998; Edwards, 1991; Simansky, 1996), with both central and peripheral administration found to decrease food intake (Hutson et al., 1988; Pollock & Rowland, 1981). The serotonergic system is therefore an obvious target in the development of appetite suppressants for the treatment of obesity.

6.1.3.1 Fenfluramine and d-FEN

Compounds that stimulate the release of serotonin, through the promotion of serotonin efflux into the synapse, such as fenfluramine and d-FEN, have been widely reported to reduce intake and bodyweight, (Blundell & Latham, 1980; Fisler et al., 1993; Gibson et al., 1993; GuyGrand, 1995; Halford & Blundell, 1993; McCann et al., 1997; Pringle et al., 2008; Vickers et al., 2000; Vickers, Easton, et al., 2003), whilst preserving the BSS in rodents (Blundell & Halford, 1998; Blundell & McArthur, 1981; Hewitt et al., 2002; Lee et al., 2004; Vickers et al., 1996; Vickers et al., 1999; Webster, 2001; however see: McGuirk, 1992; Montgomery, 1988).

Fenfluramine was approved for the treatment of obesity in 1996, after evidence showed that it reduced food intake in healthy (Blundell et al., 1979; Goodall & Silverstone, 1988; Willner, 1990) and obese (Halford et al., 2010) humans. For example, fenfluramine 60mg/day reduces food intake in one meal by ~26% (Blundell et al., 1979; Foltin et al., 1996) and fenfluramine 40mg/day reduces total daily caloric intake (Foltin et al., 1996). Furthermore, it was found to selectively reduce meal size rather than number of meals (Foltin et al., 1996), emphasising the enhanced satiation effect. Clinical research found that fenfluramine treatment results in a weight loss between 1.2 and 11.9kg (Pinder et al., 1975). Despite this, it was withdrawn from the market in 1997 due to high rates of pulmonary hypertension (Mark et al., 1997).

Similarly, d-FEN reduces self-reported food intake (Drent et al., 1995; Goodall & Silverstone, 1988; GuyGrand, 1995; Marbury et al., 1996) and produces significant reductions in hunger ratings prior to a meal in both lean and obese humans (Blundell & Hill, 1990). It has also been successful in the inhibition of intake and weight regain following a VLCD (Finer et al., 1992) and has been shown to protect against DIO (Fisler et al., 1993). Furthermore, d-FEN has demonstrated little to no tolerance in chronic studies (Vickers et al., 2000). The European INDEX (INternational DEXfenfluramine) clinical trial (Guygrand et al., 1989), found that 52% of obese volunteers taking d-FEN treatment for 1 year lost ≥10% baseline bodyweight compared to placebo with an average weight loss of 9.82kg (placebo = 7.15kg). However, following treatment cessation, rapid weight gain was reported (Guygrand, 1992) and, as with fenfluramine, d-FEN was withdrawn from the market in 1997 (FDA, 1997a), due to cardiovascular problems.

6.1.3.2 Selective Serotonin Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRIs) increase extracellular serotonin via the blockade of reuptake transporters and reduce food intake (see review: Heal, Cheetham, et al., 1998). Similarly, MAO inhibitors, that prevent the breakdown of serotonin, increase extracellular serotonin levels and reduce food intake (Feldman, 1988). SSRIs, such as fluoxetine (Clifton et al., 1989; Halford & Blundell, 1996b; Heisler et al., 1997; Lawton et al., 1995; McGuirk et al., 1992; Pijl et al., 1991; Ward et al., 1999; Willner, 1990; Yen et al., 1987); femoxetine (Halford & Blundell, 1993), fluvoxamine (Wieczorek et al., 2001), paroxetine (Halford & Blundell, 1993; Konkle et al., 2003), and sertraline (Nielsen et al., 1992; Simansky & Vaidya, 1990) have all been found to reduce intake and bodyweight, and to accelerate the BSS in rodents.

In clinical trials, fluoxetine produced greater weight loss than control after 2 weeks (Lawton et al., 1995; McGuirk & Silverstone, 1990; Pijl et al., 1991) and, in a 16 week study, reduced the mean number of eating sessions and total food intake (Ward et al., 1999). In fact, it has been reported that fluoxetine can produce weight loss of 0.5kg per week (Wise, 1992). Although this is not sustainable, evidence shows that after 60 weeks, although still producing significantly greater weight loss than placebo, maximal weight loss is not seen suggesting that weight is regained half-way through trials (Darga et al., 1991; Goldstein et al., 1995).

6.1.3.3 Sibutramine

Sibutramine is a dual serotonin and noradrenaline reuptake inhibitor (Gundlah et al., 1997; Heal, Aspley, et al., 1998; Weintraub et al., 1991) that produces hypophagia and weight loss in rodents (Connoley et al., 1995; Halford et al., 1995; Luque & Rey, 1999; Wirth et al., 2001) and humans (Barkeling et al., 2003; Chapelot et al., 2000; Halford, Boyland, Cooper, et al., 2010; Hansen et al., 1998, 1999; Rolls et al., 1998) in a behaviourally-selective manner (Halford et al., 1995; Higgs et al., 2011; Tallett et al., 2009c). Chronic studies demonstrate that the effects on weight loss continue beyond the inhibition of food intake (Connoley et al., 1995; Day & Bailey, 1998), suggesting that there may be some additional effects on energy expenditure (Hansen et al., 1998).

Approved for the treatment of obesity in 1997, the STORM (Sibutramine Trial of Obesity Reduction and Maintenance) clinical trial demonstrated that obese patients lost 11.3kg over a 6-month period following sibutramine treatment (10mg/day) with a LCD (James et al., 2000). Evidence also reports improvements in triglycerides, lipoprotein, cholesterol, uric acid, waist circumference and quality of life measures (McMahon et al., 2000). Furthermore, meta-analyses of sibutramine treatment produced an average weight loss of ~4.4kg (Arterburn et al., 2004; Padwal et al., 2003). Despite the apparent success of sibutramine (Meridia®, Reductil®) for weight loss treatment, it was withdrawn in October 2010 following evidence of serious, non-fatal cardiovascular events in the Sibutramine Cardiovascular Outcome Trial (SCOUT; James et al., 2010).

6.1.4 Selective Serotonin Receptor Subtypes

6.1.4.1 5-HT Receptor Involvement in Fenfluramine and d-FEN Anorexia

Despite the apparent utility of serotonergic drugs for the treatment of obesity, there have been problems with weight regain following treatment cessation (Darga et al., 1991) and side-effects (Guygrand et al., 1989), including cardiac valve abnormalities (Connolly et al., 1997) and pulmonary hypertension (Mark et al., 1997; Michelakis & Weir, 2001; for review see: Loke et al., 2002).

Therefore, more recent research has focused on the specific receptors that mediate the anorectic action of serotonergic drugs. For example, the simultaneous administration of selective receptor antagonists and d-FEN demonstrated that its anorectic effects are mediated by 5-HT_{1B} receptors (Dalton et al., 2006; Lee et al., 2004; Simansky & Nicklous, 2002) but not 5-$HT_{2A/2C}$ receptors (Clifton et al., 2000; Neill & Cooper, 1989; Samanin et al., 1989). In contrast, Vickers (Vickers et al., 2001) found that pretreatment with SB-242084 (a selective 5-HT_{2C} receptor antagonist), but not GR-127935 and SB-224289 (selective 5-HT_{1B} receptor antagonists) blocked d-FEN-induced hypophagia. It is now known that d-FEN and fenfluramine-induced anorexia is mediated via both 5-HT_{1B} and 5-HT_{2C} receptors (Garfield, 2009; Halford et al., 2007; Lucas et al., 1998; Simansky & Nicklous, 2002; Vickers et al., 1999). Evidence shows that d-FEN-induced reductions in meal size are attenuated by 5-HT_{1B} receptor antagonists, while d-FEN-induced reductions in feeding rate are attenuated by 5-HT_{2C} receptor antagonist (Grignaschi & Samanin, 1992; Vickers et al., 2001). Furthermore, d-FEN's major metabolite, norfenfluramine, is a full 5-HT_{2C} agonist (Curzon et al., 1997). Additionally, metergoline (a non-selective 5-$HT_{1/2}$ receptor antagonist) blocks fluoxetine-induced hypophagia (Halford & Blundell, 1996b; Lee & Clifton, 1992) and the anorectic effects of other SSRIs, (such as sertraline) can be blocked by selective 5-HT_{1B} and 5-HT_{2C} receptor antagonists (Lucki et al., 1988) . This evidence suggests that the anorectic action induced by serotonergic drugs is mediated by 5-HT_{1B} and 5-HT_{2C} receptors (Hewitt et al., 2002; Kitchener & Dourish, 1994; Lopez-Alonso et al., 2007; Schreiber et al., 2000; Vickers & Dourish, 2004)

6-1: Summary of the serotonin receptor subtypes and their impact on appetite

Adapted from Lam et al. (2010)

Subtype	Agonist action	Antagonist action	Knock-out / Deletion models
5-HT$_{1A}$	Increases food intake (Dourish, 1985)	Decreases food intake (Moreau et al., 1992)	No food intake or bodyweight phenotype (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998)
5-HT$_{1B\ (1D\beta)}$	Decreases food intake (Halford & Blundell, 1996b; Lee & Simansky, 1997)		Increased bodysize (Bouwknecht et al., 2001)
5-HT$_{1D\ (1D\alpha)}$	Decreases food intake (Boeles et al., 1997)		
5-HT$_{2A}$ (Formerly 5-HT$_2$)	Decreases food intake (Fox et al., 2010)		No food intake phenotype (Weisstaub et al., 2006)
5-HT$_{2B}$			Reduces food intake (Yadav, 2009)
5-HT$_{2C}$ (Formerly 5-HT$_{1C}$)	Decrease food intake (Kennett & Curzon, 1988) (Kitchener & Dourish, 1994; Martin et al., 1998; Schreiber & De Vry, 2002)	Increase food intake (Bonhaus et al., 1997)	Hyperphagia and increased bodyweight (Tecott et al., 1995)
5-HT$_{3A-E}$		Increase food intake (Hayes & Covasa, 2006)	No food intake phenotype (Bhatnagar et al., 2004)
5-HT$_4$	Decrease food intake (Jean et al., 2007)	Increase food intake (Jean et al., 2007)	No food intake phenotype (Compan et al., 2004)
5-HT$_{5A + 5B}$			No bodyweight phenotype (Grailhe et al., 1999)
5-HT$_6$		Decreases food intake (Heal et al., 2008)	Decreases food intake and obesity resistant (Frassetto et al., 2008)
5-HT$_7$			No bodyweight phenotype (Hedlund et al., 2003)

6.1.4.2 Intrinsic Effects of 5-HT Receptor Ligands on Appetite

The various 5-HT receptor sub-types induce differing effects on food intake and appetite regulation (see Table 1-1). 5-HT$_1$ receptors act as autoreceptors and are therefore found on cell soma dendrites/terminals as well as postsynaptically (Lanfumey & Hamon, 2004). The administration of 5-HT$_{1A}$ receptor agonists, such as 8-OH-DPAT, reduces serotonin release (Dourish et al., 1985; Hopwood & Stamford, 2001), increases intake, and disrupts the BSS (Simansky & Vaidya, 1990; however see: Lopez-Alonso et al., 2007). In contrast, the administration of 5-

HT_{1B} receptor agonists (such as CP 93129 and RU 24969) accelerates the BSS (Halford & Blundell, 1996a; Hewitt et al., 2002; Kitchener & Dourish, 1994; Simansky & Vaidya, 1990) and decreases food intake (Koe et al., 1992; Lee & Simansky, 1997; Macor et al., 1990; Torgersen et al., 1990). Conversely, pharmacological antagonism or genetic deletion via 5-HT_{1B} KO increases intake and bodyweight in rodents (Bouwknecht et al., 2001), and attenuates the anorectic response to d-FEN and other serotonin agonists (Lee et al., 2004; Lucas et al., 1998). Interestingly, 5-HT_{1B} receptors are also expressed on non-serotonergic neurons and may elicit their anorectic action via inhibition of other neurotransmitters (Barnes & Sharp, 1999).

Administration of 5-HT_{2C} receptor agonists to rodents produces an enhanced BSS profile similar to d-FEN, exhibiting a delay of initiation, reduced meal size and meal rate (Clifton, 2000; Kennett & Curzon, 1988; Martin et al., 1998; Schreiber & De Vry, 2002), whilst selectively advancing satiety (Kitchener & Dourish, 1994; Simansky & Vaidya, 1990) and producing weight loss beyond that of anorexia (Vickers et al., 2000). On the other hand, 5-HT_{2C} receptor blockade increases food intake (Bonhaus et al., 1997) while 5-HT_{2C} receptor KO models are overweight and hyperphagic (Nonogaki et al., 1998; Tecott et al., 1995), with a delayed BSS profile (Hewitt et al., 2002; Vickers et al., 1999), marked hyperactivity (Nonogaki et al., 2003; Xu, 2008) and attenuated responses to d-FEN and *m*CPP (Tecott et al., 1995; Vickers et al., 1999). See Table 1-2 for an outline of the $5HT_{1B}$ and $5HT_{2C}$ receptor agonists currently in development.

Table 6-2: Current 5HT1B and 5HT2C Receptor Agonists

Receptor Subtype	Name	Reference
a preferential 5-$HT_{1A/1B}$ receptor agonist	RU 24969	Hutson, 1988 Kennett & Curzon, 1988
a preferential 5-$HT_{1A/2C}$ receptor agonist	TFMPP	Kennett & Curzon, 1988 Kitchener & Dourish, 1994 Simansky & Vaidya, 1990
a selective 5-HT_{1B} receptor agonist	CP-93129	Lee, Aloyo, Fluharty, & Simansky & Vaidya, 1998
a selective 5-HT_{1B} agonist	CP-94253	Halford & Blundell, 1996a
a preferential 5-$HT_{1B/2C}$ receptor	*m*CPP	see Section 6.1.4.3
a selective 5-HT_{2C} receptor agonist	YM348	Hayashi et al., 2004
	Org 12962	Halford et al., 2005
	VER 3323	Miller, 2005
	Ro 4590334	Clifton et al., 2005
	Ro 60-0175	Vickers et al., 2000

More recently, attention has also been drawn to 5-HT_6 receptors (see review: Heal et al., 2008). Evidence shows that the administration of selective 5-HT_6 receptor agonists reduces food intake and increases weight loss in lean (Bentley et al., 1999; Woolley et al., 2001; Woolley et al., 2004) and obese (Caldirola & Svartengren, 2005; Shacham et al., 2005) rodents, an action thought to be related to satiety enhancement (Fisas et al., 2006).

6.1.4.3 *m*CPP

*m*CPP is a preferential 5-$HT_{1B/2C}$ receptor agonist, that is found to dose-dependently suppress food intake and weight gain, both in rodents (Clifton et al., 1993; Dalton et al., 2006; Hikiji, 2004; Kennett & Curzon, 1988, 1991; Kennett et al., 1987; Samanin et al., 1979; Vickers et al., 2000; Vickers, Easton, et al., 2003; Ward et al., 2008) and humans (Cowen et al., 1995; Sargent et al., 1997; Walsh et al., 1994). Evidence suggests that these effects are produced in a behaviourally-selective manner (Clifton et al., 1993; Hewitt et al., 2002; Kitchener & Dourish, 1994; Lee et al., 2004).

Preclinical studies have demonstrated that *m*CPP chronically reduces food intake (Ashkzari et al., 2003), and water consumption (Buczek et al., 1994; Castro et al., 2002). It also reduces taste preference (Badaue-Passos et al., 2003; Carli & Samanin, 1992; Cooper & Barber, 1994; De Gobbi et al., 2007) and attenuates the reinforcing efficacy of palatable food in rats (Ward et al., 2008; Wolff & Leander, 2000) via central action at the 5-HT_{2C} receptors (Dryden, Wang, et al., 1996; Hikiji, 2004; Kaplan et al., 1998). *m*CPP has also been shown to act synergistically, with the cannabinoid antagonist rimonabant, to reduce breakpoints in a progressive ratio schedule of reinforcement in male rats (Ward et al., 2008).

Although, *m*CPP may also agonise 5-HT_{2A} and 5-HT_{2B} receptors, the anorectic action is thought to be primarily a result of 5-HT_{2C} receptor activation. Evidence shows that pharmacological (Hewitt et al., 2002; Kennett & Curzon, 1988; Kennett et al., 1997) or genetic deletion (Dalton et al., 2006; Tecott et al., 1995) of 5-HT_{2C} receptors attenuates *m*CPP-induced hypophagia. Furthermore, pair-fed controls that exhibit a similar level of weight loss to *m*CPP-treated rodents demonstrate that the bodyweight effects of *m*CPP are secondary to its hypophagic effects (Vickers, Easton, et al., 2003).

In humans, *m*CPP has been found to reduce food intake by ~30% in lean volunteers (Cowen et al., 1995; Walsh et al., 1994) and to produce a significant

increase in weight loss in obese patients compared to placebo (Sargent et al., 1997). Disappointingly, however, there have been reports of increased in blood pressure and heart rate following *m*CPP treatment (Ferreira et al., 2005; Ghaziuddin et al., 2003).

6.1.4.4 Lorcaserin

Preclinical work with the preferential 5-HT_{2C} receptor agonists, *m*CPP and TFMPP, emphasised the importance of $5HT_{2C}$ receptors in the enhancement of satiety (Clifton et al., 2005; Hewitt et al., 2002; however see: Lopez Alonzo, 2007), and has led to the development of more selective $5HT_{2C}$ receptor agonists (such as lorcaserin) for the treatment of obesity (Bickerdike, 2003).

Lorcaserin ([1R]-8-chloro-2,3,4,5-tetrahydro-1-methyl-1H-3-benzazepine) is a selective 5-HT_{2C} receptor agonist that is thought to have an improved cardiovascular profile compared with previous serotonergic obesity treatments (Smith, Prosser, et al., 2009). Lorcaserin administration has been found to reduce food intake and inhibit DIO (Hayashi, Sonoda, et al., 2004; Vickers et al., 2000) in rodents, while producing a selective enhancement of the BSS (Higgins et al., 2012).

Approved for marketing and distribution by the FDA in 2012 (Belviq®), the lorcaserin clinical trials have demonstrated huge potential for the treatment of obesity (Bays, 2004; Hurren & Berlie, 2011; Martin et al., 2011). Early phase II trials found a significant decrease in bodyweight within the intention-to-treat (ITT) population after 12 weeks (Smith, Prosser, et al., 2009) and subsequent phase III trials found ≥35% of patients lost ≥5% of their baseline bodyweight.

The Behavioural Modification and Lorcaserin for Overweight and Obesity Management (BLOOM) trial found that one year of lorcaserin treatment (20mg/day) resulted in 47.5% of patients losing ≥5% of their baseline bodyweight (placebo = 20.3%) and 22.6% of patients losing ≥10% of their baseline bodyweight (placebo = 7.7%). Furthermore 67.9% of those who continued treatment for 2 years maintained their weight loss. There were also improvements in waist circumference, BMI, blood pressure, triglycerides and cholesterol (Fidler et al., 2011; Smith et al., 2010). The Behavioural Modification and Lorcaserin Second Study for Overweight and Obesity Management (BLOSSOM) trial found a weight loss of ≥10% in 22.6% of patients receiving lorcaserin treatment (20mg/day; placebo = 9.7%). Additionally, the BLOOM Diabetes Mellitus (BLOOM-DM) trial found a mean weight loss of 4.5% with lorcaserin treatment (20mg/day; placebo = 1.5%), however there were no significant improvements in secondary endpoints.

6.1.5 Serotonin and Non-Appetite Regulatory Behaviours

In addition to appetite regulation, serotonergic drugs have been associated with: anxiety disorders such as, generalised anxiety disorder (Rickels & Rynn, 2002), panic disorder (Neumeister et al., 2004) and obsessive compulsive disorder (Goddard et al., 2008); depression (Papakostas et al., 2008; Usala et al., 2008); aggression, for example a lack of serotonin increases aggression (Bouwknecht et al., 2001; Saudou et al., 1994); sleep and arousal, for example increased serotonin levels increase wakefulness (Trulson & Jacobs, 1979); temperature regulation (Guscott et al., 2003); sexual dysfunction; epilepsy; Alzhiemers disease; urinary incontinence; and hot flushes (see review: Bishop & Nilsson, 2003).

Despite this spectrum of activity, the majority of the serotonergic drugs reviewed above have demonstrated a behaviourally-selective induction of satiety. As previously mentioned, the serotonin releasers, fenfluramine and d-FEN, have demonstrated that they impact feeding behaviour in a manner consistent with an acceleration of the BSS (Blundell & Halford, 1998; Blundell & Latham, 1980; Blundell & McArthur, 1981; Hewitt et al., 2002; Lee et al., 2004; Vickers et al., 1996; Vickers et al., 1999; Webster, 2001). The same is true for SSRIs such as fluoxetine, sertraline, paroxetine, and femoxetine (Clifton et al., 1989; Halford & Blundell, 1993, 1996b; Kitchener & Dourish, 1994; McGuirk et al., 1992; Simansky & Vaidya, 1990; Willner, 1990), and for the dual reuptake blocker sibutramine (Tallett et al., 2009c). Additionally, preferential 5-HT_{1B} and 5-HT_{2C} receptor agonists, such as CP-94253 (Halford & Blundell, 1996a; Lee et al., 2002), *m*CPP (Hewitt et al., 2002; Kitchener & Dourish, 1994; Lee et al., 2004; Simansky & Vaidya, 1990) and TFMPP (Kitchener & Dourish, 1994; Simansky & Vaidya, 1990) have also demonstrated a behaviourally-selective enhancement of satiety. In contrast, 5-HT_2 receptor agonists, such as DOI and MK212, and 5-HT_{1B} receptor agonist RU-24969 have been found to disrupt the BSS (Blundell & Halford, 1998; Hewitt et al., 2002; Kitchener & Dourish, 1994; Simansky & Vaidya, 1990).

6.1.5.1 Hypoactivity

Despite the above reports, questions have been raised about the behavioural specificity of the serotonergic anorectic response. For example, it has been reported that 5-HT_{2C} receptor agonists concomitantly induce excessive grooming, nausea and **hypoactivity**. This behavioural profile has been reported for a wide range of 5-HT_{2C} receptor agonists, such as *m*CPP, DOI, MK212 CP-809101, lorcaserin, Ro 60-0175 and VER 23779 (Clifton et al., 2000; Halford et al., 1997; Hewitt et al., 2002; Higgins et al., 2012; Kennett & Curzon, 1988; Kennett et al.,

1997; Kitchener & Dourish, 1994; Simansky & Vaidya, 1990; Somerville et al., 2007; Stiedl et al., 2007; Vickers et al., 2000). Conversely, 5-HT_{2C} receptor antagonists have been shown to produce hyperactivity (Fletcher et al., 2013). Evidence has also shown that the hypo-locomotion is attenuated in 5-HT_{1B} KO mice (Clifton et al., 2003; Lee et al., 2004). This evidence suggests that 5-HT_{2C} receptor agonists may not produce a behaviourally-specific anorexia.

6.2 Serotonin-Opioid Interactions

Growing evidence demonstrates that a functional relationship exists between the serotonergic and opioid systems. Opioid receptors have been found on serotonin nerve terminals (Parenti et al., 1983). Furthermore, compounds that agonise μ-opioid receptors suppress the excitatory effects induced by 5-HT_{2A} receptor activation (Marek & Aghajanian, 1998), while increased serotonin levels are found to negatively regulate μ- and δ- receptors (Passarelli & Costa, 1989; Yoshioka et al., 1993) and alter β-endorphin levels (Bagdy et al., 1990; Majeed et al., 1985).

There is a known system interaction in pain regulation. For example, it has been shown that the effects of morphine treatment are enhanced by fenfluramine (Coda et al., 1993) and fluoxetine (Gatch et al., 1998; Hynes et al., 1985). Furthermore, morphine tolerance is delayed by the simultaneous administration of 5-HT_{1A} receptor agonists (Nayebi & Charkhpour, 2006) while, conversely, serotonin-induced pain tolerance is blocked by opioid antagonists (Gray et al., 1998).

In relation to appetite regulation, the co-administration of naltrexone and fluoxetine has been shown to suppress ethanol consumption in a synergistic manner (Gardell, 1997; Rezvani et al., 2000). Similarly, naloxone and fluoxetine co-administration has been shown to suppress food intake in an additive manner (Hagan et al., 1997). Interestingly, fluoxetine appears to reduce carbohydrate intake, while naloxone reduces fat intake, resulting in an additive reduction in overall consumption. Furthermore, administration of 5-HTP has been found to potentiate naloxone-induced hypophagia in food-deprived rats (Fernandez-Tome, 1988). Interestingly, the 5-HT antagonist methysergide and the 5-HT_3 antagonist ICS-205,930 also potentiates naloxone-induced hypophagia (Beczkowska, 1991) and ethanol intake (Johnson, 2000; Le, 1994), suggesting a potential role for 5-HT_3 receptors in opioid anorexia. However, it is relevant to note that, research conducted the at University of Leeds, using similar BSS methodology to that used in the present thesis, failed to find a significant interaction on food intake using a combination of sibutramine and naloxone (Tallett et al., 2010b). Interestingly, *m*CPP

has been shown to act synergistically with the cannabinoid antagonist rimonabant, to reduce breakpoints in a progressive ratio schedule of reinforcement in male rats (Ward et al., 2008). The synergistic modulation of motivation for palatable foods using *m*CPP could be further assessed with a variety of anorectic compounds, including the opioid antagonist naltrexone.

6.3 Rationale; Chapter Six

In view of the recent approval of the selective 5-HT_{2C} receptor agonist lorcaserin (Belviq®) for the treatment of obesity, in addition to the reported hypoactivity associated with preferential 5-HT_{2C} receptor agonist *m*CPP, the aim of the current chapter is to assess the anorectic efficacy and behavioural specificity of *m*CPP, alone and in combination with low-dose naltrexone treatment. As relevant dose-response data on naltrexone were already available (see Chapter 5, Experiment 5), Experiment 7 characterises the dose-response profile of *m*CPP while Experiment 8 explores the effects of combined low-dose treatment with these agents.

6.4 Experiment Seven; *m*CPP Dose-Response

6.4.1 Method

For the main methodological details, refer to General Methods (Chapter 3).

6.4.1.1 Subjects and Design

10 adult male Lister hooded rats (200.51 ± 2.34g on arrival from Charles River, U.K and 470.60 + 9.00g by the end of the study) were employed for this study. A within-subjects design was adopted whereby each subject received all four experimental conditions according to a Latin Square (with a 7 day wash out period): Vehicle (V); *m*CPP 0.1mg/kg (*m*CPP0.1); *m*CPP 1.0mg/kg (*m*CPP1.0); and *m*CPP 3.0mg/kg (*m*CPP3.0).

