Appetite and Functional Brain Responses to Cannabinoids

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Faculty of Life Sciences

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Abstract

The obesity epidemic is a major health threat affecting one in four people in the affluent western world, where high-energy foods are easily available and there is little need for exercise. To identify novel therapeutic targets for the treatment of obesity, one important step is to further define the complex circuitry in the brain which is ultimately responsible for our appetite and body weight regulation. Although complex, appetite can be thought of as having two distinct, though none mutually exclusive, aspects: the need to eat (homeostatic) and the desire to eat (hedonistic).

The need to eat, a product of energy homeostasis, is what drives the consumption of food for basic survival. In an attempt to further define the mainly “homeostatic” neuronal circuitry, we combined blood-oxygen-level-dependent (BOLD) pharmacological-challenge magnetic resonance imaging (phMRI) with c-Fos functional activity mapping to characterise “whole brain” responsiveness to an orexigenic dose of the glucose anti metabolite 2-deoxy-D-glucose (2-DG). Using these complementary methods, we demonstrated functional brain activity in a number of known glucose-sensing brain regions, including parts of the hypothalamus and brainstem, following administration of 2-DG when compared with vehicle treatment.

The desire to eat is a result of a complex interplay between the reward and motivational circuits implicated in addictive behaviours, and those which control energy homeostasis. Recent research has pointed to the endocannabinoid system, and specifically the central cannabinoid 1 (CB$_1$) receptor, as a key target mediating the functional cross talk between the two appetitive systems. To define the sites of action of cannabinoids, we used an orexigenic dose of the full CB$_1$ agonist, CP55940, to map responsive brain regions again using BOLD phMRI and whole-brain c-Fos functional activity mapping. Areas of interest demonstrated a drug interaction when the CB$_1$ receptor inverse agonist, Rimonabant was co-administered. These complementary methods demonstrated functional activity in the cortico-striatal-hypothalamic pathway, a key system in the motivational drive to eat.

The appetitive actions of synthetic CB$_1$ inverse agonists such as Rimonabant are well documented. We, however, described a putative novel endogenous CB$_1$ inverse agonist, hemopressin, which is the first identified peptide ligand of CB$_1$ receptors. We showed that hemopressin inhibits agonist-induced receptor internalisation in a heterologous cell model in vitro. When administered centrally or systemically in vivo, we found that hemopressin decreases nocturnal food intake in out-bred rats and mice, as well as in obese, leptin-deficient ob/ob mice. Importantly, hemopressin induces hypophagia without causing any apparent adverse side effects. We have also shown that the anorectic effect is absent in CB$_1^{-/-}$ mice, and that hemopressin can block CB$_1$ agonist-induced hyperphagia in male rats, providing strong evidence for antagonism of the CB$_1$ receptor in vivo.

We speculate that hemopressin may be one of a family of endogenous functional CB$_1$ receptor ligands that modulate the activity of appetite pathways in the brain.

Appetite and Functional Brain Responses to Cannabinoids
Declaration

I, the undersigned declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Garron T. Dodd

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### Abbreviations

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<tr>
<td>2-AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-Deoxy-D-glucose</td>
</tr>
<tr>
<td>Acb</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>AcbC</td>
<td>Nucleus Accumbens Core</td>
</tr>
<tr>
<td>AcbSh</td>
<td>Nucleus Accumbens Shell</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-Related Peptide</td>
</tr>
<tr>
<td>AI</td>
<td>Agranular Insular Cortex</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Arc</td>
<td>Arcuate Hypothalamic Nucleus</td>
</tr>
<tr>
<td>BCC</td>
<td>Behavioural Control Column</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed Nucleus of the Stria Terminalis</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood Oxygen Level Dependent</td>
</tr>
<tr>
<td>BSS</td>
<td>Behavioural Satiety Sequence</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium Ion</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and Amphetamine-Regulated Transcript</td>
</tr>
<tr>
<td>CB$_1$</td>
<td>Cannabinoid Receptor 1</td>
</tr>
<tr>
<td>CB$_2$</td>
<td>Cannabinoid Receptor 2</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral Blood Flow</td>
</tr>
<tr>
<td>CBV</td>
<td>Cerebral Blood Volume</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
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<td>CeA</td>
<td>Central Amygdala</td>
</tr>
<tr>
<td>CeA</td>
<td>Central Amygdala</td>
</tr>
<tr>
<td>Cg</td>
<td>Cingulate Cortex</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>Cpe</td>
<td>Carboxypeptidase E</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate Putamen</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-Releasing Hormone</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMN</td>
<td>Dorsomedial Hypothalamic Nucleus</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<td>DNQX</td>
<td>6,7-Dinitroquinoxaline-2,3-dione</td>
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<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>EMEA</td>
<td>European Agency for the Evaluation of Medical Products</td>
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<tr>
<td>FAAH</td>
<td>Fatty Acid Amide Hydrolase</td>
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<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
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<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<tr>
<td>HAB1</td>
<td>α-haemoglobin chain gene</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal Axis</td>
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<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<td>IC</td>
<td>Inferior Colliculus</td>
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<td>IC₅₀</td>
<td>Half Maximal Inhibitory Concentration</td>
</tr>
<tr>
<td>IMS</td>
<td>Imaging Mass Spectroscopy</td>
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<tr>
<td>IPAC</td>
<td>Interstitial Nucleus of Posterior Limb of the Anterior Commissure</td>
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<td>LC</td>
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<td>m/z</td>
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<td>MALDI</td>
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1.1 Introduction