6.4.1.2 Drugs

1-(3-chlorophenyl) piperazine hydrochloride (*m*CPP; Tocris Bioscience, UK) was dissolved to required concentrations in physiological saline (0.9%) which, alone, served for control injections. Doses of *m*CPP (0.1, 1.0 and 3.0 mg/kg) were selected based on previous published research (e.g. Hewitt et al., 2002; Kennett & Curzon, 1988; Kitchener & Dourish, 1994; Lee et al., 2004; Simansky & Vaidya, 1990; Ward et al., 2008). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg 30 minutes prior to testing.

6.4.1.3 Procedure

Testing occurred over four weeks, with two test days per week and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square.

6.4.2 Results

Full statistical details can be found in Appendix 8.

6.4.2.1 Habituation Phase Food Intake

Mash consumption differed significantly during habituation ($F(4,36) = 12.70$, $p < 0.001$). Intake on the first trial was lower ($p \leq 0.01$) than on trials 3-5 (trial 1: 13.14 ± 1.42g; trial 2: 15.86 ± 1.80g; trial 3: 17.62 ± 1.64g; trial 4: 18.75 ± 1.58g; trial 5: 19.00 ± 1.86g.). The development of stable intake was confirmed both by the lack of significant variation across trials 2-5, and the close similarity in scores between the final habituation trial and vehicle control in the subsequent experiment (19.26 ± 1.20g).

6.4.2.2 Test Day Bodyweight

Test-day bodyweights were equivalent across the four treatment conditions (V: 402.4 ± 12.2g; *m*CPP0.1: 407.4 ± 11.6g; *m*CPP1.0: 413.2 ± 15.1g; *m*CPP3.0: 403.2 ± 9.3g ($F(3,27) = 0.18$, $p > 0.05$).

6.4.2.3 Test Day Food Intake

Control food pot measures showed an average weight loss via evaporation of only 0.17% throughout the experimental period (0.04 – 0.49%). The effects of *m*CPP on mash consumption are shown in Figure 6-2. Treatment with the 5-HT_{2C} receptor agonist significantly influenced food intake ($F(3,27) = 33.77$, $p < 0.001$), with Bonferroni comparisons confirming significant suppression relative to vehicle control at 1.0 mg/kg (38.6% decrease; $p < 0.01$) and 3.0 mg/kg (57.4% decrease; $p < 0.001$). The intermediate ($p = 0.051$) and highest ($p < 0.001$) dose levels both differed significantly from 0.1 mg/kg but not from each other.

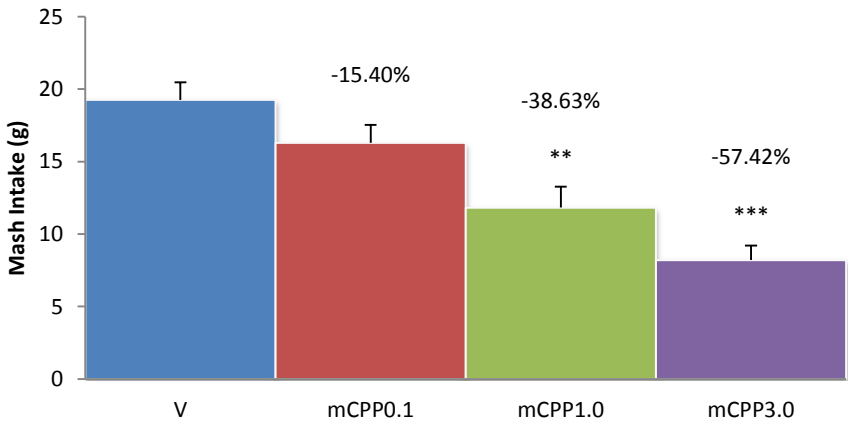


Figure 6-2: Experiment Seven. Effects of acute *m*CPP on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = Vehicle; *m*CPP0.1 = *m*CPP 0.1mg/kg; *m*CPP1.0 = *m*CPP 1.0mg/kg; and *m*CPP3.0 = *m*CPP 3.0mg/kg. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus V. See text for further details.

6.4.2.4 Total (one-hour) Behavioural Analyses

Effects of *m*CPP on feeding-related parameters (latencies, average duration of eating bouts, & average rate of eating) are summarised in Table 6-2, while effects on the total frequency and duration of ingestive and non-ingestive behaviours are shown in Figure 6-3.

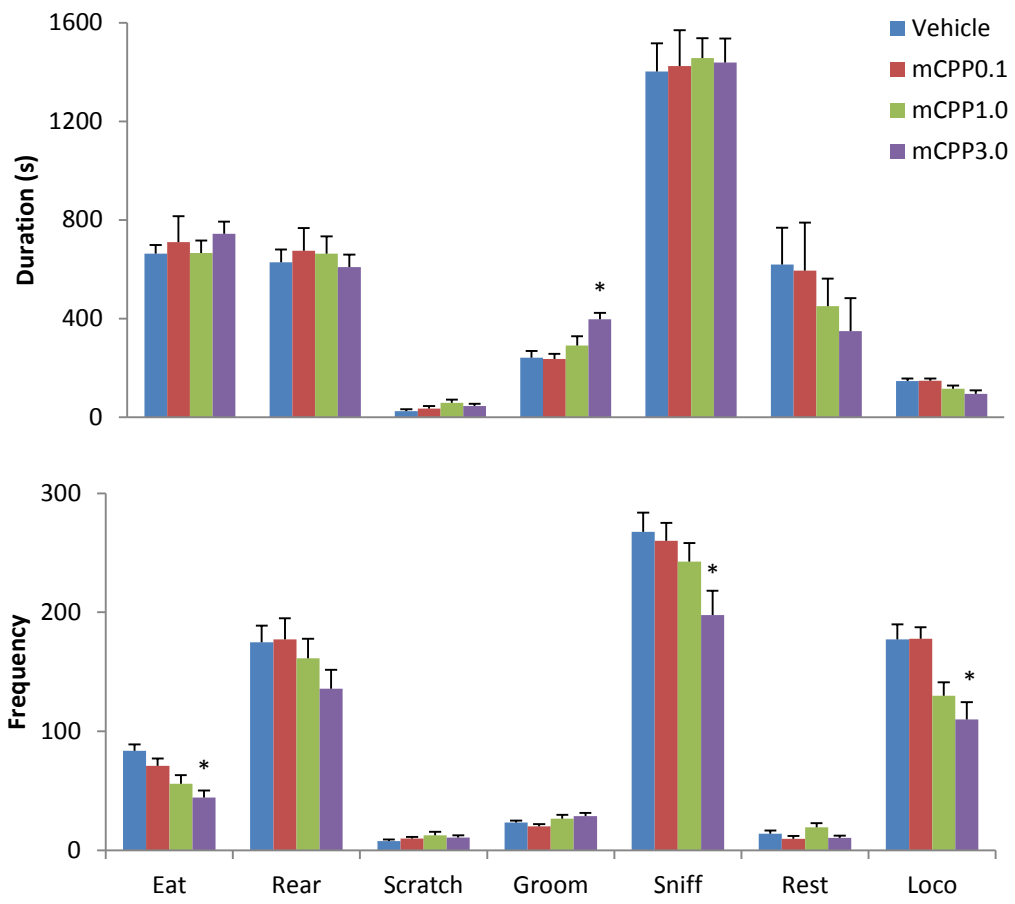


Figure 6-3: Experiment Seven. Effects of acute *m*CPP on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = Vehicle; *m*CPP0.1 = *m*CPP 0.1mg/kg; *m*CPP1.0 = *m*CPP 1.0mg/kg; and *m*CPP3.0 = *m*CPP 3.0mg/kg. * $p \leq 0.05$ versus V. See text for further details.

ANOVA revealed significant effects of *m*CPP on: latency to locate the food ($F(3,27) = 18.95$, $p = 0.001$), the average duration of eating bouts ($F(3,27) = 6.43$, $p < 0.01$) and eating rate ($F(3,27) = 40.15$, $p < 0.001$), as well as the frequency of eating ($F(3,27) = 11.60$, $p < 0.001$), sniffing ($F(3,27) = 5.83$, $p < 0.01$) and resting ($F(3,27) = 3.13$, $p < 0.05$), the frequency and duration of locomotion ($F(3,27) \geq 7.22$, $p \leq 0.001$), and the duration of grooming ($F(3,27) = 9.82$, $p < 0.001$). No other variables showed a significant effect of drug ($F(3,27) \leq 2.89$, $p > 0.05$).

As summarised in Table 6-2 and Figure 6-3, the lowest dose of *m*CPP (0.1 mg/kg) had no significant effects on behaviour. The intermediate dose of 1.0 mg/kg significantly reduced the rate of eating ($p < 0.001$) while the reduction in eat frequency and the increase in eat bout duration at this dose level closely approached significance ($p \leq 0.06$). Most treatment effects were observed at the highest dose tested (3.0 mg/kg), which, relative to vehicle control, increased the

time taken to locate the food source at the beginning of the test ($p < 0.01$) and time spent grooming ($p < 0.001$), while reducing the rate of eating ($p < 0.001$), as well as the frequency of eating, locomotion and sniffing ($p \leq 0.02$). It is worth noting that this dose also produced effects on eat bout duration (increase) and the locomotion frequency (decrease) that only just failed to reach significance ($p \leq 0.06$).

Table 6-3: Experiment Seven. Acute effects of *m*CPP on eating-related parameters

Data are presented as mean values (+ SEM). † $p < 0.06$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus V. See text for further details.

Measure	Vehicle	*m*CPP 0.1mg/kg	*m*CPP 1.0mg/kg	*m*CPP 3.0mg/kg
Latency to locate food (s)	5.75 ± 1.82	11.13 ± 3.78	9.29 ± 2.25	52.04 ± 8.90**
Latency to eat (s)	16.64 ± 2.61	15.94 ± 6.71	34.34 ± 14.70	48.50 ± 22.36†
Eat bout (s)	8.23 ± 0.73	10.18 ± 1.33	13.31 ± 1.73†	20.35 ± 4.23
Eat rate (g/min)	1.75 ± 0.08	1.52 ± 0.13	1.04 ± 0.07***	0.67 ± 0.08***

6.4.2.5 Periodic (Timebin) Behavioural Analyses

With the exception of grooming and scratching ($F \leq 1.78$, $p > 0.05$), 2-way ANOVA revealed significant main effects of time for the frequency ($F(11,99) \geq 11.29$, $p \leq 0.001$) and duration ($F(11,99) \geq 3.13$, $p \leq 0.001$) of all behavioural measures. This result confirms the typical pattern of behaviour during these 1h feeding tests which, as the session progresses, comprises a gradual reduction in active behaviours and an increase in resting (e.g. Ishii et al. 2003a&b; Rodgers et al. 2001; Tallett et al. 2009a&b; Wright & Rodgers 2013). Significant drug x time interactions were found for the frequency and duration of eating ($F(33,297) \geq 1.80$, $p \leq 0.01$), rearing ($F(33,297) \geq 1.77$, $p \leq 0.01$) and locomotion ($F(33,297) \geq 1.82$, $p \leq 0.01$), as well as the frequency of sniffing and scratching ($F(33,297) \geq 1.74$, $p \leq 0.01$), and the duration of grooming ($F(33,297) = 1.90$, $p < 0.01$).

A series of one-way ANOVAs (and Bonferroni post-hocs) within each timebin indicated that 1.0 and 3.0 mg/kg *m*CPP reduced the frequency of feeding during timebins 1-3 ($p \leq 0.01$). Furthermore, over the same early timeframe, the 3.0 mg/kg dose additionally increased time spent grooming ($p \leq 0.05$) and reduced the frequency of rearing and sniffing, as well as the frequency and duration of locomotion (all $p \leq 0.05$). Figure 6-4 illustrates the temporal effects of *m*CPP for the frequency of eating, locomotion, rearing and sniffing.

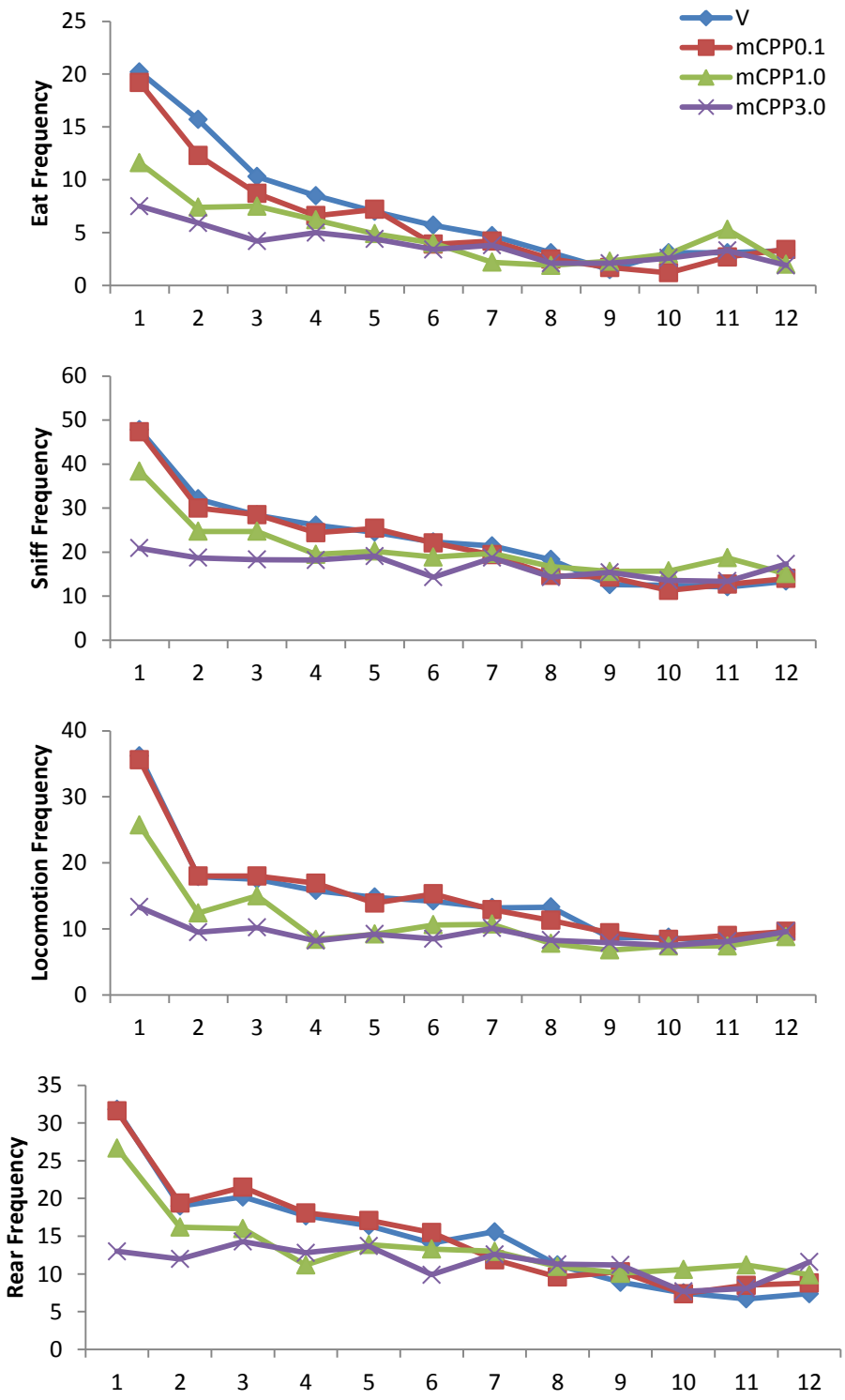


Figure 6-4: Experiment Seven. Effects of acute *m*CPP on the frequency of eating, sniffing, locomotion and rearing in male rats during a 1-h test with palatable mash

Data are expressed as the mean frequency of each behaviour in 12 x 5-min timebins. Dose-dependent suppression of all behaviours is apparent during the early part of the test session. See text for further details.

6.4.2.6 Behavioural Satiety Sequence (BSS)

Treatment effects on the BSS are summarised in Figure 6-5. The vehicle control profile shows a clear peak feeding response during the first 15-20 min of the test. Over time, feeding is seen to decline while time spent resting increases, with an eat-to-rest transition occurring just over half-way through the session (timebin 7). Although very similar normal behaviour patterns were evident with both the low and intermediate doses of *m*CPP, the highest dose of the compound tended to disrupt the BSS. Although an eat-to-rest transition is discernible (timebin 8), 3.0 mg/kg *m*CPP not only suppressed the peak feeding response but also induced an unusual behaviour pattern characterised by periodic feeding and higher-than–normal levels of grooming throughout the test session (see Figure 6-5; bottom right).

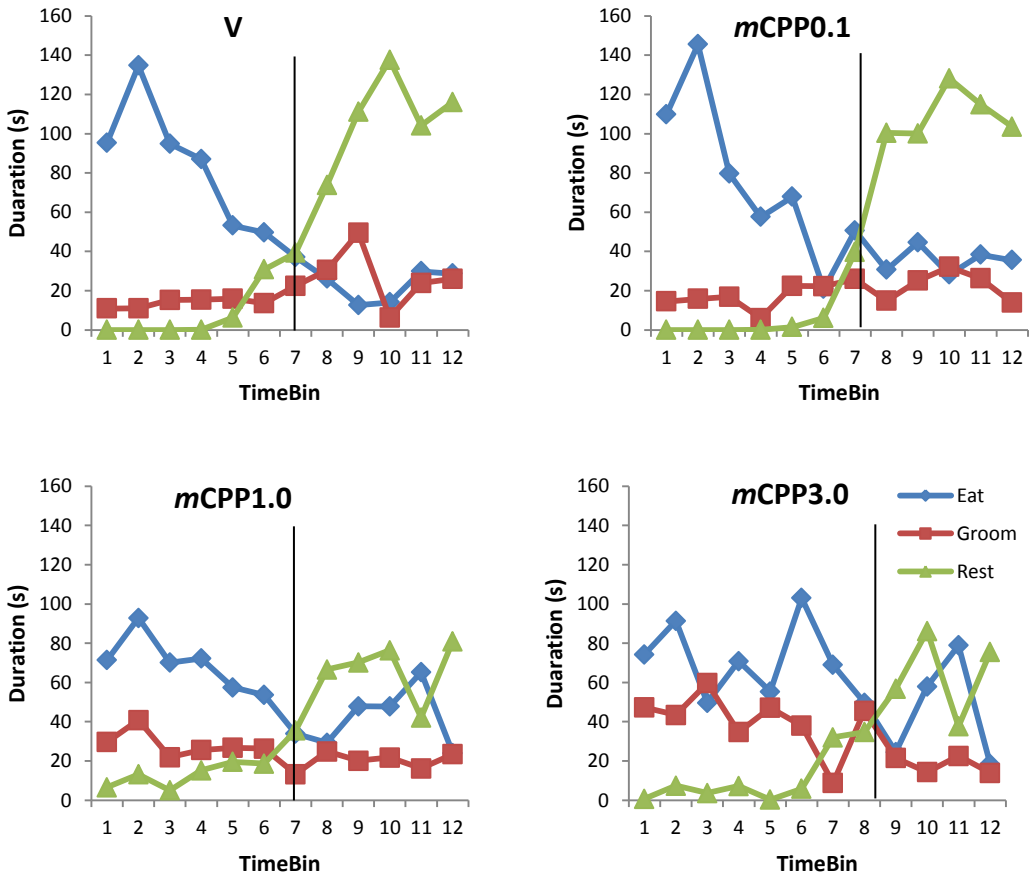


Figure 6-5: Experiment Seven. Effects of acute *m*CPP on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. V = Vehicle; *m*CPP0.1 = *m*CPP 0.1mg/kg; *m*CPP1.0 = *m*CPP 1.0mg/kg; and *m*CPP3.0 = *m*CPP 3.0mg/kg. See text for further details.

6.4.2.7 Bodyweight Gain

Data not shown. ANOVA failed to reveal any significant effect of acute *m*CPP treatment on 7-day absolute weight gain ($F(3,27) = 0.11$, $p > 0.05$). Although analysis of percent daily weight gain confirmed normal growth over time ($F(6,54) = 228.27$, $p < 0.001$), this analysis also failed to reveal a main effect for drug treatment ($F(3,27) = 0.20$, $p > 0.05$) or a drug x time interaction ($F(18,162) = 0.78$, $p > 0.05$).

6.4.3 Summary of Main Findings

The results of Experiment 7 show that acute treatment with *m*CPP dose-dependently reduces food intake and the frequency (but not duration) of feeding behaviour. *m*CPP also dose-dependently increased the time taken to find food and to commence feeding, and reduced the rate of eating.

Notably, these effects were accompanied by hypoactivity characterised by a dose-dependent reduction in the frequency (but not duration) of sniffing and locomotion. The frequency of all of the non-ingestive behaviours was suppressed from the very start of the test session and not, as would be expected with enhanced satiety, after the consumption of at least some food. Furthermore there is no indication whatsoever of an acceleration (shift to the left) in the BSS as is typical of a wide range of behaviourally-selective anorectic agents.

6.4.4 Design of Experiment Eight

Experiment 7 confirmed that acute *m*CPP 0.1mg/kg did not significantly suppress food intake, nor did it produce a suppression of active behaviours, such as sniffing, rearing or locomotion (see Figure 6-3). Therefore, a *m*CPP dose of 0.1mg/kg was selected for the interaction study.

Based on the data from the naltrexone dose-response study (Experiment 5) and the previous combination study using naltrexone (Experiment 6), two sub-maximal doses of naltrexone were selected. Experiments 5 and 6 confirmed that naltrexone 0.1mg/kg, did not significantly suppress food intake. Although Experiment 6 found a significant reduction in food intake for naltrexone 1.0mg/kg, it is pertinent to note that the actual % suppression was very similar to the non-significant reduction seen in Experiment 5. Therefore, 0.1 and 1.0 mg/kg of naltrexone were deemed appropriate for the interaction study.

6.5 Experiment Eight; *m*CPP and Naltrexone Interaction

6.5.1 Method

6.5.1.1 Subjects and Design

10 adult male Lister hooded rats (216.30 ± 1.38g on arrival from Charles River, U.K and 532.75 ± 9.58g by the end of the study) were employed in this study. A within-subjects design was adopted whereby each subject received all six experimental conditions according to a Latin Square (with a 7-day wash out period): Vehicle and Vehicle (VV); Vehicle and Naltrexone 0.1mg/kg (VNL); Vehicle and Naltrexone 1.0mg/kg (VNH); *m*CPP 0.1mg/kg and Vehicle (mV); *m*CPP 0.1mg/kg and Naltrexone 0.1mg/kg (mNL); and *m*CPP 0.1mg/kg and Naltrexone 1.0mg/kg (mNH).

6.5.1.2 Drugs

1-(3-chlorophenyl) piperazine hydrochloride (*m*CPP; Tocris Bioscience, UK) and naltrexone hydrochloride (Sigma-Aldrich, Poole, UK) were dissolved in physiological saline (0.9%) which, alone, served as a vehicle control. A low dose of *m*CPP (0.1 mg/kg) was used in combination with one of two doses of naltrexone (0.1mg/kg = NL, 1.0mg/kg = NH). All solutions were freshly prepared on test days and administered i.p. in a volume of 1ml/kg. The first injection (*m*CPP or vehicle) was given 30 minutes prior to testing with the second (naltrexone or vehicle) given 15 minutes prior to testing.

6.5.1.3 Procedure

Testing occurred over six weeks, with two test days per week, and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square.

6.5.2 Results

Full statistical details can be found in Appendix 9.

6.5.2.1 Habituation Phase Food Intake

Intake differed significantly during habituation week ($F(4,36) = 32.75$, $p < 0.001$), with intake on trial 1 significantly lower ($p \leq 0.05$) than on trials 2, 3 and 5, and intake on trial 2 significantly different from that on trials 3-5 ($p \leq 0.05$; trial 1 = 9.61 + 1.82g; trial 2 = 16.17 ± 1.79g; trial 3 = 20.22 ± 1.51g; trial 4 = 21.94 ± 1.24g; trial 5 = 22.18 ± 1.73g). The lack of significant difference across trials 3-5 indicates stabilisation of intake toward the end of the habituation period, a conclusion

confirmed by the similarity in scores between the final habituation trial and the vehicle (VV) condition (22.63 + 1.28g).

6.5.2.2 Test Day Bodyweight

Test-day bodyweights did not differ significantly across treatment conditions: VV: 451.0 ± 12.2g; VNL: 481.1 ± 14.6g; VNH: 457.2 ± 16.3g; mV: 465.1 ± 16.4g: mNL: 474.5 + 17.4g; mNH: 465.5 + 16.5g (main effect *m*CPP: $F(1,9) = 0.14$, $p > 0.05$; main effect naltrexone: $F(2,18) = 2.24$, $p > 0.05$; interaction: $F(2,18) = 0.21$, $p > 0.05$).

6.5.2.3 Test Day Food Intake

Control food pot measures showed an average weight loss via evaporation of only 0.22% throughout the experimental period (0.14 - 0.36%). Treatment effects on food intake are summarised in Figure 6-6. ANOVA confirmed significant main effects for *m*CPP ($F(1,9) = 24.21$, $p < 0.001$) and naltrexone ($F(2,18) = 29.30$, $p < 0.001$), but no significant interaction ($F(2,18) = 1.56$, $p > 0.05$).

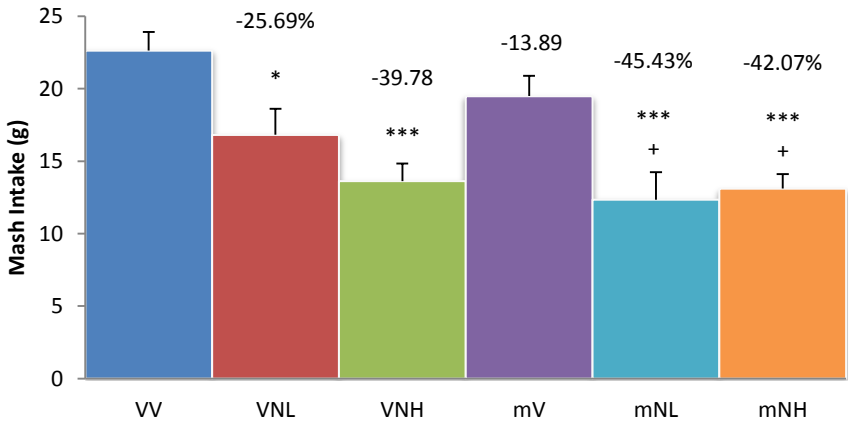


Figure 6-6: Experiment Eight. Effects of *m*CPP and naltrexone, alone and in combination, on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are presented as mean values (+ SEM). The percentages refer to intake reduction compared to vehicle. V = Vehicle, m = *m*CPP 0.1mg/kg, NL = 0.1 mg/kg naltrexone, NH = 1.0 mg/kg naltrexone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ v VV; + $p < 0.01$ v mV. See text for full details

Post-hoc comparisons revealed that, relative to vehicle control (VV), mash intake was significantly suppressed by NL ($p < 0.05$) and NH ($p < 0.001$) when given alone, and when each was given in combination with *m*CPP ($p \leq 0.001$). By contrast, *m*CPP *per se* had no significant effect on mash consumption. Importantly, appetite suppression under neither drug combination differed significantly from that seen with the opioid antagonist given alone (i.e. mNL vs VNL or mNH vs VNH).

This observation, combined with the significant differences between *m*CPP given alone and when administered with either dose of naltrexone ($p \leq 0.01$), would be consistent with a lack of meaningful anorectic interaction between the two compounds.

6.5.2.4 Total (one-hour) Behavioural Analyses

Figure 6-7 shows treatment effects on the total frequency and duration of ingestive and non-ingestive elements, while Table 6-4 summarises effects on feeding-related measures

Significant *m*CPP x naltrexone interactions were found only for the frequency and duration of locomotion ($F(2,18) \geq 3.93$, $p \leq 0.05$), while a significant main effect of *m*CPP was found only for the rate of eating ($F(1,9) = 6.70$, $p < 0.05$). By contrast, many variables demonstrated significant main effects for naltrexone: eating rate ($F(2,18) = 5.59$, $p < 0.05$); the frequency and duration of eating ($F(2,18) \geq 5.81$, $p \leq 0.05$), grooming ($F(2,18) \geq 4.73$, $p \leq 0.05$), scratching ($F(2,18) \geq 6.87$, $p \leq 0.01$) and sniffing ($F(2,18) \geq 7.18$, $p \leq 0.01$) ; the frequency of rearing ($F(2,18) = 5.36$, $p < 0.05$); and the duration of resting ($F(2,18) = 6.46$, $p < 0.01$). No other interactions or main effects were significant.

Table 6-4: Experiment Eight. Acute effects of *m*CPP and naltrexone, alone and in combination, on eating-related parameters

Data are presented as mean values (+ SEM). V = Vehicle, m = *m*CPP 0.1mg/kg, NL = 0.1 mg/kg naltrexone, NH = 1.0 mg/kg naltrexone. See text for full details.

Measure	VV	VNL	VNH	mV	mNL	mNH
Latency to locate food (s)	3.38 ± 0.45	4.01 ± 1.04	3.12 ± 0.54	2.99 ± 0.34	4.28 ± 0.99	3.81 ± 1.04
Latency to eat (s)	9.23 ± 1.92	7.83 ± 1.24	14.19 ± 4.28	18.54 ± 8.27	9.82 ± 2.66	9.27 ± 3.87
Eat bout (s)	11.09 ± 1.18	9.67 ± 1.37	8.69 ± 0.70	9.59 ± 0.80	11.51 ± 1.48	11.16 ± 1.48
Eat rate (g/min)	1.78 ± 0.06	1.72 ± 0.11	1.60 ± 0.13	1.70 ± 0.08	1.43 ± 0.11	1.48 ± 0.07

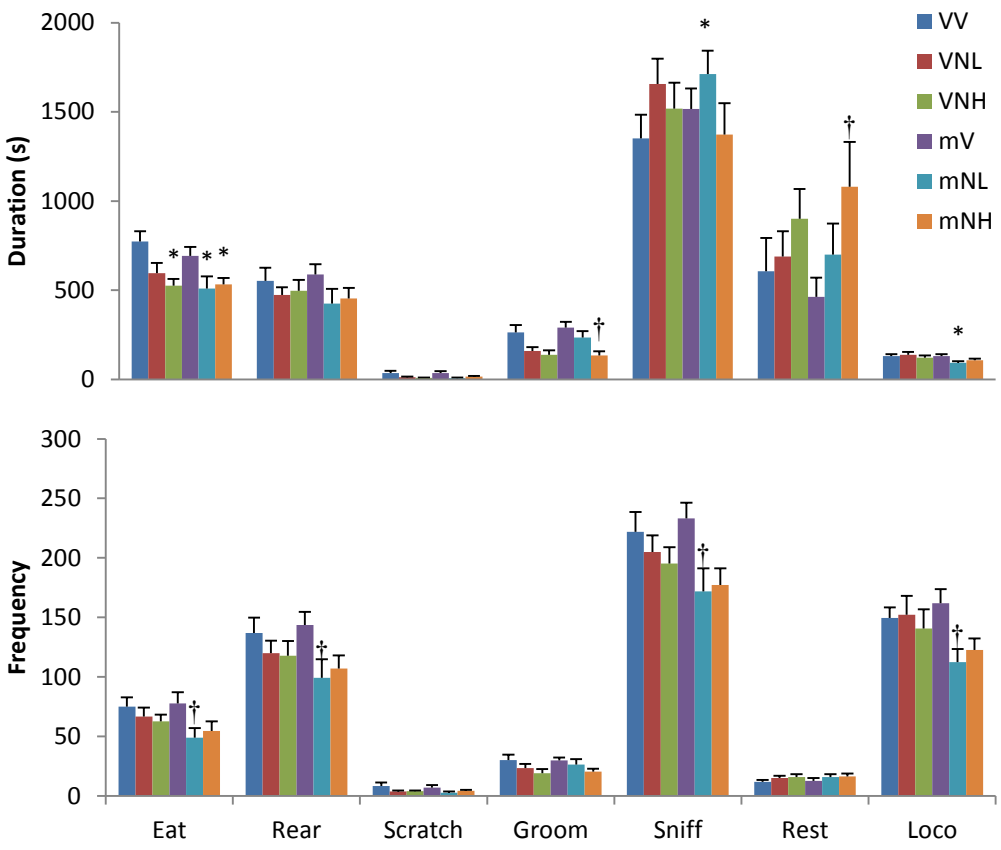


Figure 6-7: Experiment Eight. Effects of *m*CPP and naltrexone, alone and in combination, on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = Vehicle, m = *m*CPP 0.1mg/kg, NL = 0.1 mg/kg naltrexone, NH = 1.0 mg/kg naltrexone; * $p \leq 0.05$, vs VV; † $p \leq 0.05$ v mV. See text for full details

As summarised in Figure 6-7, post-hoc analyses actually revealed relatively few treatment effects compared to VV control. This outcome suggests that the ANOVA pattern of drug main effects (see above) reflects relatively weak responses that reach significance only as a result of the increased statistical power of larger sample sizes. Nevertheless, eat duration was significantly reduced by NH given alone and by both doses of naltrexone in combination with *m*CPP ($p \leq 0.05$), while the combination of *m*CPP and the lower (but not higher) dose of naltrexone significantly increased the duration of sniffing and decreased the duration of locomotion ($p \leq 0.05$). However, in only one of these instances (locomotion duration) was there any significant difference between the drug combination and either drug given alone (mNL vs VNL, $p < 0.02$). All other significant pairwise comparisons concerned differences between *m*CPP given alone and when given in combination with naltrexone. Thus, relative to the 5-HT_{2C} receptor agonist given alone (mV), the low dose combination (mNL) reduced the frequency of eating,

rearing, sniffing and locomotion ($p \leq 0.05$) while the high dose combination (mNH) significantly reduced the duration of grooming and increased the duration of resting ($p \leq 0.05$). However, as shown in Figure 6-6, none of these *m*CPP/naltrexone dose combinations differed significantly from the corresponding naltrexone only treatment conditions. This overall pattern confirms the large number of main effects for naltrexone and the minimal impact of its combination with *m*CPP

6.5.2.5 Periodic (Timebin) Behavioural Analyses

With the exceptions of the frequency and duration of grooming and scratching ($F(11,99) \leq 1.85$, $p > 0.05$), significant main effects of time were found for the frequency ($F(11,99) \geq 7.30$, $p \leq 0.001$) and duration ($F(11,99) \geq 2.31$, $p \leq 0.05$) of all behavioural measures. This profile reflects the typical pattern of behavioural change over the course of the test session. Figure 6-7 illustrates these temporal patterns for the frequency of eating, locomotion, rearing and sniffing, while the BSS charts (Figure 6-8; see below) clearly show the general increase in resting behaviour as the session progressed. Significant 3-way interactions (*m*CPP x naltrexone x time) were found for four measures: eat frequency, rest duration, and both the frequency and duration of sniffing ($F(22, 198) \geq 1.83$, $p \leq 0.05$). Additional 2-way interactions were found for eat duration and rest frequency (naltrexone x time: $F(22,198) \geq 2.34$, $p \leq 0.001$), as well as the frequency and duration of locomotion (*m*CPP x time: $F(22,198) \geq 2.08$, $p \leq 0.05$).

Significant interactions involving time were further explored by a series of two-way ANOVAs within each timebin. These analyses revealed significant drug main effects or interactions for eat frequency and/or duration in timebins 1-3, 5-6, 8 and 10 ($F(1,9) \geq 5.15$, $p \leq 0.05$; $F(2,18) \geq 3.94$, $p \leq 0.05$), locomotion frequency and/or duration in timebins 3, 6, 10 and 12 ($F(1,9) \geq 5.20$, $p \leq 0.05$; $F(2,18) \geq 3.60$, $p \leq 0.05$), rest frequency and/or duration in timebins 3, 5, 6, 7 and 12 ($F(1,9) = 5.09$, $p \leq 0.05$; $F(2,18) \geq 4.93$, $p \leq 0.05$), and sniff frequency and/or duration in timebins 2, 3, 5, 6, 7, and 12 ($F(1,9) = 5.09$, $p = 0.05$; $F(2,18) \geq 3.65$, $p \leq 0.05$). Although followed up by a series of within-timebin Bonferroni comparisons, such fine-grain analyses were associated with higher variance around each data-point and, as such, produced few significant contrasts. However, it is worth noting that (relative to VV control) eat frequency was significantly reduced by mNL in timebins 1 and 2 ($p \leq 0.05$); eat duration was decreased in timebin 2 by mV and mNL ($p \leq 0.05$) and, in timebin 3, by VNH, mV and mNH ($p \leq 0.02$).

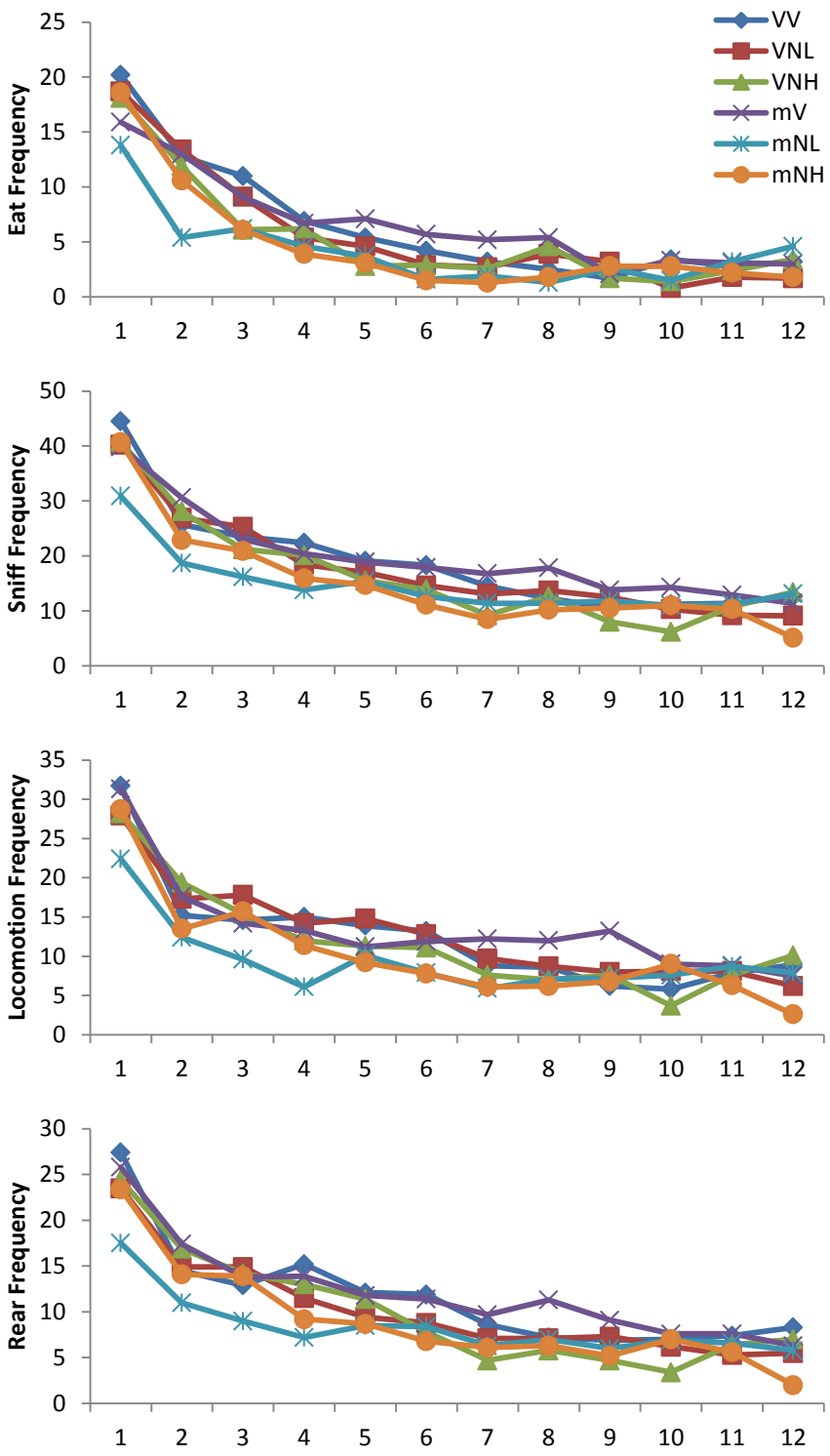


Figure 6-8: Experiment Eight. Effects of *m*CPP and naltrexone, alone and in combination, on the frequency of eating, sniffing, locomotion and rearing in male rats during a 1-h test with palatable mash

Data are expressed as the mean frequency of each behaviour in 12 x 5-min timebins. Dose-dependent suppression of behaviour is apparent during the early part of the test session. See text for further details

6.5.2.6 Behavioural Satiety Sequence (BSS)

Figure 6-9 illustrates the BSS profiles for each of the treatment conditions. The control BSS profile (VV; top left panel) indicates the typical peak feeding response in the first 15-20 min of the test. Feeding gradually gives way to grooming and resting as time progressed, with an eat-to-rest transition occurring circa half-way through the test session. Although neither dose of naltrexone given alone interfered with normal behavioural structure (centre & bottom left panels), there is a clear dose-dependent acceleration (shift to the left) of the entire sequence. *m*CPP given alone (top right panel) maintained the BSS but actually produced a modest shift to the right (delay in the eat-rest transition), whereas its combination with either dose of naltrexone (centre & bottom right) produced effects indistinguishable from those of the opioid receptor antagonist alone (centre & bottom left).

6.5.2.7 Bodyweight Gain

Data not shown. No significant main effects or interactions were found for 7-day absolute weight gain; animals typically gained 21-25g irrespective of treatment condition (main effect *m*CPP: $F(1,9) = 0.49$, $p > 0.05$; main effect naltrexone: $F(2,18) = 0.79$, $p > 0.05$; interaction: $F(2,18) = 0.39$, $p > 0.05$). Analysis of percent bodyweight change over days following treatment confirmed normal growth patterns (main effect DAY: $F(2,18) = 122.54$, $p < 0.001$), but it too failed to reveal any significant drug main effects, drug interactions, or drug x time interactions (main effect *m*CPP: $F(1,9) = 0.04$, $p > 0.05$; main effect naltrexone: $F(2,18) = 1.61$, $p > 0.05$; interaction: $F(2,18) = 0.24$, $p > 0.05$).

6.5.3 Summary of Main Findings

The results of Experiment 8 show that naltrexone had a somewhat more potent behavioural effect in the current study than has previously been seen in Experiments 5 and 6. Thus, even the lower dose (0.1 mg/kg) induced a modest though significant reduction in intake (~26%; $p < 0.05$) relative to vehicle control.

Consistent with results obtained in Experiment 7, *m*CPP (0.1 mg/kg) did not when given alone induce any significant behavioural effects when compared with vehicle (VV) control. Again, it is interesting to note that it tended to marginally delay (rather than accelerate) the BSS. Furthermore, there was little evidence that the combination of *m*CPP with either dose of naltrexone resulted in a stronger effect on intake or behaviour than seen in response to naltrexone alone. Therefore, under present test conditions and at the dose levels currently used, our results do not

support a positive anorectic interaction between the 5-HT_{2C} receptor agonist *m*CPP and the opioid receptor antagonist naltrexone.

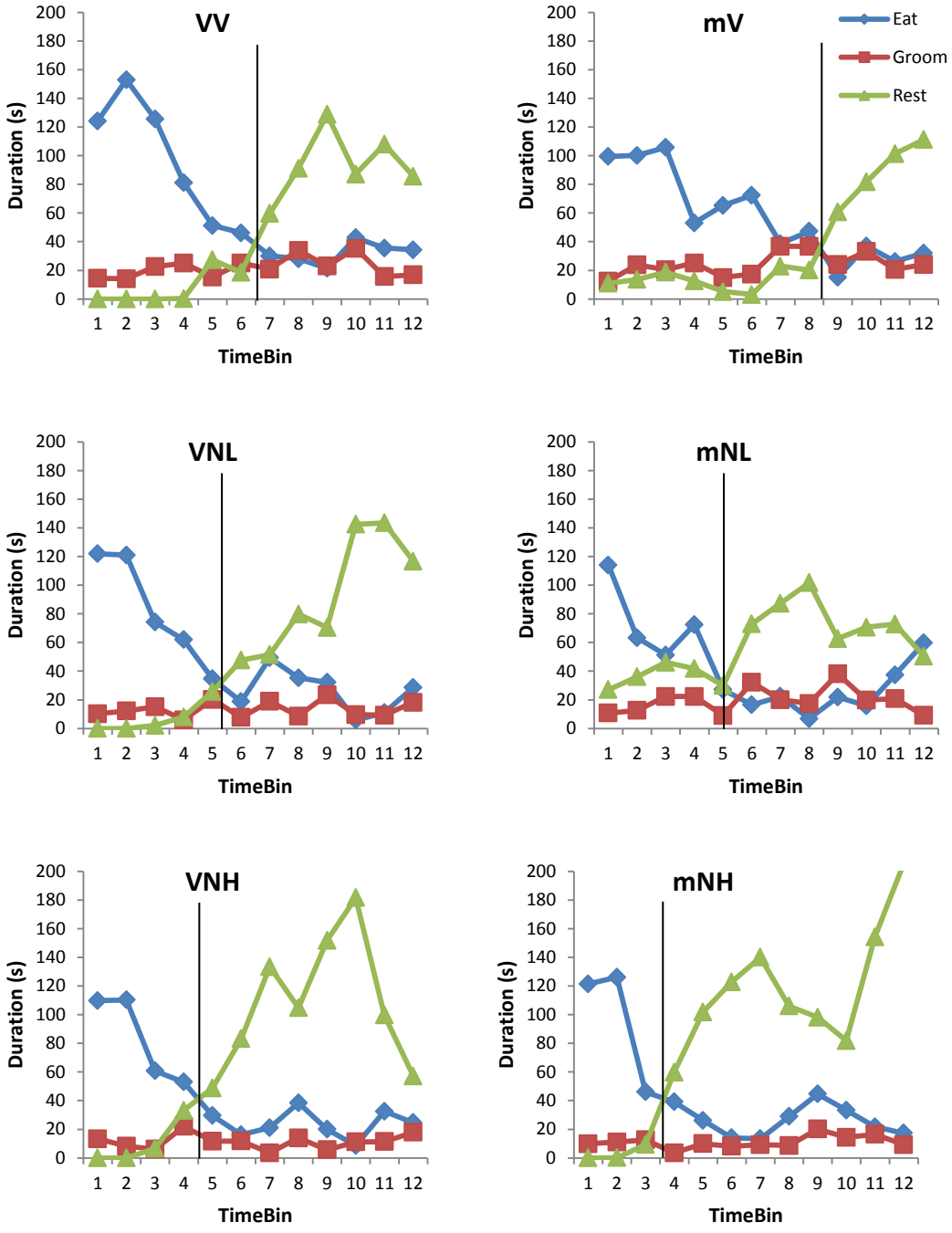


Figure 6-9: Experiment Eight. Effects of *m*CPP and naltrexone, alone and in combination, on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores (s = seconds) in each of 12 x 5min timebins comprising the 1h test period. The vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. 12 x 5-min timebins. V = vehicle, m = *m*CPP 0.1mg/kg, NL = 0.1 mg/kg naltrexone, NH = 1.0 mg/kg naltrexone. See text for details.

6.6 Chapter Six Main Findings

The aim of the current Chapter was to employ BSS methodology to assess the anorectic efficacy and behavioural specificity of combined low-dose treatment with naltrexone and *m*CPP.

- **Experiment 7** showed that acute administration of *m*CPP dose-dependently reduces food intake and the frequency of feeding behaviour in male rats. The *m*CPP-induced anorexia did not appear behaviourally-selective as it was accompanied by other behavioural changes indicative of hypoactivity, including (at the highest dose; 3.0mg/kg) disruption of the BSS.
- **Experiment 8** showed that the combination of a sub-anorectic dose of *m*CPP with one of two naltrexone doses (threshold and sub-maximal) did not produce a positive interaction on food intake or feeding behaviour.

The findings of Chapter 6 suggest that a 5-HT – opioid interaction may not prove to be a fruitful avenue of appetite / weight loss polytherapy. However, future research should perhaps assess the generality of current findings to more recently developed 5-HT_{2C} receptor agonists, such as lorcaserin, Ro 60-0175, CP-809101 and/or VER23779.

Chapter 7 Peptide and Opioid System Interactions

7.1 Incretins

Incretins, discovered in the 1900s (Bayliss & Starling, 1902), are glucose-lowering intestinal-derived molecules. The subsequent postprandial enhancement of insulin secretion, by their release in the gut, is known as the incretin effect (Creutzfeldt, 1979; Elrick et al., 1964; Nauck et al., 1986).

The first identified incretin, isolated from porcine intestine, was found to inhibit gastric secretion in dogs (Pederson et al., 1975) and was called gastric inhibitory polypeptide (GIP_a). However, it was later found that only pharmacological doses of GIP_a produced inhibition of gastric secretions, whereas a stimulating effect was seen at physiological doses (Lauritsen et al., 1980). It was therefore renamed glucose-dependent insulinotropic polypeptide (retaining the acronym; GIP). It is now known that GIP is released from K cells in proximal regions of the GIT, such as the duodenum and jejunum (Kim & Egan, 2008).

Later, it was discovered that, in addition to glucagon, the proglucagon gene (GCG), widely expressed peripherally and centrally (in the caudal brain stem and hypothalamus: Merchenthaler et al., 1999), encodes two other peptides. The two peptides, with 50% homology to GCG, were named glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2; Kreymann et al., 1987).

7.1.1 Glucagon-like Peptide 1

GLP-1 is a 30 amino-acid molecule secreted by the intestinal L cells, located predominantly in the distal ileum and colon, in response to nutrient ingestion (Brubaker, 2006) and in proportion to nutrient consumption (see reviews: Baggio & Drucker, 2007; Drucker, 2006; Holst, 2007; Parker et al., 2010). There are multiple forms of GLP-1: GLP-$1_{(1-37)}$ and GLP-$1_{(1-36)}NH_2$ (aka. GLP-$1_{(1-36)}$amide), both of which thought to be inactive; and GLP-$1_{(7-37)}$ and GLP-$1_{(7-36)}NH_2$ (aka. GLP-$1_{(7-36)}$amide), the active forms. It is interesting to note that the majority of GLP-1 in humans comprises GLP-$1_{(7-36)}NH_2$ (Orskov et al., 1994). All GLP-1 subtypes act on GLP-1 receptors (GLP-1R) which are widely expressed in the pancreatic islets, lung, heart, kidney, intestine and the CNS; hypothalamus and brainstem (Baggio & Drucker, 2007; Drucker, 2006).

The half-life of bioactive GLP-1 is less than 2 minutes due its inactivation by the enzyme dipeptidyl peptidase IV (DPP-IV; aka CD26; Deacon et al., 1995; Kieffer et

al., 1995; Mentlein et al., 1993). DPP-IV metabolises the active molecules (GLP-$1_{(7-37)}$ and GLP-$1_{(7-36)}NH_2$) to GLP-$1_{(9-37)}$ and GLP-$1_{(9-36)}NH_2$ (respectively). However, evidence shows that GLP-1 remains intact in DPP-IV null mice (Kieffer et al., 1995) suggesting that the inhibition of DPP-IV may be a potential avenue for maintaining a prolonged action of GLP-1 (see Section 7.1.5).

7.1.1.1 GLP-1 and Appetite Regulation

Initial studies in 1996 demonstrated that central administration of GLP-1 reduces food intake for a short period (TangChristensen et al., 1996; Turton et al., 1996), whereas peripheral administration had no effect (Navarro et al., 1996; TangChristensen et al., 1996; Turton et al., 1996). However, later studies found that both central (Meeran et al., 1999; Perez-Tilve et al., 2007) and peripheral administration of GLP-1 produced anorexia both in rodents (Chelikani et al., 2005; Larsen et al., 2001; Rodriquez de Fonseca et al., 2000; for review see: Barrera et al., 2011) and humans (Flint et al., 1998; Gutzwiller, Drewe, et al., 1999; Gutzwiller, Goke, et al., 1999; Naslund et al., 1999; Naslund et al., 1998; Zander et al., 2002; however see: Long et al., 1999; for a meta-analysis see: Verdich et al., 2001).

The anorectic effect of ICV GLP-1 can be blocked by pre-treatment with exendin$_{(9-37)}$, a GLP-1R antagonist (Meeran et al., 1999; TangChristensen et al., 1996; Turton et al., 1996) which, alone, significantly increases food intake and bodyweight (Meeran et al., 1999; Turton et al., 1996; however see: Larsen et al., 2001) despite a preload (Williams et al., 2009). Exendin$_{(9-37)}$ is also shown to block NPY-induced anorexia (Turton et al., 1996). More recent studies have shown that GLP-1R null mice display a hyperphagic phenotype without weight gain (Hansotia et al., 2007; however, see: Scrocchi et al., 1996), an effect recently replicated with ICV exendin$_{(9-37)}$ administration (Knauf et al., 2008).

Interestingly, there have been reports of differential effects of GLP-1 in obese and lean subjects. Evidence shows that obese humans and patients with T2DM exhibit reduced postprandial levels of GLP-1 (Ranganath et al., 1996; Vilsboll et al., 2001) due to impaired GLP-1 release (Carr et al., 2010; Muscelli et al., 2008; Vilsboll, Agerso, et al., 2003; Vilsboll, Krarup, et al., 2003). Futhermore, obese patients demonstrate an enhanced hypophagic response to GLP-1 treatment whereas, GLP-1 infusion has little effect in lean human studies (Long et al., 1999).

7.1.2 GLP-1 Mechanism of Action

GLP-1 has a complex physiological action on GLP-1R in different organs and tissues, as summarised in Figure 7-1.