Homeostasis is a key goal of many physiological processes; to maintain constancy in the internal milieu. The body requires a constant supply of metabolic fuel, usually in the form of glucose. Glucose levels in the blood are determined by neural and endocrine interplay between organs including the brain, liver and pancreas. However, the body must also store energy in the form of glycogen and fat, in order to protect against fluctuations in food availability and ensure survival through growth and reproduction. It is likely that the body maintains energy homeostasis by defending a number of different “set points”, which may include blood glucose levels, liver glycogen and adiposity (Keesey et al. 1979). Energy homeostasis can be described in very simple terms as the constant balance between food intake and energy expenditure, required to maintain body weight and deliver adequate metabolic supplies. Although a product of energy homeostasis, feeding behaviour is also a product of the brain’s balance between hunger and satiety; collectively termed “appetite.”

Appetite can also be split crudely into two, non-mutually exclusive aspects; the need to eat and the desire to eat. The need to eat, a direct effector of energy homeostasis, is what drives the consumption of food for basic survival. It features a number of interacting physiological systems, which incorporate redundancy to either increase or decrease feeding behaviour. Fundamental to adaptive responses are hormonal signals such as leptin, ghrelin, cholecystokinin (CCK), and mechanical signals such as gastric stretch (Smith et al. 1981, Smith 2000), which act to initiate or terminate feeding, or to provide a reflection of the body’s adiposity and energy balance. These signals are integrated by peripheral nerves and brain centres including the hypothalamus and brainstem, where they act to regulate central pathways and modulate appetite.

1.1.1 Central Pathways Involved in the Homeostatic Control of Appetite

The dependence of energy homeostasis on the hypothalamus was first demonstrated in a number of lesioning studies performed in the 1940s and 1950s. These studies showed that destruction of the ventromedial, paraventricular and dorsomedial regions of the hypothalamus resulted in hyperphagia (increased food intake), whereas lesioning of the lateral hypothalamus resulted in hypophagia (decreased food intake). This implied an intrinsic role for specific hypothalamic nuclei in energy homeostasis: the arcuate (Arc) and ventromedial (VMN) nuclei located in the ventromedial hypothalamus, the dorsomedial (DMN) and paraventricular (PVN) nuclei, alongside the more dispersed lateral hypothalamic
(LH) area. From these initial breakthroughs, continuing research over last half a century has shown that it is not only the specific hypothalamic nuclei that control “the need to eat”, but that neuronal circuits within, between and impinging on them, are also central to the response.

One nucleus known to play an important role in coordinating energy homeostasis via these specific neuronal circuits is the Arc (Fig 1.1). Circulating hormonal signals indicating energy status (i.e. leptin, insulin and ghrelin) can communicate directly with the brain (Broadwell RD 1976) and act on at least two distinct neuronal populations in the Arc (Fig 1.1) (Cone RD 2001). One population co-expresses the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Hahn TM 1998), and the second expresses anorexigenic peptides derived from pro-opiomelanocortin (POMC), as well as cocaine- and amphetamine-regulated transcript (CART) (Kristensen P 1998). These two populations act in a mutually antagonistic way; when one is activated, the other is inhibited, promoting net changes in the Arc’s influence on downstream “feeding-related” targets in the brain.

Both of these neuronal populations project to a number of second-order nuclei involved in the control of energy homeostasis, such as the PVN, VMN and LH. The LH contains two neuronal types that express melanin-concentrating hormone (MCH) or orexins (A and B); two peptides which increase food intake when administered centrally (Fig. 1.1) (Qu et al. 1996, Haynes AC 1999). The PVN is a complex structure containing many autonomic and neuroendocrine outputs from the hypothalamus, including three neurone types that contain the peptide transmitters corticotrophin-releasing hormone (CRH), thyrotrophin-releasing hormone (TRH) and oxytocin (OXT) (Wynne et al. 2005). However, this is a gross oversimplification in that many of these “second-order nuclei” are directly responsive to circulating factors (Balthasar et al. 2004, Dhillon et al. 2006). This simplified model has arisen mainly through a lack of knowledge regarding the identity of the neuronal populations that constitute nuclei such as the DMN and VMN. Notwithstanding, there is strong emerging evidence that neurones of the VMN, which contain pituitary adenylate cyclase activating polypeptide (PACAP) or brain-derived neurotrophic factor (BDNF), are a direct target for circulating metabolic hormones and are involved in energy homeostasis (Xu et al. 2003, Komori et al. 2006, Hawke et al. 2009).
If appetite was controlled exclusively by homeostasis, the majority of the population would be at optimum body weight, and would rate the pleasurable feeding alongside other physiological processes, such as breathing. However, certain foods are more palatable than others, and their consumption is more strongly reinforced. Humans are often motivated to pay high prices for tasty food, suggesting that if money is equivalent to work expended, then eating is rewarding. Furthermore, the rewarding nature of food is not exclusive to humans, as most mammals will eat highly-palatable food beyond their homeostatic needs (Saper et al. 2002).
1.2 The Behavioural Control Column

The hypothalamus has many other functions besides energy homeostasis, including temperature regulation, blood pressure, electrolyte balance, mood expression and behaviour (Bear MF 2001). Therefore in the context of motivated behaviours, the hypothalamus is thought not to be an isolated entity made from discrete one-function nuclei, but instead as containing the fundamental drivers that coordinate information from both the peripheral senses and higher cognitive areas. The hypothalamus is thought to integrate this information in order to select the most appropriate behavioural/motor response (Swanson 2000). This might include secretion of corticosteroids into the blood in response to fear, or increase motor activity to enhance the chance of finding food in times of starvation.