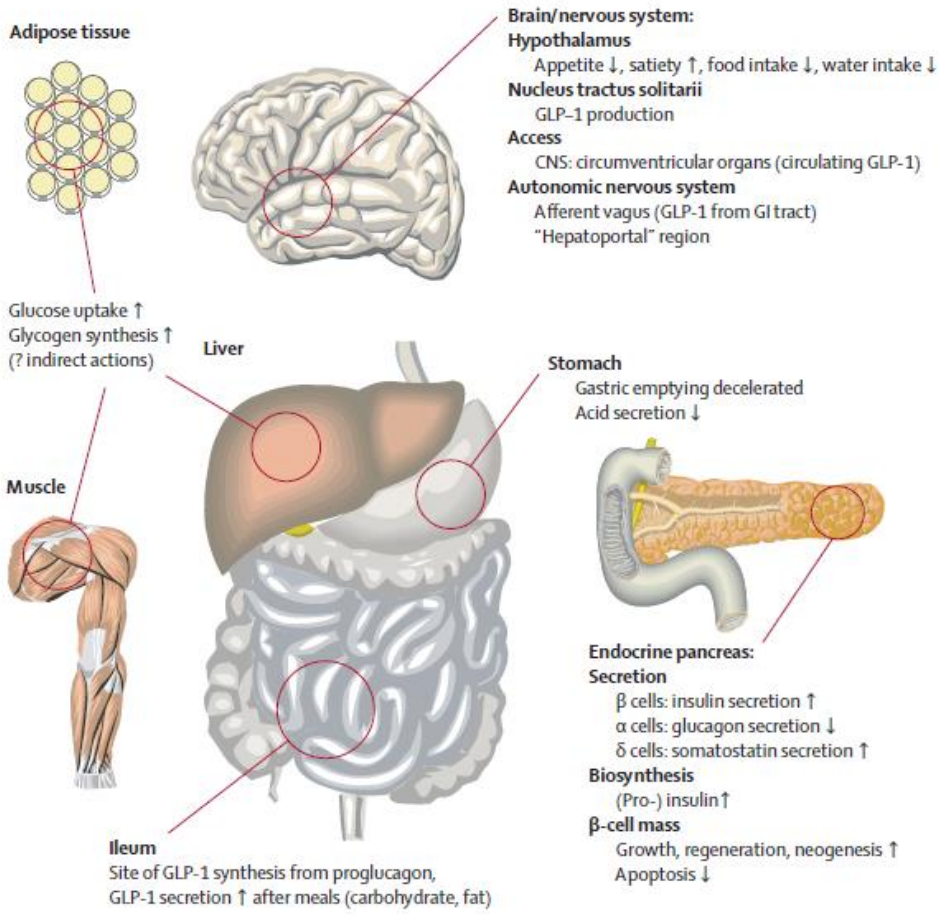


Figure 7-1: Physiology of Secretion and Action of GLP-1

Source (Drucker, 2006)

7.1.2.1 Peripheral: Insulinotropic effect

Of the two GLPs, only GLP-1 is found to stimulate the insulin receptor (Kieffer & Habener, 1999), as would be expected of an incretin (Kreymann et al., 1987). Evidence has since demonstrated that GLP-1 is also released in response to glucose administration (Unger et al., 1968), whereby it acts as an incretin to stimulate insulin secretion from pancreatic β-cells. It also acts in adipose, liver and muscle tissue to increase glucose uptake and glycogen production (Drucker, 2006; Nogueiras et al., 2009). These actions led to the development of GLP-1R agonists for the treatment of type 2 diabetes mellitus (T2DM; for review: Nielsen et al., 2004; Peters, 2010). Additional reports suggest that increased GLP-1 signalling is the mechanism by which drastic improvement in T2DM occurs following gastric bypass surgery (Bose et al., 2009).

7.1.2.2 Peripheral: Gastric slowing effects

GLP-1 is also associated with a slowing of gastric emptying and acid secretion in rodents (Talsania et al., 2005) and humans (Chelikani et al., 2005; Flint et al., 2001; Long et al., 1999; Naslund et al., 1998) via the stimulation of the sympathetic nervous system (SNS; Andrews et al., 2007; Yamamoto et al., 2002). It is proposed that this deceleration of gastric emptying attenuates increases in meal-associated blood glucose (Meier et al., 2003; Willms et al., 1996). Interestingly, however, it has been reported that this effect is not due to a direct action, but is vagally mediated (Bucinskaite et al., 2009; Rocca & Brubaker, 1999). Evidence shows that administration of exendin$_{(9-39)}$ or vagal afferent denervation blocks GLP-1-induced gastric emptying (Baggio & Drucker, 2007) and anorexia (Abbott et al., 2005; Talsania et al., 2005). Furthermore, GLP-1 release appears to be biphasic. Early release takes place ~10-15minutes postprandially, with the later release occurring after ~30-60 minutes (Herrmann et al., 1995). However, given the hypothesis of nutrient-stimulated GLP-1R activation in the distal ileum, the early release component is not fully understood. Therefore, neural mediation by GRP (gastrin releasing peptide), acetyl-choline and GIP has been proposed. This suggests that there may be a proximal-to-distal neural signalling pathway for GLP-1 secretion.

7.1.2.3 Central; Appetite regulation via the brainstem and hypothalamus

GLP-1 has been reported as a ‘neurohumoral’ agent because of its ability to act both as a hormone and transmitter in the periphery as well as the CNS (Drucker, 2006). GLP-1 is produced in the NTS, DMV, and AP, with projections to many GLP-1R-expressing brain areas involved in energy balance, such as the PVN and NAcc (Dossat et al., 2011; Drucker & Asa, 1988; Merchenthaler et al., 1999; Rinaman, 2010). Evidence shows that GLP-1 produces neuronal activation in the hypothalamus and brainstem (Abbott et al., 2005; Larsen et al., 1997; Rowland et al., 1997), while GLP-1R expression in these same brain regions is significantly altered by fasting and re-feeding (Zhou et al., 2003). GLP-1R agonists are therefore thought to reduce food intake via action at sites in PVN and hindbrain (Hayes et al., 2008; McMahon & Wellman, 1998; Schick et al., 2003) and even the HPA (Larsen et al., 1997), amygdala (Kinzig, D'Alessio, et al., 2002; Kinzig, Figueriedo, et al., 2002) and NAcc (Dossat et al., 2011). Moreover, evidence demonstrates that lesions of brainstem–hypothalamic pathways prevent peripheral GLP-induced anorexia and block hypothalamic activation (Abbott et al., 2005). Similarly, systemic pre-treatment with capsaicin, which ablates neurons and prevents vagal signalling,

has also been found to block peripheral GLP-1R agonist-induced anorexia (Talsania et al., 2005).

In contrast, although both GLP-1 and its receptor agonists (such as exendin-4; see Section 1.1.3) are small molecules able to pass the BBB (Kastin et al., 2002), the GLP-1-albumin recombinant fusion protein, that cannot cross the BBB, is still able to activate CNS and inhibit intake in mice, potentially via vagal signalling (Baggio & Drucker, 2007). Additionally, there are reports of anorexia following i.p. administration of GLP-1R agonists even with lesions to AP and subfornical organ (Baraboi et al., 2010). This evidence emphasises that GLP-1 has a complicated physiology incorporating both central and peripheral mechanisms of action (Hayes, Leichner, et al., 2011).

7.1.3 Exendin-4

Exendin-4, a 39 amino-acid peptide, is a naturally occurring form of exenatide[1] (synthetic; AC2993) isolated from the salivary secretions of the lizard, *Heloderma suspectum* (Eng et al., 1992). Its 53% amino identity with GLP-1 means that exendin-4 is not a GLP-1 analogue, but does share many actions at the GLP-1R. Additionally, it is not a substrate for DPP-IV and therefore has a much longer circulating half-life of 60-90minutes (Kolterman et al., 2005; Parkes, Jodka, et al., 2001).

Similar to GLP-1, it is known to produce a glucose-dependent enhancement of insulin release (Egan et al., 2002; Egan et al., 2003; Parkes, Pittner, et al., 2001; Young et al., 1999) and a slowing of gastric emptying (Jodka et al., 1998). Importantly, in this context, central and peripheral exendin-4 treatment is found to induce satiety and reduce intake in both rodents (Aziz & Anderson, 2002, 2003; Bojanowska & Nowak, 2007; Bojanowska & Radziszewska, 2011; Chan et al., 2013; Hayes, Kanoski, et al., 2011; Kanoski et al., 2011; Kanoski et al., 2012; Mack, Moore, et al., 2006; Perez-Tilve et al., 2007; Szayna et al., 2000; Talsania et al., 2005; for review see: Nielsen et al., 2004) and humans (Buse et al., 2004; DeFronzo et al., 2005; Edwards et al., 2001; Heine et al., 2005; Kendall et al., 2005; Ratner et al., 2006; Riddle et al., 2006).

Detailed meal pattern analysis shows that exendin-4 specifically reduces meal size (Williams et al., 2009) in human and non-human primates (Flint et al., 1998; Scott & Moran, 2007). Exendin-4 has also been shown, through reduced CPP and

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[1] Although the terms “exendin-4” and “exenatide” can be used interchangeably, the term “exendin-4” will be used throughout the present thesis.

progressive ratio operant-conditioning, to decrease the rewarding value of food (Dickson et al., 2012). Furthermore, some studies suggest that exendin-4-induced anorexia is dependent on specific nutrient intake (Aziz & Anderson, 2002, 2003; Pritchett & Hajnal, 2012).

Interestingly, i.p. injection of exendin-4 is more potent than GLP-1 at reducing plasma glucose (Bhavsar, Lachappell, et al., 1998; Young et al., 1998) and food intake (Rodriquez de Fonseca et al., 2000). A possible explanation is that exendin-4 has been found to have enhanced transport at the BBB compared to that of GLP-1 (Kastin & Akerstrom, 2003). In contrast, GLP-1R agonists that lack penetration of the BBB have also been shown to produce anorexia (Baggio et al., 2008; Baggio et al., 2004b). Therefore, the greater potency of exendin-4 compared to GLP-1 may be a result of greater plasma concentrations or differential activation of GLP-1 receptors (Barrera et al., 2009).

Human trials of glycaemic control in T2DM patients over 30 weeks of exenatide treatment (10µg) found no evidence of a weight loss plateau and, by the end of the trial, subjects had lost up to 2.8kg of bodyweight from baseline (Buse et al., 2004; DeFronzo et al., 2005; Heine et al., 2005; Kendall et al., 2005). Similarly, longer studies, over an 82 week period, have found equivalent weight loss of ~2.5kg (Ratner et al., 2006; Riddle et al., 2006).

Further developments of GLP-1R agonists include: the exenatide LAR (long acting release) formulation, which has been shown to produce 24-hour glycaemic control and weight loss with only one subcutaneous administration per week (Kim et al., 2007); CJC-1134, is a modified exendin-4 analogue conjugated to recombinant human albumin, also shown to have an extended longer half-life (Christensen & Knop, 2010); albugon (naliglutide) is a recombinant GLP-1–albumin protein found to lower blood glucose and enhance insulin, in addition to activating c-fos expression in multiple regions of the central nervous system, inhibiting gastric emptying and reducing food intake in mice following both central and peripheral administration (Baggio et al., 2004b); and Liraglutide (NN2211).

7.1.4 Liraglutide

Liraglutide is an acylated GLP-1 molecule, and therefore a full agonist of the GLP-1 receptor, which shares 97% of its amino acid sequence identity with human GLP-1. Its resistance to DPP-IV breakdown means that liraglutide has a much longer half-life (see review: Russell-Jones, 2009). Although designed for the treatment of T2DM, it is also found to reduce food intake and bodyweight in rodents (Hayes, Kanoski, et al., 2011; Knudsen, 2010; Raun, von Voss, Gotfredsen, et al., 2007;

Raun, von Voss, & Knudsen, 2007) and humans (Hansen et al., 2001; Nauck et al., 2006).

In trials of glycemia control, patients with T2DM taking 1.9mg liraglutide daily lost 2.99kg, a significant weight loss compared to placebo (Vilsboll et al., 2007). A trial of liraglutide treatment (3.0mg) in conjunction with a LCD found that participants lost significantly more weight (-6.3kg) over a 20week period compared to placebo (-2.8kg) or orlistat treatment (2.1kg; Astrup et al., 2009). The trial also found reductions in additional obesity-related parameters, such as blood pressure and the prevalence of pre-diabetes (Astrup et al., 2009). Interestingly, a comparison of liraglutide and exentatide over a 26 week trial found similar levels of weight loss (liraglutide –3.24kg and exenatide –2.87kg; Buse et al., 2009).

7.1.5 DPP-IV inhibitors

Research comparing the effects of GLP-1R agonists and DPP-IV inhibitors on the potentiation of endogenous GLP-1 and GIP have highlighted some significant differences (Lamont & Drucker, 2008). Notably, DPP-IV inhibitors do not appear to produce a significant reduction in food intake and bodyweight in rats (see reviews: Ahren et al., 2004; Raun, von Voss, Gotfredsen, et al., 2007). Furthermore, phase I trials of sitagliptin, vildagliptin, alogliptin and linagliptin (DPP-IV inhibitors) found that, although treatment significantly reduced HbA1c (a measure of inadequate glycaemic control), it had no effect on bodyweight compared to placebo over a 18 week period (DeFronzo, Fleck, et al., 2008; DeFronzo, Okerson, et al., 2008; Pratley et al., 2006; Raz et al., 2006; Taskinen et al., 2011). Therefore, DPP-IV is not currently considered a target for the development of anti-obesity agents, instead research should focus on the implications of these findings. For example, there may be an unidentified peptide or metabolite of more significance in the potentiation of endogenous GLP-1 and GIP than DPP-IV.

7.2 Behavioural Specificity

Early in the development of GLP-1R agonists, Rinaman et al., linked GLP-1R activation with illness and stress (Lachey et al., 2005; Rinaman, 1999b). For example, evidence shows that exendin-4 administration to house musk shrews induces emesis in 40% of animals (Chan et al., 2013). Unsurprisingly, then, exenatide treatment has been reported to induce nausea during clinical trials (Buse et al., 2004; Pinelli & Hurren, 2011; Ratner et al., 2006; Riddle et al., 2006). Additionally, exendin-4 has been reported to produce behavioural inhibition (Erreger et al., 2012; Mack, Laugero, et al., 2006; Mack, Moore, et al., 2006) while,

conversely, GLP-1 KO mice display increased locomotion (Hansotia et al., 2007). These effects are mirrored by reports of GLP-1-induced CTA and/or pica in animals (Chan et al., 2013; Kanoski et al., 2012). All these actions are known to suppress appetite indirectly (Halford, Boyland, Blundell, et al., 2010; Halford et al., 1998; Vickers & Clifton, 2012), and cast doubt on the behavioural specificity of GLP-1-induced anorexia.

Furthermore, lithium chloride (LiCl) has been shown to activate GLP-1 expressing neurons in the CNS (Rinaman, 1999a), triggering visceral illness or anxiogenic-like behaviour. Additionally, LiCl and GLP-1 produce similar neuronal activation patterns and behavioural profiles (Thiele & Seeley, 1998), an effect blocked by GLP-1R antagonists (Parkinson, 2009). Moreover, LiCl-induced CTA is blocked by exendin$_{(9-39)}$ (Rinaman, 1999a; Seeley et al., 2000), demonstrating a strong case for GLP-1R mediated malaise.

When considering GLP-1R agonists for the treatment of obesity, it is also pertinent to note that GLP-1Rs are located in the heart and stimulate the autonomic nervous system (ANS; Gardiner et al., 2008). It is therefore unsurprising that central and peripheral administration of GLP-1R agonists increases heart rate as well as systolic, diastolic and mean arterial blood pressure (Drucker et al., 2011). This problem may limit the potential of GLP-1R agonists as anti-obesity agents, particularly in view of the heightened awareness/concern of the FDA regarding matters of cardiovascular function.

7.3 GLP-1R Agonists in Combination Therapies

In view of the current interest in drug polytherapy for obesity, there have been several treatment combinations involving GLP-1R agonists that have shown considerable promise. For example, additive/synergistic anorectic and/or weight loss interactions have been reported for GLP-1 and glucagon (Day, 2009) as well as both GLP-1 (Paulik et al., 2011) and exenatide (Reidelberger et al., 2011b; Talsania et al., 2005) when given in combination with the gut peptide, PYY$_{3-36}$. Similarly, positive effects on food intake have been reported for exendin-4 in combination with the adiposity signal leptin (Bojanowska & Nowak, 2007; Reidelberger et al., 2011a), the amylin analogue calcitonin (Bello et al., 2010), and the cannabinoid CB1 receptor antagonist/inverse agonist AM-251 (Bojanowska & Radziszewska, 2011).

7.4 Rationale; Chapter 7

In view of evidence concerning the unwanted effects of exenatide given alone (nausea, emesis, hypoactivity), Chapter 7 aims to: (i) comprehensively profile the acute behavioural effects of the peptide in rats during tests of palatable food consumption and (ii) investigate the potential advantages of concurrently targeting satiety signalling and hedonics, by exploring the potential advantages of low-dose (sub-anorectic, sub-maximally anorectic) combinations of exendin-4 and the opioid receptor antagonist, naltrexone.

7.5 Experiment Nine; Exendin-4 Dose-Response

7.5.1 Method

For the main methodological details, refer to General Methods (Chapter 3).

7.5.1.1 Subjects and Design

10 adult male Lister hooded rats (202.53 ± 2.34g on arrival from Charles River, U.K and 467.81 ± 11.32g by the end of the study) were employed for this study. A within-subjects design was adopted whereby each subject received all four experimental conditions according to a Latin Square (with a 7-day wash out period): Vehicle (V); Exendin-4 0.025µg/kg (Exn0.025); Exendin-4 0.25µg/kg (Exn0.25); Exendin-4 2.5µg/kg (Exn2.5).

7.5.1.2 Drugs

Exendin-4 (exenatide; Tocris Bioscience, UK) was initially dissolved to a concentration of 1mg/10ml in distilled water, following which it was serially diluted to final concentrations, disbursed in 0.7ml volumes to individual aliquots, and stored at -20ºC until required. Distilled water, which alone served as vehicle control, was stored in an identical manner. On test days, the required aliquots were slowly thawed in hand to room temperature just prior to use, and the requisite volumes administered i.p. in a volume of 1ml/kg 30 minutes prior to testing.

Doses of exendin-4 (0.025, 0.25 and 2.5µg/kg) were selected on the basis of previous research on food intake to span the full range from ineffective to sub-maximal (Aziz & Anderson, 2002, 2003; Bojanowska & Nowak, 2007; Bojanowska & Radziszewska, 2011; Chan et al., 2013; Hayes, Kanoski, et al., 2011; Kanoski et al., 2011; Kanoski et al., 2012; Mack, Moore, et al., 2006; Perez-Tilve et al., 2007; Szayna et al., 2000; Talsania et al., 2005).

7.5.1.3 Procedure

Testing occurred over two weeks, with four test days per week, 5 animals tested per day, and a 7 day wash-out period. Treatment order was counterbalanced both within and between test days according to the Latin Square. It should be noted that, as animals did not show appreciable amounts of scratching during Experiment 9, data for this variables are not reported.

7.5.1.4 Error

There was an error in bodyweight collection during Experiment 9 that resulted in a loss of two data points per animal during one test week, consequently only a 3 day post-treatment % bodyweight analysis could be conducted.

7.5.2 Results

Full statistical details can be found in Appendix 10.

7.5.2.1 Habituation Phase Food Intake

Mash consumption differed significantly over the course of habituation ($F(4,36) = 13.63$, $p < 0.001$), with intake on the first trial lower than on trials 2, 3 and 5 ($p \leq 0.01$) and intake on trial 2 lower than on trial 5 ($p = 0.001$; trial 1: 14.58 ± 0.97g; trial 2: 17.00 ± 1.05g; trial 3: 19.98 ± 1.20g; trial 4: 19.35 ± 1.69g; trial 5: 22.81 ± 0.78g). The development of a stable intake pattern was confirmed by the lack of difference across habituation trials 3-5, as well as the close similarity in intake scores between those habituation trials and the vehicle condition in the main experiment (21.07 + 1.48g).

7.5.2.2 Test Day Bodyweight

Test-day bodyweights were comparable across the various treatment conditions (V: 403.6 ± 12.2g; Exn0.025: 410.6 ± 11.3g; Exn0.25: 426.3 ± 14.8g; Exn2.5: 410.3 ± 9.9g ($F(3,27) = 1.04$, $p > 0.05$).

7.5.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.29% throughout the experiment (range = 0.05 - 0.67%). Treatment effects on food intake are summarised in Figure 7-2. There was a significant main effect of exendin-4 on 1h mash consumption ($F(3,27) = 48.89$, $p = 0.001$). Bonferroni comparisons confirmed a significant anorexia ($p < 0.001$) relative to vehicle control at both 0.25µg/kg and 2.5µg/kg (35.6% and 75.6% decreases, respectively). Comparisons between dose levels further confirmed the dose-dependency of these effects with 0.25µg/kg ($p < 0.02$) and 2.5µg/kg ($p < 0.001$) differing significantly from 0.025µg/kg, and 2.5µg/kg differing significantly from 0.25µg/kg ($p < 0.01$).

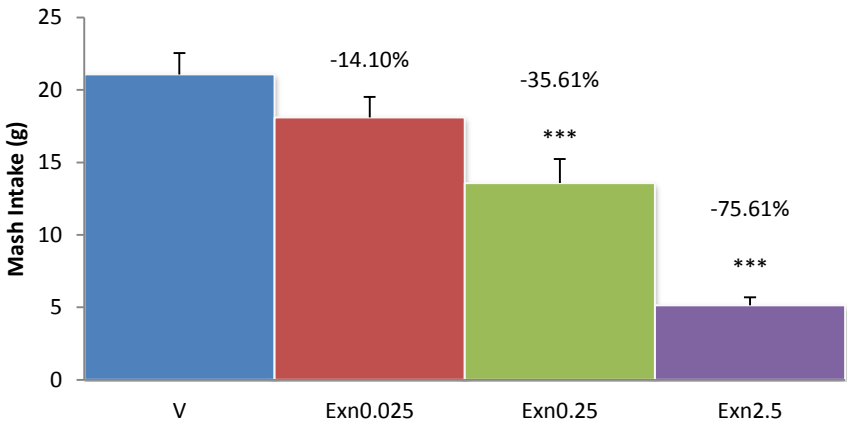

Figure 7-2: Experiment Nine. Effects of acute exendin-4 on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = Vehicle, Exn0.025 = Exendin-4 0.025µg/kg, Exn0.25 = Exendin-4 0.25µg/kg, Exn2.5 = Exendin-4 2.5µg/kg. See text for details. *** $p < 0.001$ vs V.

7.5.2.4 Total (one-hour) Behavioural Analyses

Treatment effects on total 1-h frequency and duration scores for ingestive and non-ingestive behaviours are illustrated in Figure 7-3, while data for eating-related parameters are summarised in Table 7-1.

Table 7-1: Experiment Nine. Acute effects of Exendin-4 on eating-related parameters

(Mean ± SE). V = Vehicle, Exn0.025 = Exendin-4 0.025µg/kg, Exn0.25 = Exendin-4 0.25µg/kg, Exn2.5 = Exendin-4 2.5µg/kg. † p < 0.062, * p < 0.01, ** p < 0.01, *** p < 0.001 vs V.

Measure	V	Exn0.025	Exn0.25	Exn2.5
Latency to find food (s)	2.40 ± 0.50	5.60 ± 1.77	4.10 ± 1.25	7.00 ± 1.22
Latency to eat (s)	18.00 ± 5.26	9.40 ± 3.17	4.70 ± 1.17	3.10 ± 0.80†
Eat bout (s)	8.76 ± 1.04	9.13 ± 1.47	11.86 ± 0.70*	16.68 ± 1.25***
Eat rate (g/min)	1.94 ± 0.09	2.68 ± 0.42	1.44 ± 0.08*	0.59 ± 0.27***

ANOVA revealed significant effects of exendin-4 on virtually all measures taken: latency to eat ($F(3,27) = 4.68$, $p < 0.05$), the average duration of eating bouts ($F(3,27) = 15.34$, $p < 0.001$) and eating rate ($F(3,27) = 15.18$, $p < 0.01$), as well as the frequency and duration of eating ($F(3,27) \geq 3.33$, $p \leq 0.05$), grooming ($F(3,27) \geq 18.51$, $p \leq 0.001$), resting ($F(3,27) \geq 4.43$, $p \leq 0.02$), locomotion ($F(3,27) \geq 30.88$, $p \leq 0.001$), rearing ($F(3,27) \geq 32.85$, $p \leq 0.001$), and sniffing ($F(3,27) \geq 9.42$, $p \leq 0.001$). The only variable not to be affected by drug treatment was the latency to locate the food source at the beginning of the test ($F(3,27) = 2.27$, $p > 0.05$).

As shown in Table 7-1 and Figure 7-3, Bonferroni comparisons indicated that behaviour was unaffected by the lowest dose exendin-4 (0.025 µg/kg). However, relative to vehicle control, the intermediate dose (0.25 µg/kg) increased the average duration of eating bouts ($p < 0.05$) and rest duration ($p < 0.01$), while reducing eating rate ($p < 0.05$), the frequency of eating ($p < 0.01$), grooming ($p < 0.05$), locomotion ($p < 0.02$) and sniffing ($p = 0.001$), and both the frequency and duration of rearing ($p \leq 0.01$). At the highest dose tested (2.5 µg/kg), exendin-4 increased the average duration of eating bouts ($p < 0.001$), reduced eating rate ($p < 0.001$) and almost significantly reduced eat latency ($p = 0.062$). This dose also significantly enhanced the frequency and duration of resting ($p \leq 0.02$), while strongly suppressing the frequency of eating ($p < 0.001$) as well as the frequency and duration of grooming ($p \leq 0.01$), rearing ($p \leq 0.001$), locomotion ($p \leq 0.001$) and sniffing ($p \leq 0.01$).