Based on neuro-development, gene expression, circuit connectivity, and neuronal function studies, the architecture underlying motivated behaviours has been encapsulated in a model proposed by Swanson (Swanson 2000). Swanson (2000) suggests that forebrain structures controlling motivation, communicate their influence to a “behavioural control column” (BCC) which is headed by hypothalamic nuclei (Swanson 2000). The BCC processes sensory, behavioural and cognitive input from the brain, and outputs its response to the hindbrain and spinal cord motor pattern generators, which govern the motorneurone pools innervating peripheral muscle and endocrine glands (Fig 1.2a) (Swanson 2000).

The major divisions of the BCC constitute a rostral segment containing nuclei involved in feeding, reproductive and defensive behaviours, and a caudal segment involved in general foraging and exploratory behaviours (Fig 1.2b). Within the rostral segment reside the main hypothalamic controllers of food intake described in described in Chapter 1.1, such as the PVN, DMN, VMN and Arc.

Motivational systems are triggered by specific signals such as osmotic imbalance, threatening stimuli and energy defects. These signals impinge on the BCC and initiate (and also terminate) the most appropriate behavioural/motor response. These signals input into the BCC in multiple ways (Fig 1.3). For instance, sensory information regarding food can input either directly via the retina or indirectly via olfactory, visceral, and oropharyngeal relay nuclei. Metabolic and hormonal signals (such as circulating levels of leptin, glucose,
insulin, angiotensin), can input their influence to the BCC via the Arc; and finally circadian influence can input via the suprachiasmatic nucleus.

A critically important input to the BCC is via the cerebral cortex (Fig 1.3). The cerebral cortex sends extensive direct and indirect afferents through diverse areas such as the hippocampus, amygdala, prefrontal cortex and striatum, allowing these areas to influence the BCC. In addition, the BCC also project back to the cerebral cortex either directly or indirectly via the dorsal thalamus. This feed forward hypothalamic projection to the cerebral cortex indicates the precedent for intimate connections between the basic motivational networks of the hypothalamus and the higher cognitive brain centres (Swanson 2000).

Despite an ever increasing awareness that reward and motivation play an integral role in feeding behaviour, the majority of feeding studies are limited to the hypothalamus. The above model proposed by Swanson et al (2000) therefore offers a mechanism by which cognitive circuits involved in reward and motivation can impinge on the hypothalamic control of feeding behaviour.

### 1.3 Food and Reward in the Brain

In contrast to the “need to eat”, it is conceivable that evolutionary pressure has resulted in a “wish to eat”. This allows over eating in times of plenty, or specific selection of highly-nutritious food in times of need. One of the most potent ways appetite achieves this is by eliciting a reward for food consumption; e.g. a highly satisfying palatable meal. The textures and tastes are most enjoyable when one is hungry compared with being satiated, suggesting an interaction between the “need” and “wish” to eat. It is the rewarding nature of food that is proposed to underlie the obesity epidemic seen throughout the western world, where high-energy foods are readily available and where there is little need for exercise.

Reward in the brain has been studied predominantly in the context of drug abuse, as addiction leads to reinforcement of responses/behaviours without homeostatic value. Studies have shown that drug reward may share some neural substrates with food reward, in particular, a series of interconnecting circuits linking the prefrontal cortex, amygdala, nucleus accumbens (Acb), ventral pallidum (VP) and ventral tegmental area (VTA), via a ‘neuronal super highway’ termed the medial forebrain bundle (Saper et al. 2002, Kelley et al. 2005). A possible way to further understand the onset of obesity is to view over eating as an
Chapter 1 – General Introduction

Figure 1.2 a) Model of the hierarchical motor system depicting the sensory, behavioural, and cognitive inputs on the BCC, and the hierarchical representation of the motor output (Swanson 2000). b) Horizontal overview of the rostral and caudal segments of the BCC in rat brain (Swanson 2000). Abbreviations: AHN, anterior hypothalamic nucleus; BCC, behavioural control column; DMN, dorsomedial nucleus; MAM, mammillary body; MPN, medial preoptic nucleus; PM, premamillary nuclei; PVN, paraventricular nucleus; SNR, reticular substantia nigra; VMH, ventromedial nucleus; VTA, ventral tegmental area. Figure adapted from (Swanson 2000).

Figure 1.3 Major anatomically defined, direct inputs of the rostral BCC which control feeding behaviour. There are four functionally defined classes of direct input into the BCC which initiate feeding behaviour. 1) Visceral and oropharyngeal sensory information from the glossopharyngeal (IX) and vagus nerves (X); 2) cognitive information from the cerebral hemispheres, funnelled through the striatum particularly the nucleus accumbens (Acb); 3) circadian information from the suprachiasmatic nucleus (SCH) and finally 4) humoral sensory information relayed from the blood circulation or possibly via circumventricular organs (Swanson 2000). Abbreviations: PFR, prefrontal cortex; AMY, amygdala; HIP, hippocampus; SFO, subfornical organ; NTS, nucleus tractus solitarius.
addictive behaviour and to focus research on how food reward is represented in the brain and how it influences feeding behaviour.

Although it is inherently logical that homeostatic and reward/cognitive circuits interact to control food intake, very little is known about how the two systems communicate. It is possible that rewarding brain circuits interfere with the upper adiposity limit set by the hypothalamus, a concept introduced by Keesey et al. (1979), whereby the hypothalamus imposes and regulates a set point in body weight (Keesey et al. 1979). However, it seems evident that understanding the influence of the rewarding/cognitive brain on the BCC is key to further understanding obesity. The sections below outline key experimental evidence implicating reward/cognitive brain regions as an influence on food intake, and possibilities of how they confer this influence onto the BCC.

1.3.1 Prefrontal Cortex and Amygdala

The prefrontal cortex, in particular the orbitofrontal cortex (OFC) (Fig 1.4a), is involved in the conscious experience of both the sensory properties of food (temperature, gustatory/visual cues, nutritional value, viscosity), and its hedonic impact or valence. Early studies showed that monkeys will work for electrical stimulation of the OFC when hungry, but not when satiated (Mora F 1979). In addition, OFC neurones show a decrease in electrical activity once fed to satiety, suggesting an involvement in the hedonistic reward value of taste (Rolls et al. 1989).

Human functional magnetic resonance imaging (fMRI) studies show olfactory stimulus intensity to be associated with amygdala activation, but the valency of the stimulus to be associated with OFC activation (Anderson AK 2003). This suggests that the OFC may represent the hedonic dimensions of pleasantness and unpleasantness (Anderson AK 2003). A subsequent study showed that subjective unpleasantness ratings of odours are associated with activity in the left and lateral OFC, whereas subjective pleasantness ratings of odours are associated with activation of the medial OFC, indicating a dissociable representation in the OFC of the hedonistic valency of olfactory stimuli (Rolls et al. 2003).
Figure 1.4 a) Human MRI scan showing a sagital section through the centre of the brain (OFC highlighted in green) (Wicks P 2006). b) fMRI study showing areas of BOLD activity (orange) in the OFC in coronal section that responds to the subjective pleasantness ratings of food rewards (Kringelbach ML 2003) 

Abbreviations: MRI, magnetic resonance imaging; fMRI, functional magnetic resonance imaging; BOLD, blood oxygen level dependent; OFC, orbitofrontal cortex.

A further human fMRI study showed a significant correlation between activation of the OFC and ratings of subjective pleasantness when a whole food was eaten to satiety (Fig 1.4b) (Kringelbach ML 2003). This sensory-specific activation of the OFC, correlating with subjective pleasantness, strongly indicates that the reward value of the somatosensory, gustatory and olfactory components and the subjective pleasantness of a particular food may be represented in the OFC.

The OFC and the basolateral/basomedial amygdala, (central to learning and emotion (Aggleton 1993)), have also been implicated in complementary roles of learned food experiences, such as an avoidance of certain foods, in order to guide beneficial feeding behaviour (Berthoud 2007). Food intake can be elicited in both hungry and satiated rats by repeated pairing of food presentation with a conditioned stimulus such as a light pulse (Weingarten 1983). Once this paired conditioned response is learnt, this behaviour can be attenuated by devaluing the food with lithium chloride (LiCl), making the paired conditioned response occur less frequently (Weingarten 1983). Rats lesioned in either the OFC or amygdala before conditioned learning show no impairment of their abilities to learn the task, however they are no longer capable of devaluation of the conditioned response with LiCl (Hatfield et al. 1996, Gallagher M 1999). Interestingly, when these lesions were placed after conditioned feeding behaviour was learnt, only lesions in the OFC and not amygdala, resulted in the attenuation of the conditioned response. This suggests that although the amygdala is critical to learning incentive values of food in response to experience, it is the
OFC that maintains and modifies these representations and influences the intake of beneficial foods (Pickens et al. 2003).

The mechanisms by which the OFC and amygdala exert their effects on food intake are unclear. One possible route, determined by retrograde tracing, shows that the OFC sends dense projections via the medial forebrain bundle to several hypothalamic nuclei, including the Arc, sub-PVN, DMN and LH, indicating a possible direct route to the hypothalamus (Berthoud 2002). In addition dense projections are described between the OFC and the basolateral/basomedial amygdale, which then projects heavily to the LH (Petrovich et al. 2005)(Berthoud 2002). This amygdala-hypothalamic connection appears to be able to influence feeding behaviour learnt during conditioning tasks, as severing this connection in rats trained to associate a conditioned stimulus with food presentation completely abolished the conditioned response (Petrovich et al. 2002). However, baseline feeding was not affected, suggesting that this connection is critical for this specific type of conditioned feeding behaviour to occur (Petrovich et al. 2002).