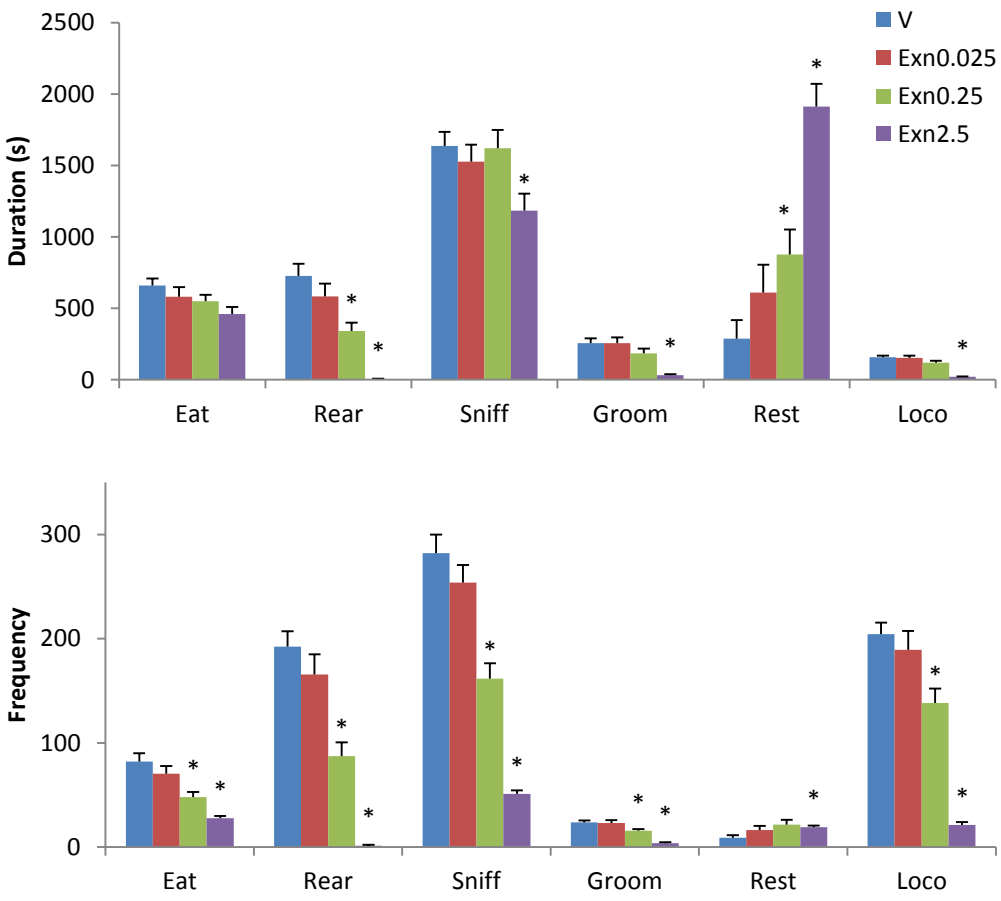

Figure 7-3: Experiment Nine. Effects of acute Exendin-4 on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). V = Vehicle, Exn0.025 = Exendin-4 0.025µg/kg, Exn0.25 = Exendin-4 0.25µg/kg, Exn2.5 = Exendin-4 2.5µg/kg. See text for further details. * $p \leq 0.05$ vs V

7.5.2.5 Periodic (Timebin) Behavioural Analyses

Timecourse analyses confirmed the typical pattern of behaviour over these 1h feeding tests, with a gradual reduction in active behaviours and increase in resting as the session progressed. Thus, with the exception of sniff duration ($F(11,99) = 1.57$, $p > 0.05$) and groom frequency ($F(11,99) = 0.85$, $p > 0.05$), significant main effects of time were found for the frequency ($F(11,99) \geq 6.31$, $p \leq 0.001$) and duration ($F(11,99) \geq 2.36$, $p \leq 0.02$) of all behavioural measures. Somewhat exceptionally, significant treatment x time interactions were found for the frequency ($F(33,297) \geq 1.56$, $p \leq 0.05$) and duration ($F(33,297) \geq 1.69$, $p \leq 0.02$) of all behavioural measures except groom frequency ($F(33,297) = 1.33$, $p > 0.05$).

One-way ANOVAs and Bonferroni post-hocs within each timebin indicated that exendin-4 (0.25-2.5 µg/kg) dose-dependently reduced the frequency of feeding at several timepoints during the first half of the test ($F(3,27) \geq 8.93$, $p \leq 0.001$), with similar though weaker effects seen on feeding duration ($F(3,27) \geq 4.91$, $p \leq 0.01$). Exendin-4 also dose-dependently enhanced the frequency, and particularly the duration, of resting over the same timeframe ($F(3,27) \geq 4.09$, $p \leq 0.02$). In addition to these effects, exendin-4 dose-dependently suppressed the frequency and duration of locomotion ($F(3.27) \geq 3.93$, $p \leq 0.02$), rearing ($F(3,27) \geq 6,17$, $p \leq 0.005$) and sniffing ($F(3,27) \geq 3.42$, $p \leq 0.05$) throughout most of the test session. Figure 7-4 illustrates the timecourse effects of exendin-4 on the frequency of eating, locomotion, rearing and sniffing.

7.5.2.6 Behavioural Satiety Sequence (BSS)

Treatment effects on the BSS are shown in Figure 7-5. Consistent with previous work in our laboratory (Ishii et al. 2003; Tallett et al. 2009a&b; see Experiments 1-8), the vehicle control profile shows a typical peak feeding response during the first 20 min of the test. Over time, resting gradually increases, with an eat-to-rest transition occurring during timebin 7. A very similar pattern of behaviour is evident with the lowest dose of exendin-4 (0.025 µg/kg) although the eat-rest transition has shifted to the left by approximately one timebin (i.e. 5 min). This acceleration of behavioural satiety is more evident at the intermediate dose (0.25µg/kg), where the eat-rest transition has moved substantially to the left (i.e. to timebin 3). Although this trend towards BSS acceleration continues with the highest dose of exendin-4 (2.5 µg/kg), where the eat-rest transition occurs in timebin 2, the structure of behaviour now appears abnormal with grooming virtually eliminated from the repertoire and resting dominating for most of the session.

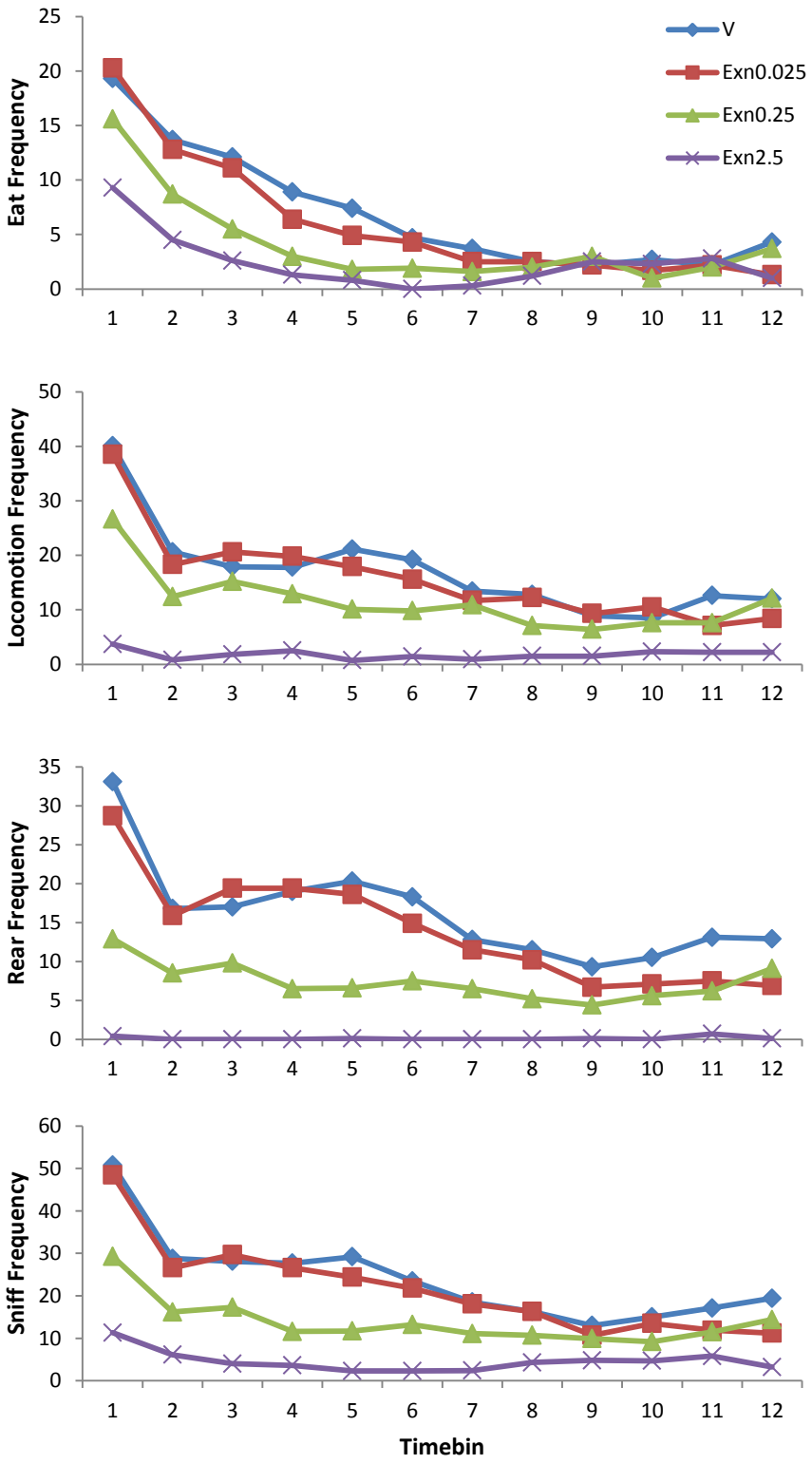

Figure 7-4: Experiment Nine. Effects of acute exendin-4 on the timecourses of eating, locomotion, rearing and sniffing frequency in male rats during a 1-h test with palatable mash

Data are expressed as the mean duration of each behaviour in 12 x 5-min timebin. V = Vehicle, Exn0.025 = Exendin-4 0.025µg/kg, Exn0.25 = Exendin-4 0.25µg/kg, Exn2.5 = Exendin-4 2.5µg/kg. See text for further details.

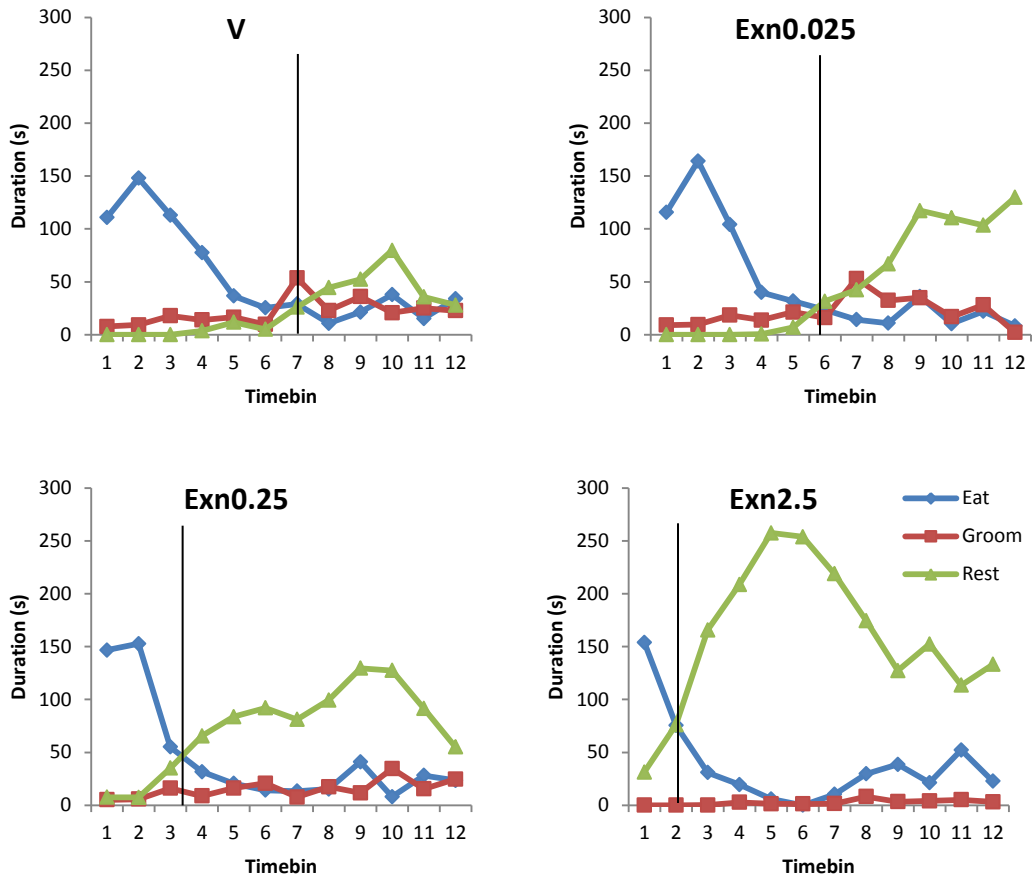

Figure 7-5: Experiment Nine. Effects of acute exendin-4 on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. V = Vehicle, Exn0.025 = Exendin-4 0.025µg/kg, Exn0.25 = Exendin-4 0.25µg/kg, Exn2.5 = Exendin-4 2.5µg/kg. See text for further details.

7.5.2.7 Bodyweight Gain

Data not shown. Although ANOVA indicated a significant treatment effect on 7-day absolute weight gain ($F(3,27) = 3.99$, $p < 0.02$), Bonferroni tests failed to detect any significant drug-vehicle differences (V = 24.95 ± 1.98g; Exn0.025 = 22.40 ± 1.00g; Exn0.25 = 19.94 ± 0.94g; Exn2.5 = 19.39 ± 1.88g). To account for minor differences in absolute test day bodyweight, datasets were converted to percent bodyweight changes from test day (test day = 100%). This finer grain analysis, possible for only 3 days post-dosing (see Section 1.3.1.4), confirmed the lack of effect of treatment ($F(3,27) = 1.57$, $p > 0.05$), day ($F(2,18) = 0.85$, $p > 0.05$), or interaction ($F(6,54) = 1.16$, $p > 0.05$).

7.5.3 Summary of Main Findings

The results of Experiment 9 showed that exendin-4 potently and dose-dependently suppressed mash consumption, with highly significant reductions (vs. control) of 26% and 75.6% at 0.25 and 2.5µg/kg, respectively. The intermediate (0.25µg/kg) and the highest doses (2.5µg/kg) were also found to significantly increase the duration of eating bouts, slow the rate of eating, and reduce the frequency (but not duration) of eating episodes. Importantly, the effects of exendin-4 (0.25 - 2.5µg/kg) were not limited to ingestive behaviour, with dose-dependent reductions in the frequency and duration of rearing, sniffing, grooming and locomotion, as well as dose-dependent increases in the frequency and duration of resting. The lowest dose of exendin-4 (0.025µg/kg) not only failed to influence mash consumption, but was also without significant behavioural effect under present test conditions.

The BSS profiles show that exendin-4 dose-dependently accelerated BSS, i.e. suppressed the peak feeding response and shifted the eat-to-rest transition to the left. However, it is important to note that, although the BSS profile of the highest dose (2.5µg/kg) could be interpreted as consistent with a strong satiety signal, there are reasons to treat this interpretation with some caution due to the gross reductions in active behaviours (see Discussion; Section 8.2.4). The results of Experiment 9 therefore raise questions about the behavioural selectivity of the acute anorectic response to exendin-4.

7.5.4 Design of Experiment Ten

Based on the data from the exendin-4 dose-response study (Experiment 9), a sub-anorectic and a sub-maximal dose of exendin-4 were selected. Experiment 9 confirmed that acute exendin-4 0.025µg/kg did not significantly suppress food intake or produce any significant behavioural effects. However, exendin-4 0.25µg/kg did significantly decrease food intake, while displaying a general reduction in active behaviours. In order to further assess the hypolocomotive and potentially sedative effect of this higher dose, alone and in combination with naltrexone, this sub-maximal dose was also selected for Experiment 10.

7.6 Experiment Ten; Exendin-4 (0.025 and 0.25µg/kg) and Naltrexone (0.1mg/kg) Interaction

7.6.1 Method

7.6.1.1 Subjects and Design

10 adult male Lister hooded rats (215.37 ± 1.63g on arrival from Charles River, U.K and 518.25 ± 13.80g by the end of the study) were employed in this study. A within-subjects design was adopted whereby each subject received all six experimental conditions according to a Latin Square (with a 7-day wash out period): Vehicle (Distilled water) + Vehicle (Saline; VV); Vehicle (Distilled water) + Naltrexone (0.1mg/kg; VN); Exendin-4 (0.025µg/kg) + Vehicle (Saline; ELV); Exendin-4 (0.025µg/kg) + Naltrexone (0.1mg/kg; ELN); Exendin-4 (0.25µg/kg) + Vehicle (Saline; EHV); and Exendin-4 (0.25µg/kg) + Naltrexone (0.1mg/kg; EHN).

7.6.1.2 Drugs

As with Experiment 9, Exendin-4 (exenatide; Tocris Bioscience, UK) was initially dissolved to a concentration of 1mg/10ml in distilled water, following which it was serially diluted to final concentrations, disbursed in 0.7ml volumes to individual aliquots, and stored at -20ºC until required. Distilled water, which alone served as vehicle control, was stored in an identical manner. On test days, the required aliquots were slowly thawed in hand to room temperature just prior to use, and requisite volumes administered 30 minutes prior to testing. Naltrexone hydrochloride (Sigma-Aldrich, UK) was dissolved in physiological saline (0.9%) which, alone, served as a vehicle control, and administered 15 minutes prior to testing. Both drugs were administered i.p. in a volume of 1ml/kg. Doses of exendin-4 (0.025 and 0.25µg/kg) and naltrexone (0.1mg/kg) were selected from data obtained in Experiments 9 and 5 (respectively).

7.6.1.3 Procedure

Testing occurred over four weeks, with two test days per week, and 5 animals tested per day. Treatment order was counterbalanced both within and between test days according to the Latin Square. It should be noted that, as animals did not show appreciable amounts of scratching during Experiment 10, data for this variables are not reported. Please also note, for this Experiment only, an additional (6th) day was employed during the habituation period. Due to timetabling reasons, and to ensure animals had no more than 4 days between the habituation and experimental phases, an additional habituation trial was necessary.

7.6.2 Results

Full statistical details can be found in Appendix 11.

7.6.2.1 Habituation Phase Food Intake

ANOVA revealed significant differences in mash consumption over the 6-day habituation period ($F(5,45) = 18.63$, $p \leq 0.001$), with intake on the first trial lower than on all other trials ($p \leq 0.01$) and intake on trial 2 lower than on trials 4, 5 and 6 ($p \leq 0.01$; trial 1: 9.55 ± 1.29g; trial 2: 14.71 ± 1.44g; trial 3: 18.05 ± 1.71g; trial 4: 19.83 ± 1.46g; trial 5: 22.04 ± 1.59; trial 6: 20.30 + 1.90). However, the development of a stable intake pattern was confirmed by the lack of difference across habituation trial 3-6, as well as the close similarity in intake scores between the final few habituation trials and the vehicle condition in the main experiment (20.19 + 1.11g).

7.6.2.2 Test Day Bodyweight

Test-day bodyweights did not differ across treatment conditions: VV = 447.10 + 13.8g; VN = 456.8 + 19.5g; ELV = 449.4 + 18.1g; ELN = 460.5 + 14.5g; EHV = 449.2 + 17.8g; EHN = 453.5 + 15.3g (main effect of naltrexone: $F(1,9) = 0.36$, $p > 0.05$; main effect exendin-4: $F(2,18) = 0.08$, $p > 0.05$; interaction: naltrexone x exendin-4: $F(2,18) = 0.03$, $p > 0.05$).

7.6.2.3 Test Day Food Intake

Control food pot measurements showed an average weight loss via evaporation of only 0.16% throughout the experiment (range = 0.07 - 0.25%). Treatment effects on mash intake are summarised in Figure 7-6. ANOVA revealed a significant exendin-4 x naltrexone interaction ($F(2,28) = 21.38$, $p < 0.001$), with post-hoc comparisons confirming significant reductions in intake versus VV control in all treatment groups ($p \leq 0.001$) except the low dose of exendin-4 given alone (ELV). Importantly, there were no significant differences between naltrexone when given alone (VN) and when given in combination with the higher dose of exendin-4 (0.25µg/kg; EHN). Although a significant difference ($p \leq 0.001$) was seen between the lower dose of exendin-4 given alone (0.025µg/kg; ELV) and when co-administered with naltrexone (ELN), this effect was due solely to the intrinsic influence of the opioid receptor antagonist (Figure 7-6). Relative to VV control, ELV reduced intake by < 10%, EHV by 41%, and NV by 35%. The observed reductions of 38.7% and 34.5% (vs. calculated composite reductions of 45% and 76%) for ELN and EHN, respectively, fully confirm the lack of an additive interaction between the two agents.

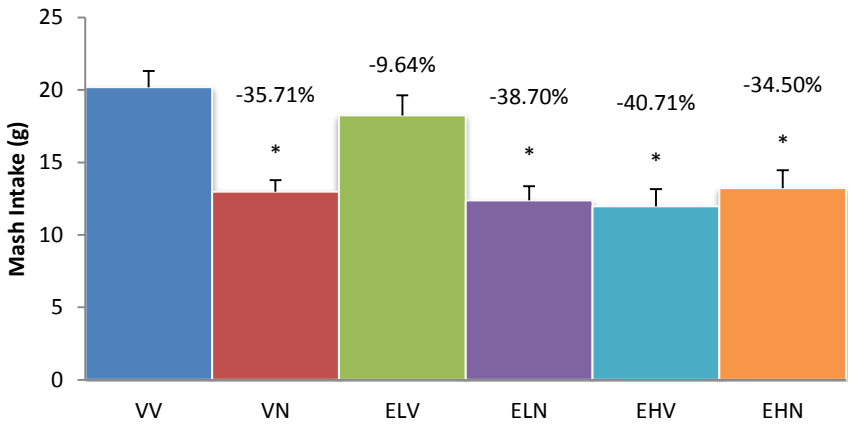

Figure 7-6: Experiment Ten. Effects of acute exendin-4 and naltrexone, alone and in combination, on mash intake by non-deprived male rats during a 1-h test with palatable mash

Data are mean values (± S.E.M). The percentages refer to intake reduction compared to vehicle. V = vehicle (distilled water or saline), N = naltrexone 0.1mg/kg; EL = exendin-4 0.025µg/kg; EH = exendin-4 0.25µg/kg; * $p \leq 0.05$ vs V. See text for full details.

7.6.2.4 Total (one-hour) Behavioural Analyses

Treatment effects on the total frequency and duration of ingestive and non-ingestive behaviours are shown in Figure 7-7, while data for feeding-related parameters (latencies, average duration of eating bouts, & average rate of eating) are summarised in Table 7-2.

Table 7-2: Experiment Ten. Effects of exendin-4 and naltrexone, alone and in combination, on mash intake and feeding-related parameters in male rats exposed for 1h to palatable mash

Data are mean values (± S.E.M). V = vehicle (distilled water or saline), N = naltrexone 0.1mg/kg; EL = exendin-4 0.025µg/kg; EH = exendin-4 0.25µg/kg; See text for full details

Measure	VV	VN	ELV	ELN	EHV	EHN
Latency to locate food (s)	4.87 ± 0.61	8.26 ± 2.32	5.29 ± 1.29	4.85 ± 1.56	8.94 ± 1.94	10.37 ± 5.25
Latency to eat (s)	14.94 ± 4.16	16.91 ± 6.61	14.25 ± 3.24	10.57 ± 2.43	10.33 ± 4.12	8.34 ± 1.46
Eat bout (s)	8.56 ± 1.15	8.19 ± 1.20	7.70 ± 1.13	6.74 ± 0.71	8.61 ± 1.13	8.24 ± 1.20
Eat rate (g/min)	2.15 ± 0.14	2.02 ± 0.13	1.99 ± 0.16	2.08 ± 0.15	1.83 ± 0.14	1.84 ± 0.18

ANOVA revealed significant exendin-4 x naltrexone interactions for the frequency and duration of eating ($F(2,28) \geq 20.97$, $p \leq 0.001$) but for no other variables ($F(2,18) \leq 2.37$, $p \geq 0.05$). Significant main effects of exendin-4 were found for the

frequency of sniffing ($F(2,18) = 9.80$, $p = 0.001$) and for both the frequency and duration of resting ($F(2,18) \geq 5.76$, $p \leq 0.02$) and rearing ($F(2,18) \geq 6.35$, $p \leq 0.01$).

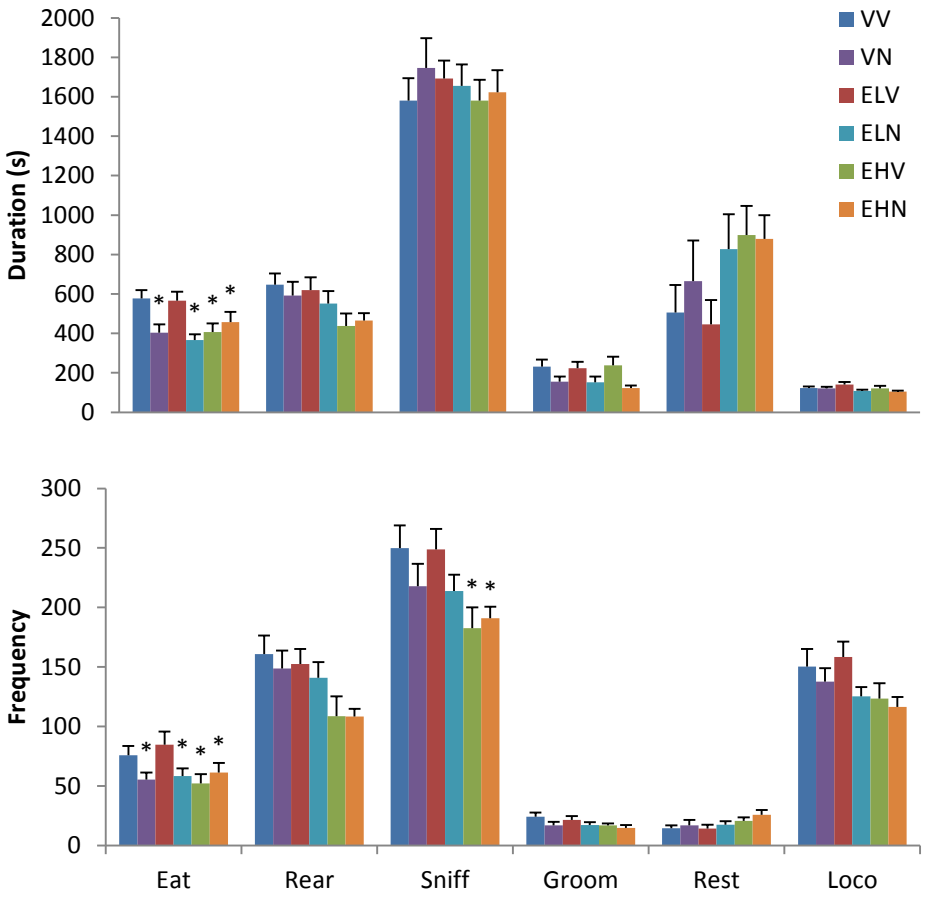

Figure 7-7: Experiment Ten. Effects of exendin-4 and naltrexone, alone and in combination, on the duration (upper panel) and frequency (lower panel) of behaviours displayed by non-deprived male rats during a 1-h test with palatable mash.

Data are mean values (± S.E.M). V = vehicle (distilled water or saline), N = naltrexone 0.1mg/kg; EL = exendin-4 0.025µg/kg; EH = exendin-4 0.25µg/kg; * $p \leq 0.05$ vs V. See text for full details.