### 1.3.2 Nucleus Accumbens

The Acb is situated in the ventromedial striatum and is made up of two distinct regions, the shell and the core. The Acb has long been proposed to play a role in motivation and reward; possessing a convergence of inputs from the VTA, amygdala, prefrontal cortex and hippocampus and forming a major part of the mesolimbic system (Moore RY 1978, Corbit LH 2001). The Acb has also been implicated in goal-seeking behaviour which drives addiction and abuse of recreational drugs, such as cocaine, nicotine, and alcohol (Baldwin AE 2000, Berthoud 2002).

The Acb is of particular interest to feeding behaviour as its modulation, by selective receptor blockade or activation, induces robust feeding responses driving preferential consumption of highly-palatable foods (e.g. sucrose) in rats, even when they are satiated (Stratford et al. 1999, Saper et al. 2002, Zhang M 2002). Blockade of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and kainate glutamate receptors with 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Maldonado-Irizarry et al. 1995), or activation of GABA_A receptors with muscimol (a GABA_A receptor agonist), in the accumbens shell (AcbSh), but not core (AcbC), significantly increases feeding in rats, suggesting different functional roles for the shell and the core in the context of feeding behaviour (Basso AM 1999).
The shell and the core also differ in connectivity. Generally, the Acb receives dopaminergic input from the substantia nigra (SN) and VTA, serotonergic input from arousal nuclei such as the dorsal raphé, and glutamatergic projections from the amygdala and prefrontal cortex (Fig 1.5) (Canteras et al. 1995, Groenewegen et al. 1999). The Acb output is via GABAergic medium spiny neurones, both directly to the LH, and indirectly via the VP (Fig 1.5) (Kelley et al. 2005). In return, the LH sends a reciprocal projection (possibly via MCH-expressing neurones) back to the AcbSh (Saito et al. 2001, Saper et al. 2002).

This accumbens-hypothalamic connection appears to be central to AcbSh-mediated feeding behaviour. Inhibition of the LH by microinjections of muscimol completely abolishes the hyperphagic effects of glutamate receptor blockade in the AcbSh (Stratford & Kelley 1999). Furthermore, intra-AcbSh administration of muscimol induces c-Fos immunoreactivity in neurones co-expressing the orexigenic peptides orexin and NPY, in the LH and Arc respectively, whilst decreasing c-Fos in Arc neurones co-expressing POMC and CART (Zheng et al. 2003, Baldo BA 2004). This suggests that the AcbSh potentially influences food intake by removing a tonic inhibitory tone on specific neuropeptide feeding circuits within the hypothalamus (Kelley et al. 2005).

As intra-LH injection of GABA\textsubscript{A} receptor antagonist does not affect feeding behaviour, the putative inhibitory tone exerted by AcbSh efferents may synapse at a site interposed between the AcbSh and the LH (Stratford & Kelley 1997). One possible key intermediate could be the VP, as it receives direct GABAergic input from the AcbSh, and sends potentially excitatory projections to the LH (Stratford et al. 1999, Stratford TR 1999, Kelley et al. 2005). Importantly, blockade of GABA\textsubscript{A} receptors in the VP results in a marked hyperphagic response suggesting an integral role of the VP as a key intermediary in this functional pathway (Stratford et al. 1999).

With reference to the BCC (described in Chapter 1.2), it is possible that the AcbSh, via its influence/projections to the LH, may have unique access among the basal ganglia to the feeding related parts of the BCC. The AcbSh may, therefore, act to negatively modulate the BCC. When this inhibitory tone is artificially released by pharmacological inhibition the AcbSh output, the activation threshold for the feeding-specific aspects of the BCC is decreased, thus, biasing an animal towards feeding behaviour even when homeostatic needs are met (Kelley et al. 2005).
Figure 1.5 Translating motivation into behaviour via cross talk between the hypothalamus and reward circuits. The simplified schematic highlights the involvement of the Acb, VP, amygdala and prefrontal cortex in the control of food intake, and how these are able to communicate with the hypothalamus and BCC. Neurotransmitters involved in the functional communication between areas are also highlighted. The dotted line represents feedback to the cortex from the outcome of the behavioural action. Abbreviation: Acb, nucleus accumbens; BCC, behavioural control column; GABA, γ-aminobutyric acid; GLUT, glutamate; LH, lateral hypothalamus; OFC, orbitofrontal cortex; SN, substantia nigra; VP, ventral pallidum; VTA, ventral tegmental area.
This implies a possible functional gateway by which reward and cognitive circuits can influence the hypothalamic driving force directing feeding behaviour. As already mentioned, recent research into food and reward has led to the concept that reward can be split into two discrete psychological components termed “liking” or conscious pleasure, and “wanting” or incentive salience (Berridge KC 2003).

### 1.3.3 “Liking” or “Conscious Pleasure” (A Role for Opioid Transmission)

The subjective affective reaction termed ‘liking’, or ‘conscious pleasure’, allows animals to engage in feeding behaviours that are beneficial and avoid feeding behaviours that are detrimental to survival (Berridge KC 2003). Liking can be determined by assessing orofacial expressions elicited in response to tastes **(Fig 1.6)** (Steiner et al. 2001). For instance, administration of sweet tastes (e.g. sucrose) elicits positive facial “liking” expressions, whereas administration of bitter tastes (e.g. quinine) elicits a “disliking” expression; homologous features in humans, primates, and rodents (Berridge 2000, Steiner et al. 2001). Although the neuronal pathways necessary for taste perception are well characterised, the mechanisms underlying this hedonic ‘liking’ are less understood (Berridge KC 2003).

One key component of ‘liking’ appears to involve opioid neurotransmission, particularly within the striatum (Berthoud 2007). It has long been known (even before the discovery of endogenous opioids) that opioids play a role in food intake (Cota et al. 2006). Several experiments have shown systemic administration of opioid agonists and antagonists to increase and decrease food intake, respectively (Glass MC 1999, Hayward et al. 2002, Barbano MF 2006, Olszewski & Levine 2007). Opioids and opioid receptors are expressed widely throughout the brain, including the hypothalamus, brainstem, amygdala and striatum (Pert et al. 1976). The striatum shows predominantly high β-endorphin, β-endorphin, and mu-opioid receptor mRNA levels, with particularly dense expression within the Acb (Cota et al. 2006). Injections of morphine directly into different regions of the striatum show that the most robust increases in feeding are elicited following intra-Acb administration, implicating its involvement in opioid-induced feeding behaviour (Kelley AE 2002, Pecina et al. 2006).
The opioid effect on food intake appears to be mediated by the mu-opioid receptor. Intra-AcbSh injections of the mu-receptor agonist DAMGO significantly increases chow intake compared with administration of delta and kappa receptor agonists (Kelley et al. 2005), and increases intake preferentially of high-fat over high-carbohydrate chow (Zhang & Kelley 2000, Will et al. 2003, Kelley et al. 2005). It also increases facial “liking” reactions (Pecina & Berridge 2000) and, opposite to the GABA\(\alpha\) agonist muscimol, increases progressive ratio responding during operant learning tasks for sucrose pellets (Kelley et al. 2005). These studies indicate that opioid transmission influences feeding behaviour by increasing the “liking” aspect of reward, resulting in preferential intake of highly nutritious food.

Initial research into reward mechanisms in the brain led to the belief that ‘liking’ or conscious pleasure was mediated by dopamine in the mesolimbic system (Berridge KC 2003). However, this has proved not to be the case. Instead, the mesolimbic dopaminergic system appears to be involved in a totally dissociable aspect of reward, termed “wanting”, or incentive salience (Berridge KC 2003).

### 1.3.4 “Wanting” or “Incentive Motivation” (A Role of Dopamine Transmission)

The dopaminergic system, in particular the mesolimbic dopaminergic neurones arising in the VTA and projecting to the Acb, has long been known to play an important role in the

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**Figure 1.6** Examples of a) ‘liking’ and b) ‘disliking’ reactions. Expressions of liking (sucrose) and disliking (quinine) are homologous in infant humans, orangutans, and rats (Berridge 2000).
reinforcing and reward properties of both drugs and food (Balleine B 1994, Saper et al. 2002, Wynne et al. 2005). Mice which lack dopamine, due to an absence of the tyrosine hydroxylase gene, become fatally hypophagic (Szczypka et al. 2001). These mice resume feeding following dopamine replacement into the caudate putamen (CPu) and Acb (Szczypka et al. 2001). Interestingly, dopamine replacement solely into the CPu restores feeding, although no preference is shown for highly palatable foods. Replacement in both the CPu and Acb replenishes food preference suggesting an integral combined role of the CPu and Acb towards dopaminergic input in food reward (Szczypka et al. 2001).

“Liking” expressions for sweet tastes are unaffected by modulation of the mesolimbic dopamine system (Berridge KC 1998, Pecina & Berridge 2000, Wyvell & Berridge 2000, Robinson & Berridge 2003, Will et al. 2003). Even extensive lesioning of the mesolimbic dopaminergic system with 6-hydroxydopamine, although resulting in a profound aphagia, is incapable of affecting ‘liking’ responses in rats following a sweet taste (Berridge KC 1998). This research suggests that although the dopaminergic system plays a role in reward, it is not involved in “liking” the reward. Initial evidence showed that following instrumental training for a cue-triggered sucrose reward, intra-AcbSh administration of amphetamine (inhibits dopamine reuptake at synapses) increases cue-elicited lever pressing, without affecting the “liking” reactions for a sucrose reward (Wyvell & Berridge 2000). This research suggests that although dopamine is not involved in hedonic “liking”, Acb dopamine controls the ability of reward cues to trigger the “wanting” or incentive salience for their associated rewards.

BCCs for motivated behaviours, such as feeding, are regulated by cascading projections from the telencephalon (Swanson 2000). The BCC contains elements of the brainstem which control the mechanics of feeding and is headed by the hypothalamus. Projections from the cortex to the BCC send collaterals to the striatum, whose outputs send collaterals to the BCC. This cortico-striatal-hypothalmic circuit offers a possible link in which reward and motivational circuits can impinge on hypothalamic control of feeding homeostasis, by interfering with the upper adiposity set limits determined by the hypothalamus (Keesey et al. 1979).

Modulation of this link could, therefore, provide a further insight into how the brain coordinates complex feeding behaviour and help refine future potential targets for anti-obesity therapies. One such potential modulator of this link between reward motivation and
feeding behaviour is a relatively recently discovered neurotransmitter system termed the cannabinoid system (Cota et al. 2006).