Significant main effects of naltrexone were found only for the frequency and duration of grooming ($F(1,9) \geq 6.42$, $p \leq 0.03$), although the effect of the opioid antagonist on the duration of locomotion just missed significance ($F(1,9) = 4.97$, $p = 0.053$). While no treatment or interaction effects were found for feeding-related parameters (latency to identify food; latency to commence eating, eating rate, or eat bout duration: Table 7-2), it should perhaps be noted that the main effect of exendin-4 on the average duration of eating bouts closely approached significance ($F(2,18) = 3.38$, $p < 0.06$). Confirming the results of the dose-response study, Bonferroni comparisons indicated that, relative to VV control, behaviour was completely unaffected by the lower dose of exendin-4 (0.025µg/kg; ELV). In

contrast, the higher dose of the peptide (0.25µg/kg; EHV) significantly reduced both the frequency and duration of eating ($p \leq 0.001$), and the frequency of sniffing ($p \leq 0.01$). Naltrexone by itself (VN) also significantly reduced both the frequency and duration of eating compared with vehicle control ($p \leq 0.002$). For the frequency and duration of eating, and as seen for mash consumption, there were no significant differences between naltrexone given alone (VN) and when given in conjunction with the higher dose of exendin-4 (EHN). Furthermore, as the lower dose of exendin-4 had no effects on these measures by itself, the significant difference between ELV and ELN ($p < 0.001$) can simply be attributed to the anorectic effect of the opioid receptor antagonist (Figure 7-7). Interestingly, the inhibitory effect of the higher dose of exendin-4 on sniff frequency was also seen when the peptide was given with naltrexone (EHN; $p < 0.03$) and, since there was no difference between the peptide in the presence or absence of naltrexone, this further confirms the lack of behavioural interaction between the two agents. Since no other pairwise contrasts versus VV control were significant, the additional main effects of exendin-4 and naltrexone reported above can be interpreted as weak effects arising from the larger sample sizes/reduced variances associated with main effects analyses.

7.6.2.5 Periodic (Timebin) Behavioural Analyses

As seen in Experiment 9, significant main effects of time were found for the frequency ($F(11,99) \geq 11.99$, $p \leq 0.001$) and duration ($F(11,99) \geq 3.45$, $p \leq 0.001$) of all behavioural measures except grooming ($F(11,99) < 1.80$, $p > 0.05$). The only 3-way interaction (exendin-4 x naltrexone x time) obtained was for rear duration ($F(22,198) = 1.80$, $p < 0.05$), with additional 2-way interactions for eat duration (exendin-4 x time: $F(22,198) = 3.25$, $p < 0.001$; naltrexone x time: $F(11,99) = 2.59$, $p < 0.01$), eat frequency (exendin-4 x time: $F(22,198) = 3.25$, $p < 0.001$), and rest frequency (naltrexone x time: $F(11,99) = 2.31$, $p < 0.02$).

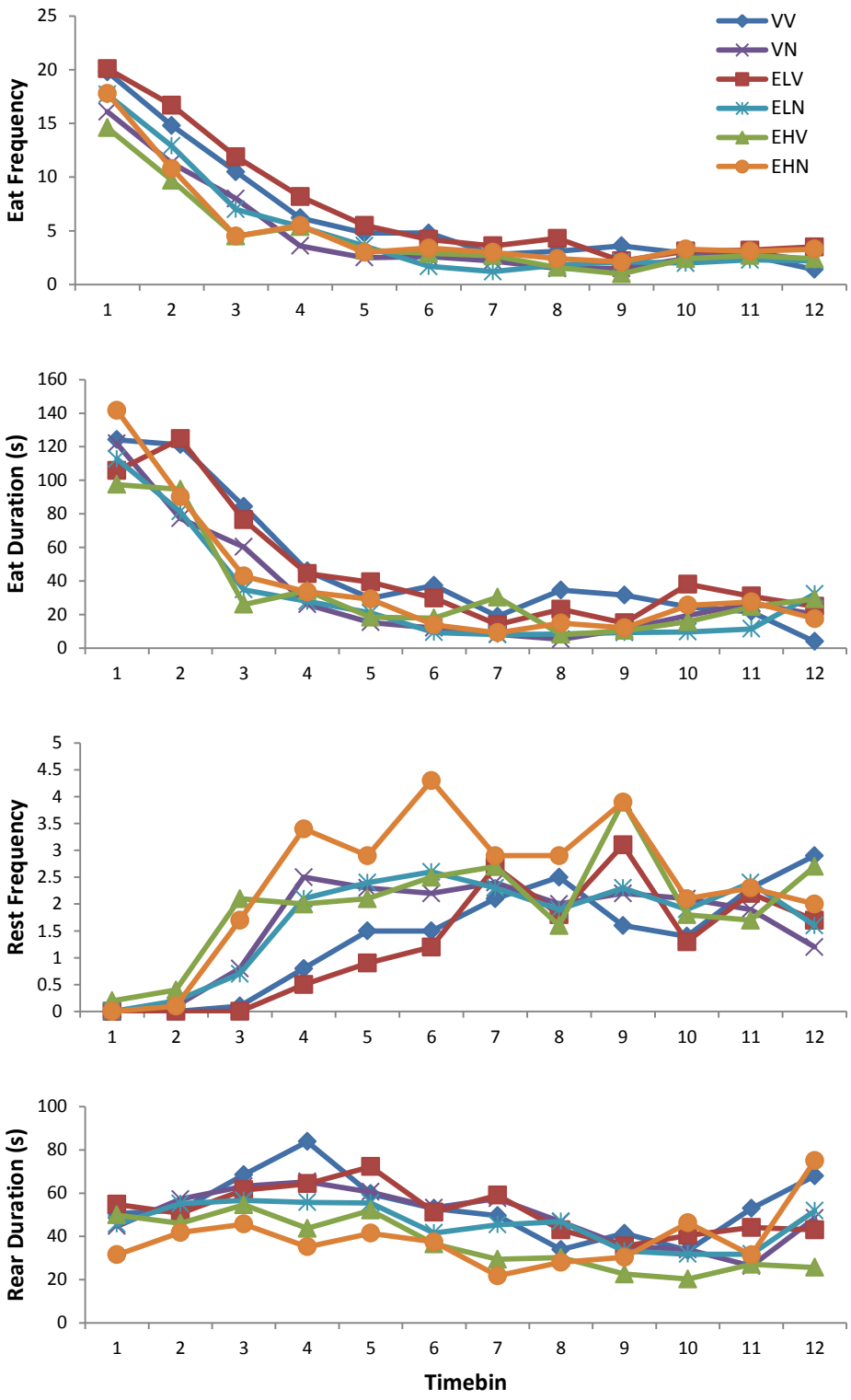

Figure 7-8: Experiment Ten. Effects of acute exendin-4 and naltrexone, alone and in combination, on the frequency and duration of eating, the frequency of resting and the duration of rearing and in male rats during a 1-h test with palatable mash

Data are expressed as the mean frequency of each behaviour in 12 x 5-min timebins. V = vehicle (distilled water or saline), N = naltrexone 0.1mg/kg; EL = exendin-4 0.025µg/kg; EH = exendin-4 0.25µg/kg. See text for further details.

All significant 2- or 3-way interactions involving the time factor were further interrogated by a series of 2-way ANOVAs within each timebin. Significant drug main effects or interactions were found for: (i) eat duration in timebins 2, 3, and 6-9 inclusive ($F(1,9) \geq 7.57$, $p \leq 0.03$; $F(2,18) \geq 3.64$, $p \leq 0.05$), reflecting a main (suppressant) effect of naltrexone; (ii) eat frequency in timebins 1-3, 6 and 8 ($F(1,9) = 7.65$, $p < 0.03$; $F(2,18) > 4.32$, $p < 0.03$) and rear duration in timebins 2-7 inclusive ($F(2,18) \geq 4.31$, $p \leq 0.03$), both reflecting a dose-dependent suppressant effect exendin-4; and (iii) rest frequency in timebins 3, 4, 6 and 9 ($F(2,18) \geq 5.01$, $p \leq 0.02$), reflecting a dose-dependent enhancement by exendin-4. These temporal effects of treatment are illustrated in Figure 7-8. Consistent with other current observations, there were no instances where a significant difference was found between test compounds given alone and when given in combination.

7.6.2.6 Behavioural Satiety Sequence (BSS)

Figure 7-9 illustrates the BSS profiles for each of the 6 treatment conditions. Consistent with numerous reports from our laboratory over the past 15 years (Halford et al. 1998; Rodgers et al 2010), the behavioural profile in the vehicle control condition (VV; top left) shows a peak feeding response during the first 20 min of the test with an eat-rest transition in timebin 6 (i.e. circa half-way through the test). A very similar (i.e. unaltered) pattern of behaviour is evident with the lower dose of exendin-4 (0.025µg/kg; ELV; centre left). However, while retaining the normal structure of feeding behaviour, the higher dose of the peptide (0.25µg/kg; EHV; bottom left) was associated with a suppression of the peak feeding response and an acceleration (shift to the left) in the eat-rest transition to timebin 4. The latter behavioural signature was essentially replicated by naltrexone given alone (VN; top right) and in combination with either dose of exendin-4 (ELN & EHN; centre & bottom right).

7.6.2.7 Bodyweight Gain

Data not shown. No significant main effects or interactions were found for 7-day absolute weight gain. The animals typically gained 18-21g irrespective of treatment condition (main effect exendin-4: $F(2,18) = 0.08$, $p > 0.05$; main effect naltrexone: $F(2,18) = 0.36$, $p > 0.05$; interaction, naltrexone x exendin-4: $F(2,18) = 0.02$, $p > 0.05$). Analysis of percent bodyweight change over days following treatment

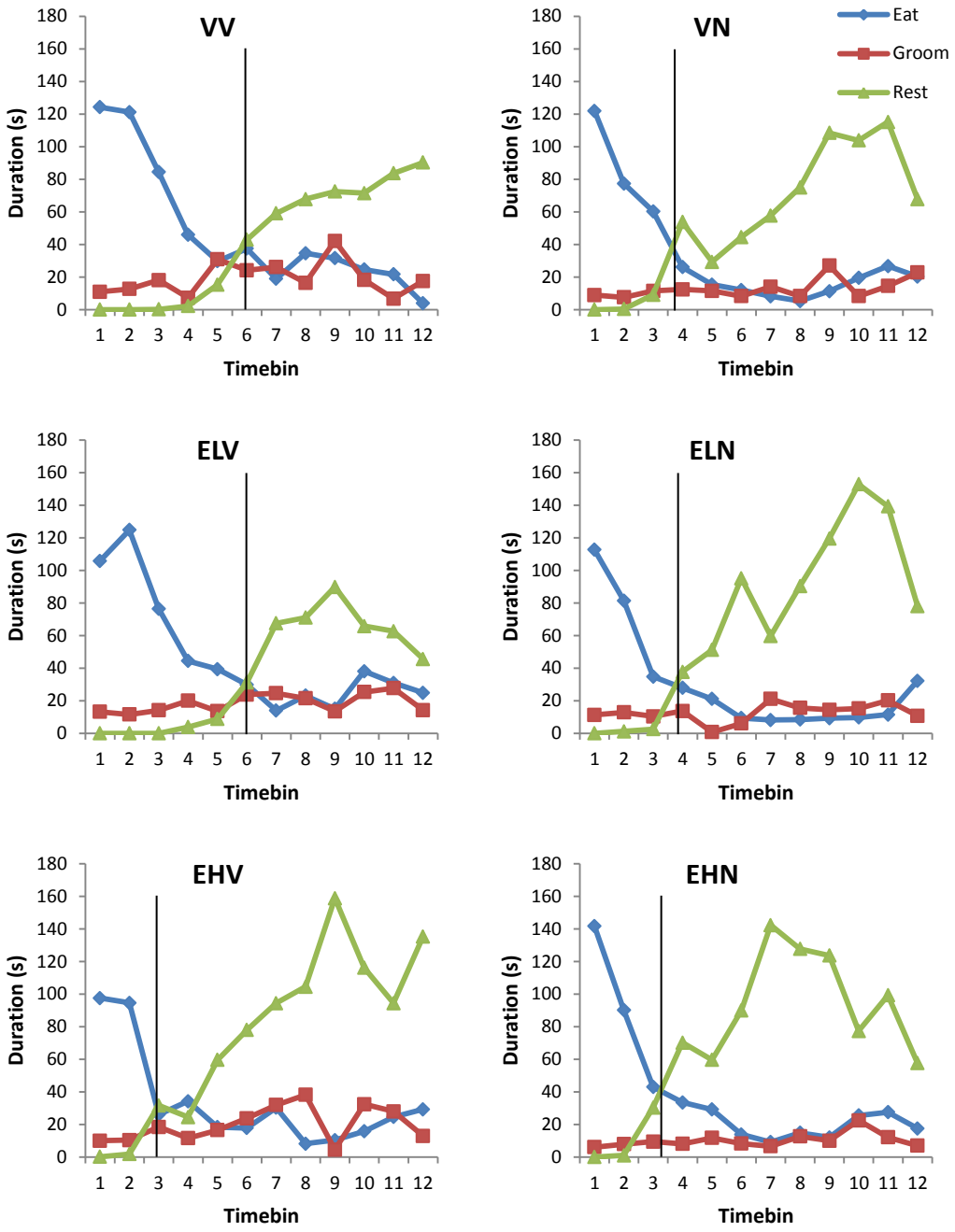

Figure 7-9: Experiment Ten. Effects of acute exendin-4 and naltrexone, alone and in combination, on the behavioural satiety sequence (BSS)

Data are expressed as mean duration scores in each of 12 x 5min timebins. The solid vertical line bisecting the x-axis is merely an aid to visualisation of the transition between eating and resting. V = vehicle (distilled water or saline), N = naltrexone 0.1mg/kg; EL = exendin-4 0.025µg/kg; EH = exendin-4 0.25µg/kg. See text for further details.

confirmed normal growth patterns (main effect day: $F(2,18) = 31.01$, $p < 0.001$), but it too failed to reveal any significant drug main effects, drug interactions, or drug x time interactions (main effect exendin-4: $F(2,16) = 0.40$, $p > 0.05$; main effect

naltrexone: $F(1,8) = 0.48$, $p > 0.05$; drug interactions, naltrexone x exendin-4: $F(2,18) = 0.77$, $p > 0.05$; drug x time interactions: $F(12,96) > 0.80$, $p > 0.05$)

7.6.3 Summary of Main Findings

Consistent with Experiment 9, Experiment 10 showed that, when given alone, the lower dose of exendin-4 (0.025µg/kg) had no significant effects on mash intake or behaviour, whereas the higher dose (0.25µg/kg) alone reduced consumption (41% vs vehicle), the frequency and duration of eating, and the frequency of sniffing. Alone, naltrexone (0.1mg/kg) also significantly reduced mash consumption (36% vs. control), and the frequency and duration of feeding behaviour. Both the higher dose of exendin-4 and naltrexone, when administered alone, suppressed the peak feeding response relative to vehicle control and modestly accelerated the BSS without disrupting normal behavioural structure.

However, the combination of exendin-4 and naltrexone failed to produce any sign of a positive interaction. The results showed a near identical anorectic effect of combined treatment versus treatment with either agent alone. The higher dose of exendin-4 (0.25µg/kg) suppressed intake by 41%, naltrexone suppressed intake by 36%, but their combination suppressed intake only by 35% (vs a predicted 77% based on simple addition of the anorectic responses to the constituent elements). These treatment effects on intake were mirrored by changes in the frequency and duration of feeding behaviour. Furthermore, although the higher dose exendin-4 and naltrexone each produced a modest acceleration in the BSS, their combination did not produce any further acceleration in this measure of behavioural satiety.

7.7 Chapter Seven Main Findings

The current chapter aimed to comprehensively profile the behavioural effects of the GLP-1R agonist, exendin-4, in rats during tests of palatable food consumption and to explore possible advantages of low-dose (sub-anorectic, sub-maximally anorectic) combinations of exendin-4 and the opioid receptor antagonist, naltrexone

- **Experiment 9** showed that exendin-4 dose-dependently reduces food intake and feeding behaviour in male rats. However, the exendin-4-induced anorexia did not appear to be behaviourally-selective as it was accompanied by other behavioural changes comparable to LiCl-induced hypoactivity and/or malaise.
- **Experiment 10** showed that, although exendin-4 and naltrexone each have intrinsic anorectic effects, co-treatment does not lead to an additive interaction on food intake or feeding behaviour.

The work reported in Chapter 7 does not offer any support for the anti-obesity potential of an exendin-4/naltrexone combination. However, it does highlight the potential behavioural selectivity issues associated with exendin-4 anorexia.

Chapter 8 Discussion

8.1 Overview of Thesis Aims

Neurobiological research has identified a large number of peripheral & central signalling pathways involved in appetite regulation and energy homeostasis (see Chapter 1). These advances have informed the development of treatments for obesity and, recently, interest has focused on the therapeutic potential of drug polytherapy or combination treatments, whereby two neurobiological pathways are concurrently targeted (see Chapter 2). Polytherapy may permit the use of lower doses and opens up the possibility of (i) additive/synergistic effects on food intake and weight gain and (ii) the reduction/elimination of side-effects normally associated with higher doses of the constituent agents. The vast majority of behavioural research on appetite tends to focus on simple outcome measures or endpoints. However, outcomes can be reached either directly by an action on the normal physiological regulation of appetite or indirectly via a host of non-specific mechanisms. Therefore, the current research employed a BSS methodology (see Chapter 3) to assess the extent to which individual effects of cannabinoid, opioid, monoaminergic and peptidergic compounds suppress food intake in a behaviourally-selective and physiologically-relevant manner. More specifically, the acute anorectic effects in male rats of individual treatment with the general opioid receptor antagonist naltrexone (Experiment 5), the noradrenaline and dopamine reuptake inhibitor bupropion (Experiment 4), the serotonin 5-$HT_{1B/2C}$ receptor agonist *m*CPP (Experiment 7), and the naturally-occurring GLP-1R mimetic exendin-4 (Experiment 9). A detailed analysis of both feeding and non-feeding parameters determined whether drug-induced reductions in 1hour palatable mash intake were a result of behaviourally-selective suppression of appetite or possibly an indirect drug effect that interferes or competes with feeding behaviour. Furthermore, the present thesis explored the acute anorectic response to co-treatment with these compounds and an opioid antagonist (naloxone; Experiments 1-3 / naltrexone Experiments 6, 8 and 10). More specifically, the acute anorectic effects in male rats of co-treatment with rimonabant and naloxone (Experiments 1-3), bupropion and naltrexone (Experiment 6), *m*CPP and naltrexone (Experiment 8), and exendin-4 and naltrexone (Experiment 10).

8.2 Individual Chapter Discussions

As only the main findings were outlined within each of the four empirical chapters (Chapters 4 - 7), Chapter 8 will focus on present results within the context of the current literature and comment upon the broader implications of this research.

8.2.1 Chapter Four; Cannabinoid and Opioid System Interactions

In view of the potential significance of pruritus to the future development of CB1 receptor antagonists, Experiments 1-3 aimed to test a clear prediction of the response competition hypothesis (Hodge et al., 2008; Tallett et al., 2007b); namely, that low dose naloxone treatment should attenuate both the pruritic and anorectic responses to a moderate dose of rimonabant.

The results reported in Chapter 4 confirmed earlier findings that rimonabant alone reduces time spent feeding and supresses mash intake by 45-50% (Colombo et al., 1998; De Vry & Jentzsch, 2004; Freedland et al., 2001; Gomez et al., 2002; Hildebrandt et al., 2003; Hodge et al., 2008; McLaughlin et al., 2006; McLaughlin et al., 2003; McLaughlin et al., 2005; Rowland et al., 2001; Thornton-Jones, 2005; Verty, McGregor, et al., 2004a; Verty et al., 2003; Vickers, Webster, et al., 2003; Werner & Koch, 2003; Wiley et al., 2005; Williams & Kirkham, 2002), whilst dramatically increasing both the frequency and duration of scratching and grooming behaviours (Janoyan et al., 2002; Jarbe et al., 2002; Jarbe et al., 2003; Jarbe et al., 2004; Jarbe et al., 2006; Tallett et al., 2007a, 2007b, 2007c). At the doses used (0.1 and 0.01mg/kg), naloxone by itself did not produce any significant effects on feeding behaviour, thereby indirectly confirming a threshold anorectic dose of circa 1.0 mg/kg for this opioid receptor antagonist (as seen under similar test conditions; Tallett et al., 2008a, 2008b, 2009a; 2010b; and references therein). Of primary interest, co-administration with naloxone was found to attenuate rimonabant-induced scratching and grooming, but not the anorectic response to rimonabant.

8.2.1.1 Food intake and Feeding Behaviour

Interestingly, in Experiment 1, the results of the combined treatment appeared to differ dependent upon the dose of naloxone used. Although the higher dose of naloxone (0.1mg/kg) failed to impact food intake or feeding behaviour, the combination of rimonabant with the low dose of naloxone (0.01mg/kg) seemed to produce an attenuation of rimonabant-induced effects on food intake and feeding duration. However, indicative of a weak statistical effect, the reduction of food

intake with the low dose combination remained significantly different from control, and did not significantly differ from rimonabant given alone. In contrast, time spent feeding did not significantly differ from control, nor did it differ from rimonabant given alone (see Figure 4-2 and 4-3). Follow-up experiments (Experiments 2 and 3) were therefore required to allow a meaningful conclusion regarding the impact of naloxone on rimonabant anorexia. Due to the unusual vehicle profile seen in Experiment 2 (see Chapter 4; Section 4.6), and the post-hoc discovery of food contamination (see Appendix 3), the results of Experiment 2 were considered suspect. Instead, the results of Experiment 3 will be used to elaborate upon the findings of Experiment 1. The intermediate naloxone dose (0.05mg/kg) used in Experiment 3 confirmed the results of the high dose naloxone seen in Experiment 1, in that this dose failed to produce any significant effects on rimonabant anorexia (see Figure 4-10).

In contrast to previous research (Chen, Huang, et al., 2004; Kirkham & Williams, 2001; Rowland et al., 2001; Tallett et al., 2008b, 2009a), the present work provides no evidence of an additive or synergistic effect of the cannabinoid-opioid interaction on food intake or feeding behaviour. In theory, the cannabinoid antagonist should reduce the incentive value of food to delay the onset of feeding (Maccioni et al., 2008; Rasmussen & Huskinson, 2008; Sanchis-Segura et al., 2004; Ward et al., 2008), whilst the general opioid antagonist should suppress the palatability of food following the initiation of feeding, to reduce meal duration and the maintenance of feeding (Apfelbaum & Mandenoff, 1981; Barbano & Cador, 2006; Cleary et al., 1996; Cooper et al., 1985; Cooper & Turkish, 1989; Giraudo et al., 1993; Glass et al., 1999; Glass et al., 2001; Hayward et al., 2006; Islam & Bodnar, 1990; Levine & Billington, 2004; Markskaufman et al., 1984; Sanger & McCarthy, 1981a, 1982). The proposed combination of centrally-mediated reductions in the incentive value or palatability of food (and peripherally-mediated alterations in lipogenesis and glucose metabolism) should result in supra-additive/synergistic effects on food intake and weight gain. The lack of such interaction in the present studies may relate to significant differences in methodology, with previous research employing sub-anorectic combinations of the constituent compounds.

8.2.1.2 Non-feeding Behaviour

In terms of non-ingestive behaviour, Experiments 1-3, fully confirmed the dramatic and statistically significant elevation of scratching and grooming behaviour following treatment with a moderate dose of rimonabant (1.5mg/kg). Furthermore,

the timecourses of scratching and grooming behaviour were similar to previous work (Tallett et al., 2007a, 2007b, 2007c), in that scratching was particularly prominent over the first half of the session and grooming in the second (see Figures 4-4 and 4-12). The present data show that these effects were attenuated by co-treatment with naloxone, a finding consistent with the use of opioid receptor antagonists to successfully treat various human pruritic disorders (Bernstein et al., 1982; Bigliardi et al., 2007; Cies & Giamalis, 2007; Friedman & Dello Buono, 2001; Phan et al., 2010; Terra & Tsunoda, 1998; see also Chapter 4; Section 4.2.3.1).

Interestingly, previous reports have suggested that rimonabant-induced scratching is more sensitive to naloxone (Tallett et al., 2008b), suggesting that the grooming and scratching behaviours induced by CB1 receptor antagonist/inverse agonists may be regulated via different mechanisms. In contrast, the current work showed a stronger naloxone-attenuation of grooming behaviour. This discrepancy may be a result of the lower levels of grooming produced by the sub-anorectic rimonabant doses used in the Tallett et al. (2008b) study. In view of these findings, it is possible that CB1 receptor antagonist/inverse agonists increase the release of endogenous opioids to induce scratching. However, the precise mechanism underlying these processes is poorly understood, as contradictory evidence demonstrates that cannabinoid receptor agonists increase the synthesis and release of endogenous opioids, at least in the CNS (Manzanares et al., 1999). An alternative hypothesis is that naloxone can prevent CB1 receptor antagonist/inverse agonists from binding to neuronal CB1 receptors (Schoffelmeer et al., 2006), potentially due to the existence of a μ-opioid/CB1 cannabinoid receptor heterodimeric pairing between opioid and cannabinoid receptors, which can create pharmacological properties distinct from either receptor alone (Christie, 2006; Rios et al., 2006). Furthermore, opioid antagonists and CB1 receptor antagonist/inverse agonists have been shown to allosterically prevent each others' binding to their own receptors (Schoffelmeer et al., 2006). Therefore, if rimonabant and naloxone act at the same heterodimeric receptor pairings, they may actually compete at this site. This also possibly explains why the combined drug effects on food intake are not as great as would have been predicted.

The scratching and grooming syndrome aside, Experiments 1-3 reported very few non-ingestive behavioural effects of treatment, the exception being a significant decrease in locomotor duration by rimonabant, alone and in combination with naloxone (0.5mg/kg; Experiment 3). As only two conditions across three experiments identified such an effect, this may well be an anomaly or statistical

artefact. However, previous behavioural studies of rimonabant (Tallett et al., 2007b, 2007c) have also shown evidence of decreased locomotive behaviour at higher doses (1.5-3.0mg/kg). This would be consistent with an acceleration of the BSS, resulting in an earlier transition from feeding to resting which, logically, leads to a reduction in general activity levels.

8.2.1.3 The Behavioural Satiety Sequence

The 'classical' BSS profile (see Chapter 3) was found to be largely disrupted following rimonabant administration, even at the moderate dose used (1.5mg/kg; Tallett et al., 2007b, 2007c). It is clear that grooming is the predominant behaviour for most of the test session, resulting in a barely discernible (see Figures 4-5 and 4-13; dashed lines) eat-to-rest transition. The results do, however, show a clear naloxone-induced dose-dependent (0.01, 0.05, 0.1mg/kg) 'normalisation' of the temporal structure of the BSS. The attenuation of grooming by naloxone permits the re-emergence of resting in the second half of the session, allowing for a much clearer eat-to-rest transition. Interestingly, despite the gradual rescue of the 'classical' BSS profile, naloxone co-administration does not impact the rimonabant-induced suppression of the peak feeding response. This is true even for the lowest dose of naloxone (0.01mg/kg), which further supports its effect on food intake and feeding behaviour as a statistical artefact. Additionally, despite the lack of effect of the co-treatment on mash consumption and feeding measures, the BSS profiles demonstrate a modest naloxone-induced acceleration of the eat-to-rest transition. Therefore, the combination treatment may have an added benefit of enhancing behavioural satiety.