1.4 Cannabinoids

*Cannabis sativa* is a flowering herb found throughout the world’s temperate and tropical zones. Cannabis cultivation has occurred for over 6000 years for utilisation as both a textile fibre and to provide extracts for medical and recreational use (Green 2002). Over the past century, this herb, and in particular its buds, has become the western world’s most used illicit drug (Aggarwal *et al.* 2009). Furthermore, despite cannabis prohibition, scientific and social communities have investigated its apparent therapeutic potential for almost 50 years (Burstein 1997, Di Marzo *et al.* 1998). Investigations into the active components of cannabis led to the identification of the chemical Δ⁹-tetrahydrocannabinol (Δ⁹-THC) as its main psychoactive component (Mechoulam & Gaoni 1965). However, the definitive breakthrough into the integral importance of the cannabinoid system arose by the discovery of endogenous cannabinoid receptors and endogenous ligands.

1.4.1 Cannabinoid Receptors

Using a reverse pharmacological approach, Δ⁹-THC binding was used to identify and clone the first cannabinoid receptor, termed cannabinoid receptor type one (CB₁), in 1990 (Matsuda *et al.* 1993), and the second receptor type (CB₂) three years later (Munro *et al.* 1993). The receptors are encoded by the *CNR1* and *CNR2* genes, respectively (Mackie 2008).

Cannabinoid receptors are distributed throughout the body. Typically, CB₁ is widely distributed in the central nervous system (CNS), and has long been considered the ‘brain-type’ cannabinoid receptor (Matsuda *et al.* 1993). CB₂ is mainly expressed in immune cells, and was previously considered to be its ‘peripheral’ counterpart (Munro *et al.* 1993). However, recent studies have shown CB₁ to be present in the periphery (Cota *et al.* 2006) and CB₂ to be present in brain-derived immune cells (Porter & Felder 2001).

CB₁ and CB₂ belong to the seven trans-membrane G protein-coupled receptor super-family (Howlett 1998). CB receptors use the G-inhibitory (Gᵢ/ₒ) protein subunit to induce (1) inhibition of adenylate cyclase; (2) activation of several types of K⁺ channels; (3) decreased opening of voltage gated Ca²⁺ channels; and (4) modulation of signal-regulated kinases
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(Howlett 2005, Dalton et al. 2009). CB$_1$ is considered to be the most abundant G protein-coupled receptor in the mammalian brain, with a density comparable to that of γ-aminobutyric acid (GABA) or glutamate receptors (Freund et al. 2003).

Using autoradiography binding studies, Herkenham et al (1991) found that CB$_1$ receptors are heterogeneously expressed through the brain. There are high levels of specific binding in the basal ganglia and its outflow nuclei (the SN and globus pallidus), the cerebral cortex, hippocampus and cerebellum, and with moderate to low levels in the ventral striatum and hypothalamus (Herkenham et al. 1991).

Numerous anatomical and electrophysiological studies have provided evidence for presynaptic localisation of CB$_1$ in various brain regions and on the axon terminals of several distinct cell types (for review, see (Freund et al. 2003)). In the amygdala, hippocampus, and neocortex, CB$_1$ is highly expressed on axon terminals of GABAergic interneurones expressing CCK. In the basal ganglia, CB$_1$ mRNA is co-localised in neurones positive for GABA, prodynorphin and proenkephalin, and GABAergic interneurones containing parvalbumin. CB$_1$ is also present on glutamatergic neurones in the hypothalamus (Freund et al. 2003). Due to the presynaptic localisation of CB$_1$ in the CNS, this receptor is placed to modulate transmission of several neurotransmitters, including GABA, dopamine, noradrenaline, glutamate and serotonin (Schlicker & Kathmann 2001).

The varied behavioural effects of Δ$^9$-THC administration (analgesia, euphoria, impaired cognitive function and hyperphagia) are thought to be mediated by the CB$_1$ receptor, as receptor expression is present in many of the brain areas implicated in these functions (Navarro et al. 1995, Witkin et al. 2005, Moreira & Lutz 2008, Cooper & Haney 2009). This theory is supported by further studies describing an absence of these behavioural effects in CB$_1^{-/-}$ mice (Valverde et al. 2005).

1.4.2 Endocannabinoids and Cannabinoid Ligands

The identification of specific receptors mediating the actions of Δ$^9$-THC initiated a great deal of interest to pursue the endogenous CB$_1$ ligands. The first endogenous cannabinoid, anandamide, was discovered from the porcine brain in 1992 (Devane et al. 1992). Anandamide is a polyunsaturated fatty acid derivate, which binds to both CB$_1$ and CB$_2$ receptors (with higher affinity for CB$_1$ (Felder et al. 1995)), and is endogenously expressed in
highest concentrations in the hippocampus, cortex, thalamus, and cerebellum (Felder et al. 1996). Importantly, anandamide is capable of producing many of the behavioural effects of $\Delta^9$-THC, and shares the same G protein-mediated actions on adenylate cyclase and Ca$^{2+}$ channels, and yet the two cannabinoids are not structurally related (Di Marzo et al. 1998).