8.2.1.4 Conclusions, Implications and Future Research

In conclusion, the experiments reported in Chapter 4, fail to support the competition hypothesis (Tallett et al., 2007b, 2007c), but instead support Hodge and colleges (2008) who, albeit using an alternative methodology, also found that the grooming and scratching syndrome does not fully account for rimonabant-anorexia. Therefore, Experiments 1-3 indicate that the anorectic and pruritic response to rimonabant treatment are independent phenomena.

Although it is currently unclear whether this grooming/scratching syndrome is centrally or peripherally initiated, previous research has suggested that it may be centrally mediated (Pavon et al., 2006). Thus, LH-21, a cannabinoid receptor antagonist that is less brain-penetrant than rimonabant, inhibits food intake without inducing scratching/grooming. This would suggest peripheral mediation of the

anorectic response (Gomez et al., 2002) but central mediation of the scratching/grooming syndrome. CB1 receptor antagonists, including rimonabant, are known to increase basal ganglia function (Gueudet et al., 1995; Rubino et al., 1998) in addition to c-Fos expression throughout the nigrostriatal system (Rodriguez De Fonseca et al., 1997). It is therefore possible that the scratching and grooming syndrome is mediated via activation or disinhibition of neural circuits that control these motor sequences (Tallett et al., 2007c). In contrast, rimonabant may induce scratching and grooming by blocking the functioning of peripherally-located CB1 receptors, creating local irritation (Maekawa et al., 2006; Rukwied et al., 2003). This hypothesis is supported by research that has identified ear-swelling (Karsak et al., 2007) and hyperalgesia (Richardson et al., 1997) as behaviours caused by CB1 receptor deletion or antagonism. Furthermore, the efficacy of peripherally acting opioid antagonists, such as methyl naltrexone (Friedman & Dello Buono, 2001) and topical naltrexone (Bigliardi et al., 2007), in the treatment of human pruritic conditions supports a peripheral site of action (Cluny et al., 2010; Gomez et al., 2002; Randall et al., 2010; Schlosburg et al., 2011). Consistent with this proposal, the findings from the current series of experiments suggest that the pruritic response is peripherally-mediated, involving both CB1 and μ- receptors (Stander et al., 2005; Yamamoto & Sugimoto, 2010). Future research should further assess if the naloxone-induced attenuation of rimonabant scratching/grooming is centrally or peripherally mediated, and this could be directly investigated using ligands that are unable to cross the BBB.

In relation to the therapeutic potential of CB1 receptor antagonist/inverse agonists, the presence of the pruritic response in newer ‘neutral’ CB1 receptor antagonists (Addy et al., 2008; Kirkham, 2008) suggests that the itch response may be unavoidable. Therefore, a simple opioid antagonist treatment strategy similar to other pruritic conditions should be employed (Ballantyne et al., 1988; Kuraishi et al., 2000; Miyamoto et al., 2002). The work presented Chapter 4 demonstrates that drug polytherapy can be therapeutically advantageous since co-treatment results in the suppression of an unwanted side-effect of one treatment by the other.

8.2.2 Chapter Five; Monoamine and Opioid System Interactions

In view of a gap in the literature concerning Contrave™, Experiments 4-6 employed BSS methodology to systematically and comprehensively profile the

behavioural effects of bupropion and naltrexone, alone and in combination, within a feeding context.

8.2.2.1 Behavioural Specificity of Bupropion

The results of the bupropion dose-response study (Experiment 4) confirmed that bupropion dose-dependently reduces mash intake (Billes & Cowley, 2007; Greenway, Whitehouse, et al., 2009; Stairs & Dworkin, 2008; Zarrindast & Hosseininia, 1988), as well as time spent feeding and the duration of feeding bouts (statistically significant only at 40mg/kg). However, our findings also question the behavioural specificity of bupropion and supports evidence of bupropion-induced psychomotor stimulation (Billes & Cowley, 2007; Carrasco et al., 2004; Cooper et al., 1980; Gomez et al., 2008; Nielsen et al., 1986; Paterson et al., 2010; Redolat, Gomez, et al., 2005; Redolat, Vidal, et al., 2005; Santamaria & Arias, 2010; Soroko et al., 1977; Zarrindast et al., 1996; Zarrindast & Hosseininia, 1988).

The anorectic dose of bupropion (40mg/kg) in Experiment 4 produced significant increases in rearing, sniffing and locomotor behaviours. This increased activity even extended to a significant increase in feeding frequency, despite reductions in feeding duration. Furthermore, although non-significant, sub-anorectic doses of bupropion also demonstrated a trend towards this hyperactive behavioural profile. It is pertinent to note that the bupropion timecourse profiles both for eat duration and other behavioural measures are almost identical (see Figure 5-3). As bupropion appears to impact all behaviours from the start of the session, this may suggest that the profile is not a result of enhanced satiety, but rather an indirect behavioural effect. For example, in a test condition of finite duration major increases in locomotor activity would allow less time for feeding behaviour. This suggests a possible response competition hypothesis, similar to that suggested for rimonabant (Tallett et al., 2007b). Moreover, the psychomotor stimulation induced by bupropion produces a BSS profile similar to that seen with the administration of amphetamines, d-FEN (Blundell & McArthur, 1981; Halford et al., 1998) and cocaine (Cooper & Vanderhoek, 1993), compounds that are known to produce behaviourally non-specific reductions in intake.

Bupropion is a selective dopamine and noradrenaline reuptake inhibitor, the psychomotor stimulant effects of which are thought to be mediated via the dopamine system. Previous research has demonstrated that dopamine infusions in the NAcc produce increased locomotor activity (Barnes et al., 1986), and that pharmacological blockade of dopamine receptors blocks amphetamine-induced

locomotion (Vaccarino et al., 1986). Furthermore, the locomotor effects of bupropion can be blocked by chronic destruction of dopamine neurons (Cooper et al., 1980) and by D_1 and D_2 antagonists (Yamada et al., 2004). Therefore, it would seem logical that the bupropion-induced increases in striatal dopamine concentrations (however see; Nielsen et al., 1986; Waldmeier, 1982) result in the reported psychomotor stimulation.

Interestingly, despite evidence that both the anorectic and psychostimulant effects of bupropion are typically produced at similar dosages, it has been suggested that they are actually independent phenomenon (Billes & Cowley, 2007). Billes and Cowley (2007) emphasise that increasing doses of bupropion that caused incremental increases in activity (0–10 and 20–40 mg/kg) did not also significantly decrease food intake to the same extent. Conversely, incremental reductions in food intake (seen between 10 and 20 mg/kg) were not correlated with a significant increase in activity levels. However, it is pertinent to note that no statistical correlations were reported, and the hypothesis would assume equivalent sensitivity of the measures of food intake and locomotor activity. This would seem improbable considering the major differences in the units of measurement and base rates for intake and activity. However, it is acknowledged that enhanced locomotor activity is not always found to reduce food intake (Cooper & Vanderhoek, 1993; Hodge et al., 2008; Vanrossum & Simons, 1969). Due to the unclear nature of the current results, future research should attempt to dissociate the two bupropion-induced effects to establish if bupropion is acting specifically on appetite mechanisms to reduce food intake (see Section 8.2.2.3). This is especially important within the context of its current status as one component of a potential polytherapy for obesity.

8.2.2.2 Behavioural Specificity of Naltrexone

The results of the naltrexone dose-response study (Experiment 5) confirmed a dose-dependent reduction in mash consumption and feeding behaviour (frequency and duration) over the one-hour test session. Although only the highest dose used (3.0mg/kg) produced a statistically significant suppression, the intermediate dose (1.0mg/kg) demonstrated a (non-significant) 22% reduction in intake, and significantly reduced the frequency of eating behaviour. This suggests that 1.0mg/kg may be close to the anorectic threshold for naltrexone. The current findings also suggest that naltrexone, while longer-acting, may be less potent than naloxone. Findings using similar methodology, within the same laboratory, found a threshold dose of 1.0mg/kg for naloxone (Tallett et al., 2008a), compared to

3.0mg/kg for naltrexone as shown in Experiment 5. The present study found no significant effects of naltrexone on the additional eating-related parameters; time taken to locate the food source, eat latency, eat bout duration and eat rate. This would suggest that the initial motivation for food remained unaffected by naltrexone. This is consistent with evidence that naltrexone reduces motivation to eat post-ingestion (Kirkham & Blundell, 1986) and that naloxone also fails to induce changes in these parameters (Glass et al., 2001; Kirkham & Blundell, 1986, 1987; Tallett et al., 2008a).

Surprisingly, main effects analysis showed a naltrexone-induced effect on most elements of behaviour, potentially questioning the behavioural selectivity of naltrexone anorexia. However, further post-hoc analysis revealed that, compared with vehicle, naltrexone (3.0mg/kg) only significantly increased rest duration, and decreased the frequency of locomotion and sniffing. Furthermore, closer examination of the timecourses revealed an effect on intake and feeding behaviours during the first half of the session, but an effect on the other behavioural parameters during the second half of the session (see Figure 5-7). This profile is consistent with a ‘normal’ BSS, with elevated resting and reduced general activity towards the end of the session. Although the elevated resting and reduced activity may be explained by naltrexone-induced sedation, the fact that resting occurred after, but not before, food consumption lends to the satiating effect of naltrexone. It is interesting to note that significant increases in resting behaviour have also been seen with higher doses of naloxone (Tallett et al., 2008a). The modest naltrexone-induced acceleration of the eat-to-rest transition (V = timebin 6; Ntx3.0 = timebin 4) is further evidence of satiety enhancement and appetite suppression rather than a non-specific behavioural disruption.

Interestingly, the findings of Experiment 5 suggest that naltrexone is less potent than naloxone and yet produces a wider behavioural activity. These differences may reflect significant variation in pharmacokinetics and pharmacodynamics (Goldstein & Naidu, 1989; Magnan et al., 1982; Raynor et al., 1994; Tepperman et al., 1983). Despite such differences, however, it is still fair to conclude that the inhibition of intake by naltrexone is a consequence of a behaviourally-selective anorectic action.

8.2.2.3 The combination

8.2.2.3.1 Food intake and Feeding Behaviour

Despite the use of ‘sub-anorectic’ doses (based on Experiments 4 and 5), when given alone, both bupropion (20mg/kg) and naltrexone (1.0mg/kg) significantly reduced food intake. However, it is pertinent to note that the actual % suppression is very similar to that seen in the dose response studies (issues regarding drug variability across experiments will be discussed later; see Section 8.3.1). The doses used in Experiment 6 can therefore be considered threshold or sub-maximal doses.

Experiment 6 demonstrated an additive effect of a bupropion-naltrexone combination on food intake (Greenway, Whitehouse, et al., 2009). The combination reduced food intake by 38-41%, suppression greater than that seen with either of the individual compounds given alone (14-25%). This effect is indicative of an additive interaction, in that the reductions seen in response to combined treatment (BNL = 38%, BNH = 41%) closely matched those simply calculated by the addition of reductions in response to the constituent agents (BV = 25%, NL = 14%, NH = 24%). This is consistent with previous reports of an additive interaction seen in lean animals (Greenway, Whitehouse, et al., 2009). Furthermore, this would not be inconsistent with the proposal that co-administration of an opioid antagonist, such as naltrexone, inhibits the opioid-mediated negative feedback effect on POMC neurons in the ARC, augmenting the effect of bupropion treatment (Greenway, Whitehouse, et al., 2009).

However, neither combination produced significant reductions in feeding behaviours (frequency or duration), demonstrating that objective reductions in food intake can be observed without changes in feeding related parameters. However, the combination did produce dose-dependent reduction in eat rate (significant only at BNH) which, most likely, is the cause of reduced mash consumption.

8.2.2.3.2 Non-Feeding Behaviour and the Behavioural Satiety Sequence

The behavioural parameters confirmed some of the findings from Experiments 4 and 5, notably, the psychomotor stimulation seen with bupropion alone. Most surprisingly, co-treatment with naltrexone attenuated bupropion-induced psychomotor stimulation. Thus, the significant increases in rear and sniff frequency following bupropion administered alone, were significantly reduced by co-administration of high-dose naltrexone (1.0mg/kg). Moreover, bupropion-induced elevated locomotion levels, failed to differ significantly from vehicle plus naltrexone co-treatment. This suggests that, similar to recent reports for CB1 receptor antagonist/inverse agonists (Tallett et al., 2007a; 2008b; see Chapter 4), co-

treatment with opioid antagonists, such as naltrexone, counters the unwanted effects of bupropion. It may also support reports that bupropion-induced psychomotor stimulation and its anorectic effects are independent phenomenon (Billes & Cowley, 2007). For example, naltrexone co-administration dose-dependently restored the structural integrity of the BSS by attenuating the psychomotor response and yet did not restore the bupropion-induced suppressed peak feeding response.

It is important to note that, opioid agonists (e.g. morphine) are known to increase locomotor activity in rodents (Iwamoto, 1981) when infused into the region of the dopamine cell bodies of the VTA and NAcc (Broekkamp et al., 1979; Kalivas et al., 1983; Stinus et al., 1980; Vezina et al., 1987), an effect blocked either by opioid antagonism (Iwamoto, 1981) or dopamine depletion (Churchill et al., 1995). The crossover between opioid and dopaminergic action in the regulation of locomotion in these areas may therefore suggest a reciprocal relationship whereby opioid blockade (via naltrexone) may act to suppress dopaminergic action and ultimately attenuate this psychomotor activity.

8.2.2.4 Conclusions, Implications and Future Research

In conclusion, findings reported in Chapter 4 support an additive interaction for the combination of bupropion and naltrexone (constituent elements in Contrave™). Experiments 4 and 5 have shown that bupropion produces a psychomotor stimulation that is attenuated by co-treatment with naltrexone. It is important to emphasise that this attenuation is seen concurrently with additive effects on food intake, suggesting that bupropion-induced anorexia and psychomotor stimulation are independent phenomena (Billes & Cowley, 2007).

Future research should investigate the bupropion-induced locomotor activity in more detail to confirm that this effect is truly independent of its anorectic properties. Although co-treatment with naltrexone appears to negate such behavioural effects, it is concerning, given the current status of Contrave™, that basic preclinical work such as this is only now being completed. In this context, it is emphasised that a thorough preclinical assessment of all novel treatments should be completed before such therapies progress to human testing.

Additionally, evidence suggests that the strength of interaction between naltrexone and bupropion varies dependent upon the obesity model used. For example, Greenway, et al. (2009) observed an additive bupropion-naltrexone interaction in lean mice, yet reported a stronger (possibly synergistic) interaction in obese mice.

The reason for this discrepancy is not immediately clear, but the greater effect of the combination in obese mice is certainly consistent with effects reported by the same authors for weight loss in obese humans. Therefore, the use of a rodent obesity model with a BSS methodology may help to provide further detail about the behavioural effects of this particular anorectic combination.

8.2.3 Chapter Six; Serotonin and Opioid System Interactions

Despite the current literature on the behavioural effects of the preferential 5-$HT_{1B/2C}$ receptor agonist *m*CPP within a feeding context, Experiment 7 assessed the behavioural specificity of its acute anorectic response under local laboratory conditions in order to select an appropriate dose for the proceeding combination study. Experiment 8 then employed BSS methodology to assess the anorectic efficacy and behavioural specificity of combined low-dose treatment with *m*CPP and naltrexone, with the intention of targeting both homeostatic satiety and hedonic mechanisms.

8.2.3.1 Behavioural specificity of *m*CPP

The results of the *m*CPP dose-response study (Experiment 7) are consistent with evidence (Clifton et al., 1993; Dalton et al., 2006; Hikiji, 2004; Kennett & Curzon, 1988, 1991; Kennett et al., 1987; Samanin et al., 1979; Vickers et al., 2000; Vickers, Easton, et al., 2003; Ward et al., 2008) that acute *m*CPP dose-dependently reduces food intake up to circa. 57% (3.0mg/kg; significant at 1.0-3.0mg/kg). Current results also support previous research (Kitchener & Dourish, 1994; Simansky & Vaidya, 1990) in that *m*CPP appears to dose-dependently reduce the frequency (but not the duration) of eating bouts (significant at 3.0mg/kg) and the rate of eating (significant at 1.0-3.0mg/kg). This type of feeding suppression is also seen with other 5-HT compounds, such as the indirect agonist sibutramine (Stricker-Krongrad et al., 1995; Tallett et al., 2009c).

*m*CPP significantly increased the time taken to locate the food source and, at the highest dose (3.0mg/kg), almost significantly increased eat latency. This is consistent with previous reports that *m*CPP increases the latency to feed at 2.5mg/kg (Clifton et al., 1993) and that it produces a dose-dependent increase in latency to initiate lever pressing in operant studies (Wallis & Lal, 1998). Furthermore, research suggests that other 5-HT-related compounds, such as sibutramine, increase the latency to the first meal in baboons (Foltin, 2006).

Unsurprisingly, *m*CPP was also found to decrease the frequency of sniffing and locomotor behaviour. This reduction in active behaviours is indicative of hypoactvity, as widely reported for a wide range of 5-HT_{2C} receptor agonists including *m*CPP (Clifton et al., 2000; Halford et al., 1997; Hewitt et al., 2002; Higgins et al., 2012; Kennett & Curzon, 1988; Kennett et al., 1997; Kitchener & Dourish, 1994; Simansky & Vaidya, 1990; Somerville et al., 2007; Stiedl et al., 2007; Vickers et al., 2000; see Chapter 6). Interestingly, the timecourse analyses both for the eating parameters and other behavioural measures are almost identical. In other words, *m*CPP is impacting all behaviours from the start of the session (see Figure 6-5), suggesting that the profile may not be a result of enhanced satiety, but rather an indirect behavioural effect. Furthermore, at a clear anorectic dose of 3.0mg/kg, *m*CPP did not demonste an acceleration in the BSS, which would be indicative of enhanced satiety. In fact, if anything, there is a shift to the right, typically indicative of delayed satiety and seen with orexigenic agents.

Experiment 7 found that, alongside the reduction in active behaviours, *m*CPP increased grooming levels (however see: Hewitt et al., 2002; Kitchener & Dourish, 1994). This finding is consistent with evidence that serotonergic agents induce scratching in both rats (Berendsen & Broekkamp, 1991; Eison et al., 1992; Kuraishi et al., 2008) and humans (Fjellner & Hägermark, 1979; Weisshaar et al., 1997; however see: Ständer et al., 2009). It is possible the increased grooming levels are a result of the ability of 5-HT_{2C} receptor agonists to increase thermogenesis (Hayashi, Suzuki, et al., 2004). Furthermore, it is interesting to note that there is a species-specific difference regarding the impact of *m*CPP on grooming behaviour. In rats, *m*CPP increases grooming (Bagdy et al., 1992; Bagdy & Makara, 1995; Kitchener & Dourish, 1994), whereas, in mice, it reduces grooming (Hewitt et al., 2002; Lee et al., 2004; Ständer et al., 2009)

Compared to Chapter 5, there is an asymmetry of findings regarding this grooming effect, whereby bupropion was found to increase locomotion and decrease grooming while, in Experiment 7, *m*CPP was found to decrease locomotion and increase grooming. This may be explained by response competition. For example, within a test session of finite duration, any enhancement of active behaviours leaves a limited amount of time for other behaviours such as grooming. In contrast, when active behaviours are suppressed, more sedentary behaviours (such as grooming) may be elevated.

Worryingly, despite some differences (lithium increases the duration of feeding time and maintains the structural integrity of the BSS, whereas *m*CPP decreases

the frequency of feeding bouts and disrupts the BSS), the behavioural profile of *m*CPP holds some similarity to that of LiCl (Ishii et al., 2004). For example, both agents reduce intake by 40-50%, reduce eating rate and reduce active behaviours such as locomotion and sniffing. This is potentially unsurprising considering the links between the 5-HT_{2C} receptor and nausea (Cowen et al., 1995; O'Neil et al., 2012; Sargent et al., 1997). Additionally, the periodic bouts of eating seen with *m*CPP are reminiscent of the profile following quinine-adulterated diet, which is characterised by digging and repeated sampling over the test session (Ishii, Blundell, Halford, & Rodgers, 2003; Ishii et al., 2004). Current findings therefore suggest that *m*CPP may reduce food intake via the induction of mild nausea and/or altered taste perception.

8.2.3.2 The combination

8.2.3.2.1 Food Intake and Feeding Behaviour

As the results of Experiment 7 suggested that *m*CPP may have produced a non-specific anorectic effect at the high dose, the lowest dose was chosen for the interaction study (Experiment 8). As with Experiment 7, *m*CPP (0.1mg/kg) alone failed to significantly impact any feeding or non-feeding behavioural measures compared to vehicle. However, it is important to emphasise the significant main effects of the currently-used dose on food intake and the rate of eating. These (larger sample) effects would suggest that 0.1 mg/kg was close to the anorectic threshold for *m*CPP (Kennett & Curzon, 1988; Ward et al., 2008), and a good choice for a sub-anorectic/threshold dose. However, it should be noted that the doses of naltrexone used (0.1 and 1.0mg/kg) both produced a significant reduction in mash consumption and a dose-dependent reduction in eat duration (significant at 1.0mg/kg). The variation of naltrexone sensitivity across experiments will be specifically discussed later (see Section 8.1.3).

The combination of *m*CPP with both doses of naltrexone significantly reduced one-hour food intake compared to vehicle. However, the co-treatment failed to provide evidence of an additive effect (mV = -40.71% vs. a calculated -45.35%; mN = -34.50% vs. a calculated -76.42%). Previous research has found that serotonin release increases opioid receptor density in hypothalamic regions associated with appetite regulation, as a potential compensatory mechanism against a serotonin-mediated reduction in opioidergic tone (Churruca et al., 2004). This would suggest that co-treatment with naltrexone (an opioid antagonist) should prevent the stimulation of opioid neurons and this negative feedback system, thereby

intensifying the appetite suppressant effects of *m*CPP alone. However, the present data on food intake and feeding parameters (eat duration and frequency) are inconsistent with this view, and provide little evidence of either dose combination producing an effect beyond that of naltrexone alone. One could go so far as to say that the results can be largely, if not totally, understood in terms of the naltrexone component.

8.2.3.2.2 Non-feeding Behaviour and the Behavioural Satiety Sequence

In Experiment 8, the increase in grooming seen with *m*CPP (significant at 3.0mg/kg in Experiment 7) appears to be attenuated by co-treatment with naltrexone. This is consistent with evidence that serotonin-induced biting and itch-related behaviour is inhibited by subcutaneous and intracisternal naloxone administration (Kuraishi et al., 2008). However, since the elevation of grooming by *m*CPP was not significant in this study, the extent to which it could be suppressed is limited; therefore, this may be spurious finding. Nevertheless, it may also suggest that, similar to recent reports for CB1 receptor antagonist/inverse agonists (Tallett et al. 2008b, 2009a; see Chapter 4) and bupropion (see Chapter 5), naltrexone co-treatment may counter at least some of the unwanted effects of higher doses of *m*CPP.

Furthermore, the decreased sniff frequency and locomotion frequency, in addition to the increased rest duration, seen with the combination treatments are patterns similar to that seen in earlier work with naltrexone (Experiment 5), albeit at higher doses. This further emphasises the point that the effects of the drug combination are primarily due to naltrexone action. It is important to note at this point that variations in these parameters can be seen at different periods during the timecourse (feeding at the beginning and decreased active behaviours towards the end). Therefore, the profile for naltrexone, alone and in combination, remains indicative of a ‘normal’ BSS profile.

It is noteworthy that the BSS profile of *m*CPP (0.1mg/kg), given alone, appears to shift to the right, suggesting a delay in the onset of satiety, which is a similar pattern to that seen in Experiment 7 with the highest dose of *m*CPP (3.0mg/kg). However, this is not the case for the combination treatments, where, as with the other parameters, the eat-to-rest transition and BSS profiles mimic those seen with naltrexone given alone.

8.2.3.3 Conclusions, Implications and Future Research

Although Experiment 7 confirmed the anorectic efficacy of *m*CPP, it brought into question the behavioural specificity of the compound. In light of the hypoactivity seen at the highest dose (3.0mg/kg), further study would be needed to confirm if *m*CPP is a selective anorectic agent, or if it is producing reductions in food intake as an indirect consequence of hypoactivity and/or increased grooming. Furthermore, at the highest dose used, the BSS profiles were not accelerated, thereby suggesting that satiety may not have been enhanced.

In summary, the combination of a sub-anorectic dose of the preferential 5-$HT_{1B/2C}$ receptor agonist *m*CPP and threshold/sub-maximal doses of the opioid antagonist naltrexone produced an infra-additive (less than additive) effect on food intake and feeding measures. Any suppression of food intake and feeding duration appeared to be purely a result of a naltrexone-induced anorexia, which largely remained unaltered by co-treatment with *m*CPP. This negative outcome is supported by the lack of a positive anorectic interaction between naloxone and sibutramine (Tallett et al., 2010b). However, it is pertinent to note that sibutramine is a dual (serotonin- and noradrenaline-) reuptake inhibitor, whereas combinations of naloxone with fluoxetine (Hagan et al., 1997) and 5-HTP (Fernandez-Tome, 1988), agents that specifically increase overall 5-HT levels, have been reported to produce an additive/synergistic interaction. It may be that the preferential 5-$HT_{1B/2C}$ receptor agonist *m*CPP could not activate the same targets. It would therefore be interesting to assess the generality of present findings using a more specific 5-HT_{2C} receptor agonist (such as lorcaserin) in combination with an opioid antagonist.

8.2.4 Chapter Seven; Peptide and Opioid System Interactions

Experiments 9 and 10 were designed to (i) assess the anorectic efficacy and behavioural specificity of GLP-1 agonist, exendin-4, with a focus on locomotor and non-ingestive behaviours and (ii) assess the combination of a compound thought to primarily influence satiety signalling, exendin-4 with one believed to primarily impact reward mechanisms, naltrexone.

8.2.4.1 Behavioural specificity of Exendin-4

The findings of Experiment 9 confirmed previous reports (Aziz & Anderson, 2002, 2003; Bojanowska & Nowak, 2007; Bojanowska & Radziszewska, 2011; Chan et

al., 2013; Hayes, Kanoski, et al., 2011; Kanoski et al., 2011; Kanoski et al., 2012; Mack, Moore, et al., 2006; Perez-Tilve et al., 2007; Szayna et al., 2000; Talsania et al., 2005) that exendin-4 (0.25 and 2.5µg/kg) potently and dose-dependently reduces intake. The intermediate dose (0.25µg/kg) reduced intake by 26% and the high dose (2.5µg/kg) reduced intake by 76%. Although current results appear to demonstrate more potent effects than previously reported (see review: Nielsen et al., 2004), this may be accounted for by differences in the palatability of the test diet (Dickson et al., 2012) and/or the duration of the observation period (Kanoski et al., 2012; Talsania et al., 2005).

The lowest dose used (0.025µg/kg) failed to produce any significant effects on mash consumption, eating-related parameters or any non-ingestive behavioural measures. In contrast, there was a significant suppression of food intake and feeding behaviour by 0.25 and 2.5µg/kg, suggesting a threshold between 0.025 and 0.25µg/kg. This is consistent with literature suggesting an ED_{50} for exendin-4 of 0.15 - 0.25µg/kg (Nielsen et al., 2004; Young et al., 1999). At the anorectic doses, exendin-4 significantly increased eat bout duration, and reduced eat rate. The highest dose also produced a near significant paradoxical reduction in eat latency. The impact of exendin-4 on these eating-related parameters may indicate some disruption to basic perceptual or motivational mechanisms, such as the induction of malaise or nausea. This is consistent with reports of emesis and nausea following exenatide treatment in human trials (e.g. Buse et al., 2004).

Dramatically, exendin-4 dose-dependently reduced both the frequency and duration of locomotion, rearing, sniffing, and grooming near to the point of elimination. Furthermore, frequency and duration measures for resting were profoundly increased, with the highest dose of exendin-4 producing resting levels >7 times greater than control. This could be interpreted as the profile of a strong satiety signal; indeed, a suppression of peak feeding following some food consumption and the acceleration of the BSS can be seen in Figure 7-5. However, whilst the suppression of the active behaviours could be perceived as the enhancement of satiety, timecourse assessments reveal that all ingestive and non-ingestive behaviours were reduced from the beginning to the end of the test session. The present ‘hypoactive’ exendin-4 profile is consistent with research (Erreger et al., 2012; Talsania et al., 2005) showing a suppression of general activity at very high doses (30 or 120µg/kg). Moreover, there is evidence of locomotor suppression at doses as low as 0.5µg/kg (Mack, Moore, et al., 2006). Additionally, exendin-4 (3µg/kg) has been reported to suppress water intake

independent of food intake (McKay et al., 2011). Such findings are consistent with behavioural non-specificity even at relatively low dose levels.

Taken together, the behavioural signature of exendin-4 seen in Experiment 9 is reminiscent of that seen following administration of LiCl, a common emetic (Ishii et al., 2004). Previous research, using a similar methodology, within the same laboratory, found that administration of LiCl reduced intake of palatable mash by 42%, whilst reducing eat rate and suppressing the frequency and duration of locomotion, rearing, sniffing, and grooming. Although not dissimilar to the current exendin-4 profile, LiCl increased the duration of time spent feeding (with no impact on frequency measures), whereas exendin-4 decreased the frequency of eating bouts (with no impact on duration measures). It should also be noted that LiCl produced no effects on resting behaviour, unlike the notable effects seen with exendin-4. However, these profile differences could be at least partly misleading as LiCl doses of equi-anorectic strength to that of 2.5µg/kg exendin-4 may well exert a more intense behavioural profile.

Current behavioural observations are consistent with the literature (discussed earlier, see Section 7.2) suggesting the overlap in neuronal activation following LiCl and GLP-1 administration. For example, systemic administration of LiCl is found to activate GLP-1 neurons in the caudal brainstem including those projecting to the PVN and other areas of the forebrain associated with appetite regulation (Parkinson et al., 2009; Rinaman, 1999a; Thiele et al., 1998). Further research has found that both the neuro-excitatory and aversive behavioural effects of LiCl can be significantly attenuated by GLP-1 receptor blockade (Rinaman, 1999a; Seeley et al., 2000; Thiele et al., 1998). Together with the current findings, the data suggest that the anorectic effects of GLP-1R agonist exendin-4 are at least partially due to visceral illness and potentially transmitted via a nausea circuitry in common with LiCl. However, it should be noted that some work has demonstrated that anorectic doses of exendin-4 fails to induce kaolin consumption (a measure of malaise; Mack, et al., 2006), suggesting that the two effects may be independent of one another, and/or occur at different doses.

8.2.4.2 The combination

8.2.4.2.1 Food Intake and Feeding Behaviour

Consistent with Experiment 9, the results of Experiment 10 demonstrated that, when given alone, the lower dose of exendin-4 (0.025 µg/kg) produced no significant effects on food consumption, whereas the higher dose (0.25 µg/kg),

given alone significantly reduced consumption by 41% (vs. vehicle), and reduced both the frequency and duration of eating. Naltrexone (0.1mg/kg) also significantly reduced mash consumption (by 36% relative to control), as well as the frequency and duration of feeding behaviour. These results conflict with the findings in Chapter 5, but are consistent with the effects obtained against a similar basal intake in Chapter 6 (see also Section 8.3.1.2).

Despite the intrinsic efficacy of the high dose of exendin-4 (0.25µg/kg) and of naltrexone (0.1 mg/kg) on intake and behaviour, the combination treatments failed to evidence any signs of a positive interaction. The near-identical effects of individual vs. combined treatment on food intake is most clearly seen with the high dose of exendin-4 and naltrexone (VN = 34.9%, EHN = 34.4% vs. a predicted 75.7%). The effects on mash consumption mirror those seen on the measures of feeding behaviour. This is consistent with previous work that failed to demonstrate an additive effect on food intake when administering GLP-1 and naloxone to neonatal chicks (Bungo et al., 1999). However, the lack of additive interaction with these compounds stands in contrast to evidence of additive and/or synergistic anorectic and/or weight loss interactions reported for GLP-1 agonists in combination with other agents such as glucagon (Day et al., 2009), PYY_{3-36} (Paulik et al., 2011; Reidelberger et al., 2011b; Talsania et al., 2005), leptin (Bojanowska & Nowak, 2007), calcitonin (Bello et al., 2010), and AM-251 (Bojanowska & Radziszewska, 2011).

8.2.4.2.2 Non-feeding Behaviour and the Behavioural Satiety Sequence

Consistent with Experiment 9, exendin-4 was found to reduce the frequency of sniffing, while less potently (seen only in main effects analysis) increasing resting and reducing locomotor activity. Interestingly, naltrexone also exerted weak (again, main effects analysis only) inhibitory effects on grooming and locomotion.

Consistent with effects on intake, although the high dose of exendin-4 and naltrexone individually produced a modest acceleration in the BSS, their combination failed to produce any further acceleration in this measure of behavioural satiety. The BSS profiles thus did not provide any evidence that co-treatment with these agents led to an additive interaction.

8.2.4.2.3 Evidence of an Additive Anorectic Interaction

It is pertinent to note that, as Experiment 10 neared completion, a report was published demonstrating an additive anorectic interaction between exendin-4 and

naltrexone in rats (Liang et al., 2013). Consistent with the present findings, the authors found significant dose-dependent anorectic effects with exendin-4 (1 - 10µg/kg) and naltrexone (0.32 – 3.2µg/kg), when given alone. Interestingly, however, the effects were much less potent than those found in the present study. However, in contrast to the current work, significant additive anorectic effects were observed, over 10-fold dose ranges, when these agents were given in combination. It is also interesting to note that, in a second experiment on CTA, Liang et al. (2013) not only confirmed the aversive effects of low doses of exendin-4 (1.0 & 3.2 µg/kg) but also showed that these effects were not countered (may have even been exacerbated) by the addition of naltrexone

The reason for the significant discrepancy in the combination efficacy are not immediately clear, especially since the two studies differed on so many methodological dimensions (see Table 8-1). Many factors can influence drug effects on behaviour, including age (Tirelli et al., 2003), strain (Porsolt et al., 1978) and housing conditions (Hughes & Syme, 1972). Therefore, any one or combination of these factors may have been responsible for the differential outcome. However, attention is specifically drawn to the potential significance of between-study differences in the method of drug co-administration. Consistent throughout the present thesis, including dose-ranging studies on exendin-4 (Experiment 9) and naltrexone (Experiment 5), animals in the current interaction experiment (Experiment 10) received two individual injections separated by a 15 minute interval, with the start of testing delayed by a further 15min (see Chapter 3). In contrast, Liang et al. (2013) dissolved the two drugs together for a single injection 10-15minutes prior to testing. This difference in co-administration (sequential versus simultaneous) becomes of interest when considering that, in Experiment 10, exendin-4 was administered well before naltrexone. The data from the current experiment demonstrates that the effects of the combined treatment are statistically indistinguishable from those seen with either agent given alone. Given this evidence, it would seem parsimonious to conclude that the effect of one of the compounds (exendin-4?) was ‘dominating’ that of the other. Although it is theoretically possible that the two drugs interfered with one another’s pharmacokinetics and/or pharmacodynamics, this would not easily account for the additive anorectic profile reported by Liang et al. (2013). Instead, it is suggested that the effects of the agent administered first (exendin-4) may dominate those of the agent administered second (naltrexone). This hypothesis may be further supported, in this case, by the aversive effects of exendin-4-induced malaise, and

Table 8-1: Summary of methodological differences that may explain the discrepancies in findings between to Liang et al. (2013) and Experiment 10

Highlighting methodological differences such as genetic strain, nutritional status, test diet, environment & duration, dose ranges, number of treatment conditions, and inter-test interval.

	Liang et al. (2013)	Experiment 10
Subjects	Sprague Dawley	Lister Hooded
Number of subjects	8	10
Weight	250-275g	215–518g
Regular Food	Prolab RMH 1000	BK No.1 Rodent Breeder and Grower (Pellet Diet)
Test Food	Prolab RMH 1000	BK No.1 Rodent Breeder and Grower (Mash)
# Treatment Conditions	18	6
Naltrexone doses	0, 0.32, 1.0, 3.2 mg/kg^{-1}	0, 0.01, 0.1 mg/kg
Exendin-4 doses	0, 1.0, 3.2, 10 $\mu g/kg^{-1}$	0, 0.025, 0.25, 2.5 µg/kg
Combination	Dissolved together in saline	15minute injection interval
Injection-Testing-Interval	10-15minutes	30min and 15min
Time of Day	Dark Phase	Light Phase
Pre-feeding	4hour food restriction	Free feeding
Test Duration	1h, 4h and 20h	1h
Wash-out period	2 days	7 days

especially when also considering that naltrexone is presumed to influence reinforcement mechanisms after the ingestion of food. In other words, the profile seen with the exenin-4 high dose combination treatment may predominantly reflect the influence of exendin-4, thereby explaining the lack of difference versus the peptide given alone.

8.2.4.3 Conclusions, Implications and Future Research

In conclusion, present results confirm that exendin-4 and naltrexone have individually intrinsic anorectic effects but that co-administration of these compounds does not produce an additive effect. This pattern differs from another recent study (Liang et al., 2013), although this discrepancy may be explained by differences in the method of drug co-administration. Interestingly, the two studies agreed on the ultimate conclusion, that exendin-4 induces a lithium-like behavioural profile in tests of food intake and that co-treatment with naltrexone does not counteract this, but may in fact worsen the aversive effects (Liang et al., 2013). Neither study supports the role of an exendin-4 plus naltrexone combination for the treatment of obesity, yet both illustrate the value of assessing the

behavioural selectivity of novel treatments/treatment combinations at an early stage in the drug development process.

Future research should assess if polytherapy could benefit the side-effect profile of exendin-4. For example, co-treatment of GLP-1 agonists with glucagon (Day, 2009), PYY_{3-36} (Paulik et al., 2011; Reidelberger et al., 2011b; Talsania et al., 2005), leptin (Bojanowska & Nowak, 2007; Reidelberger et al., 2011a), the amylin analogue calcitonin (Bello et al., 2010), and the cannabinoid CB1 receptor antagonist/inverse agonist AM-251 (Bojanowska & Radziszewska, 2011) has previously been shown to produce an additive/synergistic effect on food intake/weight gain. However, none of these studies has involved in-depth behavioural analysis such as that employed in the current thesis.

8.3 Issues Arising

8.3.1 Drug Variability/Sensitivity

The experiments of the present thesis have identified some sensitivity differences to particular compounds. Despite no obvious differences in the experimental subjects, the laboratory, the methodologies, or the researcher, potency variations have been identified both for the opioid antagonist naltrexone, and the dopamine and noradrenaline reuptake inhibitor bupropion.

8.3.1.1 Variability in Vehicle Control Profile

Some variation in baseline control group intake was seen across experiments (see Figure 8-1). This could be readily explained by batch differences in appetite and feeding patterns. Interestingly, seasonal effects on laboratory rodent behaviour (Ferguson & Maier, 2013) may have influenced between-study variation in intake and general activity levels. However, neither the variation in food intake (Figure 8-1) nor seasonal changes appear to map onto variations in other non-ingestive behaviours such as locomotion and/or resting. Nevertheless, the between-study variation in intake may help to, at least partially, account for the variation in drug sensitivity. For example, significant reductions in mash consumption are more likely to be detected when control levels of intake are high.

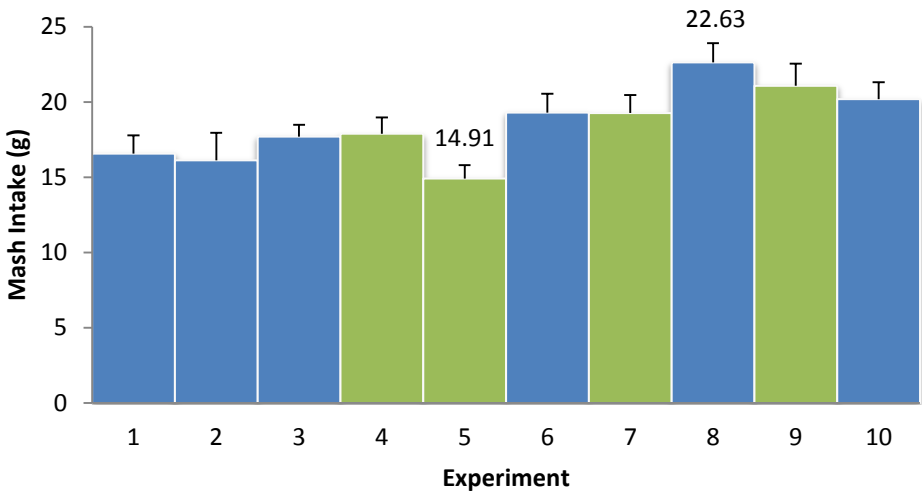

Figure 8-1: Effects of baseline control treatment on mash intake by non-deprived male rats during a 1-h test with palatable mash, across all 10 Experiments

Data are mean values (± S.E.M). Minimum and Maximum mean values are reported. V = vehicle (distilled water or saline). Green = Dose-response studies (aka. single injection studies), Blue = Interaction studies (aka. double injection studies). See text for full details

8.3.1.2 Variability of Naltrexone Sensitivity

Throughout the current thesis, the highest variability of drug sensitivity has been seen with naltrexone. Data from Experiment 5 showed that the suppressant effects of naltrexone on food intake and feeding duration were statistically significant at the highest dose (3.0mg/kg) only. In contrast, Experiment 6 demonstrated that a lower dose (1.0mg/kg) significantly reduced mash consumption and time spent feeding. Although it should be noted that this dose actually reduced intake by 21.68% (and significantly decreased the frequency of eating episodes) in Experiment 5, a reduction similar to that seen in Experiment 6 (25.98%). This suggests that the variability in drug response is most likely due to the influence of variation in baseline intake on the statistical threshold. This explanation may also explain the statistical reduction in mash intake with naltrexone 1.0mg/kg in Experiment 8 and 10. The baseline control in Experiment 8 was one of the higher intake values seen in the thesis (as seen in Figure 8-1; 21.07g ±1.47) whereas the baseline control of Experiment 5 was atypically low (14.91g ± 0.90). Therefore, Experiment 5 produced a seemingly larger naltrexone suppression of intake (39.78%), yet all three experiments (5, 6 and 8) produced similar absolute mash intakes (11.68 ± 1.05, 14.28 ± 1.36, and 13.63 ± 1.20, respectively).

Most dramatically, the low dose naltrexone (0.1mg/kg) which produced only a 4.89% reduction in mash intake in Experiment 5 and a non-significant 14.12%

reduction in Experiment 6, produced significant 25.69% and 35.71% reductions in Experiments 8 and 10, respectively. Again, however, the absolute mash intakes were quite similar (Exp5, 14.19 ± 0.92; Exp6, 16.57 ± 1.46; Exp8, 16.81 ± 1.80; Exp10, 12.98 ±0.80), suggesting that variability in baseline intake is the root cause of the inconsistency in dose-sensitivity.

It is also important to note that the interaction drug studies involved a double injection procedure which may have altered the stress background and therefore the drug response. However, it is pertinent to note that the mean baseline control intake across dose-response studies (18.75g) is almost identical to that seen in the interaction studies (18.28g).

8.3.1.3 Variability of Bupropion Sensitivity

In contrast, it is unclear as to why bupropion (20mg/kg) failed to significantly suppress intake in Experiment 4, and yet produced a significant reduction in consumption in Experiment 6. Both Experiments (4 and 6) were characterised by a mid-range baseline control intake (17.89 ± 1.08 and 19.30 ± 1.24, respectively). Furthermore, there is no evidence of a floor effect; in fact, Experiment 4 shows that bupropion (20mg/kg) marginally increased intake from vehicle to 18.85g (± 0.81), whereas Experiment 6 actually showed a significant reduction from vehicle to 14.84g (± 1.42).

Overall, the observed differences in drug sensitivity across studies indicate that previous research is not a fool-proof guarantee of the behavioural outcome for a specific drug dose. This could be a consequence of batch differences, baseline levels of behaviour, strain of rat, and the exact methodologies employed. Therefore, caution must always be exercised when using previous research to guide dose selection and/or when comparing drug effects across papers/laboratories.

8.3.2 The Implications for the Interpretation of the Behavioural Satiety Sequence and Behavioural Specificity

A key theme that runs through the current thesis is the interpretation of the BSS in the context of behavioural specificity. BSS methodology is typically used to provide behavioural profiles to confirm whether a treatment-induced reduction in food intake is reached directly by an action on the normal physiological regulation of appetite <u>or</u> indirectly via a host of non-specific mechanisms (Halford et al., 1998; Rodgers et al., 2010). Interestingly, the current work has highlighted that a non-

specific behavioural profile does not necessarily allow for conclusions to be drawn regarding the causation of the anorexia.

For example, in Chapter 4 (Experiment 1-3), the induction of compulsive scratching and grooming by rimonabant may be an unwanted drug effect but it does not appear to account for the concurrent suppression of appetite (see Figure 4-13). The naloxone-induced blockade of rimonabant-induced scratching and grooming, but not of rimonabant anorexia, demonstrates that a non-selective drug effect is not automatically the cause of anorexia. Similarly, in Chapter 5 (Experiment 6), the induction of hyperlocomotion by bupropion could lead to the conclusion that the anorectic response to this drug is mediated by some non-specific mechanism, such as increased locomotion (see Figure 5-4). In contrast, current data suggest that bupropion independently produces anorexia in addition to psychomotor stimulation. Thus, the hyperlocomotive effects of bupropion were attenuated by naltrexone co-treatment without impacting the bupropion-induced suppression of peak feeding (see Figure 5-11).

The findings of Chapters 4 and 5 therefore imply that while providing comprehensive behavioural profiles, BSS methodology should not be the only tool used when assessing the behavioural specificity of drug effects on food intake. The need for additional testing should also be considered following the interpretation of “selective” anorectic profiles, such as that seen with exendin-4 in Chapter 7. As discussed earlier, the BSS profile for exendin-4 (2.5µg/kg) could be interpreted as the profile of a strong satiety signal (see Figure 7-5). However, an assessment of the timecourse reveals that all ingestive and non-ingestive behaviours were reduced from the beginning to the end of the test session, which may alternatively suggest that exendin-4 is producing a sedative action. Further research is therefore required to assess the causation of this exendin-4 behavioural profile.

8.4 Future directions

In addition to the directions discussed in individual chapters, future research in this field should focus on clarification of current findings through the application of additional research methodologies Furthermore, the agents used in this thesis have not exhausted the anorectic compounds (see Chapter 2) that could potentially be assessed, alone and in combination, for their therapeutic potential as anti-obesity agents.

8.4.1 Additional methodologies

It is important to emphasise that the methodology used in the current thesis is but one approach designed to play a part in a broader battery of tests needed to provide a comprehensive understanding of drug effects on food intake. Ideally, BSS analysis should be conducted initially on all new treatments and/or combinations with the most promising approaches then subject to multiple behavioural assessments such as meal patterning analysis (e.g. Clifton, 2000), macronutrient preference (e.g. Berthoud et al., 2012), dietary-induced hyperphagia (e.g. Hariri & Thibault, 2010) and genetic obesity models (e.g. Kurtz et al., 1989). Additional studies, investigating hedonics and motivation, can also be conducted. BSS methodology employs measures of eating-related parameters, such as the ability to seek food, the initial motivation for food, and the rate of food consumption, effects on which may provide evidence relevant to basic perceptual or motivational mechanisms. However, it can be argued that a non-significant effect on food intake does not negate a possible treatment effect on food motivation, as assessed for example by breakpoint analysis (e.g. Somerville et al., 2007). Therefore, further assessment of these motivational mechanisms may enhance our understanding of appetite suppressing agents.

Although the eat-to-rest transition, used to assess the acceleration/delay of satiety, is not mathematically or statistically derived, other tests would allow for a more mathematical assessment of drug interaction, e.g. isobolographic or dose-addition analysis. Isobolographic analysis (Loewe & Muischnek, 1926; cited in Tallett, 2009) assesses the efficacy of a drug combination on food intake by graphically representing the data. The ED_{50} for the individual drugs (calculated by regression analysis) is plotted on a graph, and these values are then connected and surrounded by their 95% confidence limits to outline the “dose-additive” region. The ED_{50} is then calculated and plotted for the combination. If this falls within that region, it is defined as additive. If it is above the region, it is considered infra-additive and, if below, it is considered supra-additive (see: Roth et al., 2010). Dose-addition analysis, however, is a less formal approach of that taken by isobolographic analysis (see: Liang et al., 2013; Ward et al., 2008)

Although this methodology would not be suitable for the large range of behavioural measures employed in the current thesis, they could be exploited to confirm the nature of any drug interactions identified with BSS analysis. The same point would apply to chronic testing and the use of rodent models of obesity.

In conclusion, more thorough preclinical work, starting initially with BSS analysis, should result in a greater understanding of how agents actually produce the endpoints outlined by the FDA and EMEA (Rodgers et al., 2010). This should ultimately help to avoid 'after-the-fact' withdrawals of compounds, seen most recently with rimonabant and sibutramine.

8.4.2 Methodological Limitations

8.4.2.1 Metabolic Assessment

The BSS is not a suitable methodology for assessing metabolic effects of anorectic agents. However, throughout the thesis, in all experimental conditions, none was associated with a reduction in total post-treatment weight gain, the principle measure used to account for this short-coming. Experiments 1-10 also failed to reveal any significant main effects of treatment or significant interactions on daily percent body weight gain. This is undoubtedly due to the acute nature of the treatment, the relatively low dosages involved (sub-anorectic, threshold and sub-maximal) and the relatively short biological half-lives of the compounds used. It is also possible that the animals may have compensated for the reduction in food intake during testing by increasing consumption throughout the rest of the day. This would readily explain why there was no significant suppression of bodyweight gain even 24hours after testing. It is therefore proposed that BSS methodology should be accompanied by post-treatment weight gain analysis and the measurement of home cage intake at regular intervals (e.g. 1h, 4h, 12h, and 24h) over the same period.

8.4.2.2 Alternative methods of co-treatment

The method of drug co-treatment used throughout the present thesis, whereby the individual agents were administered i.p. individually 15minutes apart, has limitations. Although, this technique accounts for the pharmacokinetic and pharmacodynamics differences in the compounds used, the dual injection method causes increased stress to the animals and may result in increased arousal and "independent" effects on food intake (as in Chapter 5 and 7). It may also cause problems with drugs competing for the same receptor site, as discussed in Chapter 7. It is proposed that future studies should assess the impact of administration method on food intake to inform the further use of BSS analysis. For example, a follow up study based on the design of Experiment 10; a within-subjects design whereby each subject receives six experimental conditions according to a Latin Square (with a 7-day wash out period): Vehicle (Saline; V); Naltrexone (0.1mg/kg)

in saline (N); Exendin-4 (0.025µg/kg) in saline (EL); Exendin-4 (0.025µg/kg) + Naltrexone (0.1mg/kg) in the same saline solution (ELN); Exendin-4 (0.25µg/kg) in saline (EHV); and Exendin-4 (0.25µg/kg) + Naltrexone (0.1mg/kg) in the same saline solution (EHN). Comparing the results of an exendin-4/naltrexone co-treatment with the use of combined injection to the results of Experiment 10 should provide a better understanding of the co-treatment effects.

8.4.3 Potential Neurobiological Targets for Future Research

As a number of recent reviews have stressed the lack of true detail on the behavioural effects of novel anti-obesity agents (see Table 2-3), alone or in combination (Adan, 2013; Chugh & Sharma, 2012; Colon-Gonzalez et al., 2013; Rodgers et al., 2012), it is important that future research behaviourally profiles these treatments using BSS and related methodologies. Given the huge variety of polytherapies currently in early-stage development (see Table 2-5), four polytherapy strategies (outlined in Chapter 2) should serve to guide further work: (1) satiety peptide plus satiety peptide, (2) adiposity signal plus satiety peptide, (3) small molecule agent plus adiposity signal or satiety peptide, and (4) small molecule agent plus small molecule agent. Specific attention should be given as soon as possible to those agents and/or combinations that have recently been licensed, or are likely to be licensed soon, for example Qsymia® (topiramate and phentermine) and Empatic™ (bupropion and zonisamide).

It is also important to note the recent focus in drug development towards agents that reduce the severity of metabolic disorders rather than having a sole focus on appetite and/or bodyweight reduction. Therefore, while BSS analysis may be a good starting point for the assessment of preclinical agents, future research should also profile the effects of novel agents on cardiovascular function as well as metabolic parameters such as cholesterol, insulin resistance and circulating triglycerides.

8.5 Overall Conclusions

In light of the limitations of lifestyle and surgical options for the treatment of obesity, the search for safe novel drugs for weight loss is of utmost importance. Disappointingly, previous and current anti-obesity drugs are limited in terms of maximal weight loss, adverse side-effects and/or long-term resistance (see Chapter 2). Although we do not yet fully understood the neurobiology of appetite, there is a huge array of interacting systems that regulate intake and bodyweight and which could be potential targets for an anti-obesity agent (see Chapter 1). However, given the industry track-record, it is paramount that these pharmacological actions are not only effective but also selective.

The current thesis has furthered our understanding of the acute effect of individual treatment with the general opioid receptor antagonist naltrexone (Experiment 5), the noradrenaline and dopamine reuptake inhibitor bupropion (Experiment 4), the serotonin 5-$HT_{1B/2C}$ receptor agonist *m*CPP (Experiment 7), and the naturally-occurring GLP-1R mimetic exendin-4 (Experiment 9), on food intake, behaviour and bodyweight gain. Furthermore, the assessment of the acute anorectic response to co-treatment with sub-maximal doses of these compounds and an opioid antagonist (naloxone; Experiments 1-3 / naltrexone Experiments 6, 8 and 10) has emphasised some of the benefits of polytherapy; the use of lower drug doses, possible synergistic but at least additive reductions in food intake, and importantly the reduction/elimination of side-effects normally associated with higher doses of the constituent agents. Finally, the current thesis has once again emphasised the value of detailed behavioural analysis of drug effects on appetite.