Since the discovery of anandamide, several other cannabinoids have been characterised, such as 2-arachidonoylglycerol (2-AG), noladin ether and virodhamine (Hanus & Mechoulam 2010). As these ligands act as functional agonists at cannabinoid receptors, they have been collectively termed endocannabinoids. Due to the high lipophilicity of endocannabinoids, they appear not to be stored in synaptic vesicles as with ‘classical’ neurotransmitters, but instead are thought to be produced de novo or ‘on demand’ through phospholipid precursors formed from the cell membrane (Di Marzo et al. 1998). For example, anandamide is derived from the membrane phospholipids, N-arachidonoyl phosphatidyl ethanolamine, following cleavage of the phosphodiester bond by a Ca$^{2+}$ activated-phospholipase D (Di Marzo et al. 1998). The ability of endocannabinoids to be released on demand further emphasises a neuromodulatory role in neuronal transmission.

Despite this fundamental difference, endocannabinoids act like ‘classical’ neurotransmitters in that they are released into the synaptic cleft following both membrane depolarization and Ca$^{2+}$ influx, and are inactivated by reuptake (Di Marzo et al. 1998). Although endocannabinoid deactivation mechanisms are unclear, there appears to be an integral hydrolysis step catalyzed by the enzyme fatty acid amide hydrolase (FAAH) and monoglyceride lipase (Dinh et al. 2004, Petrosino & Di Marzo 2010).

Endocannabinoids make up only one of four cannabinoid ligand classes. Various synthetic ligands have been produced, all of which exhibit different affinities and potencies in cannabinoid receptor activation (Hanus & Mechoulam 2010). Synthetic ligands which are structurally related to $\Delta^9$-THC, such as HU-120, are termed ‘classical’ cannabinoids (Mechoulam et al. 1988). “Non classical” cannabinoids, such as CP-55940, comprise bi- and tricyclic analogy to $\Delta^9$-THC (Howlett et al. 1990). The final class consists of aminoalkyloids, the most notable of which is WIN-55,212 (for review, see (Cota et al. 2006)).

Pharmacological manipulation of the endocannabinoid system has placed emphasis on the development of specific antagonists of cannabinoid receptors (Rodriguez de Fonseca et al. 2010).
To date, most studies employ Rimonabant, a Sanofi-Avensis compound previously known as SR 141716A, and a derivative called AM251. Both compounds are highly CB₁ specific (Rinaldi-Carmona et al. 1994). It is important to note that these compounds are considered to be ‘inverse agonists’ as opposed to ‘pure’ antagonists; the difference being that unlike antagonists, which block the effects of other ligands by simply occupying the receptors, inverse agonists bind to the receptor but have the opposite intracellular effects. As the CB₁ receptor is constitutively active, inverse agonists can have an opposing action when administered alone (Porter & Felder 2001, Rodriguez de Fonseca et al. 2005).


### 1.4.3 Physiological Role of the Cannabinoid System in Appetite

The earliest reference to cannabis and appetite is found as early as 300AD in the Hindu Raja Nirghanta, which proposes cannabis smoking as a remedy for appetite loss (Abel 1975). The hyperphagic actions of cannabis are widely acknowledged, and users are often quoted referring to “the munchies”; the experience of a voracious appetite and increased food enjoyment. This effect was explored by the US military in 1933, who reported that soldiers “high” on cannabis possessed an “intense hunger” and ate more food than controls (Siler et al. 1933).

It was not until 1971 that the first, scientifically controlled study on cannabis and feeding was undertaken (Hollister 1971). Standardised doses of Δ⁹-THC were administered to volunteers in either fed or fasted conditions. In both states, the treated group ate more than control, although the effect was only significant in the fed condition, suggesting that Δ⁹-THC induced hyperphagia may involve non-homeostatic mechanisms (Hollister 1971). This is
supported by a further human study showing that smoking cannabis not only induces hyperphagia, but also increases the desire to heat highly-palatable food (Abel 1975).

In animal studies, hyperphagia was demonstrated following administration of $\Delta^9$-THC or anandamide in animals given palatable food (Williams & Kirkham 1999, Koch 2001). Furthermore, increased motivation for sucrose intake and beer consumption was observed in rats following administration of cannabinoid CB$_1$ specific agonists (Gallate et al. 1999). Selectively blocking the CB$_1$ receptor with Rimonabant reduces the motivation for sucrose, beer and alcohol intake, indicating that incentive and/or motivational processes could be under a permissive control specifically by CB$_1$ action.

1.4.4 Cannabinoids and Reward

As discussed in Chapter 1.3, overeating may be viewed as an addictive behaviour. The underlying mechanisms are unclear, although they may involve the “hijacking“ of the brain reward systems. Limbic levels of dopamine and endocannabinoids strongly correlate with cravings for palatable food, indicating a potential co-modulatory role with dopamine in reward-driven feeding (Tanda & Goldberg 2003). Also, the co-expression of serotonin receptors 5-HT$_{1B}$ with CB$_1$ (particularly in the CPu and Acb) points to interaction between the serotoninergic and endocannabinoid systems (Hermann et al. 2002).

In areas of the limbic forebrain, CB$_1$ mRNA expression is down regulated in rats fed a palatable diet (Harrold et al. 2002). This effect could be explained by increased limbic anandamide levels shown following consumption of a high-energy diet (Berger et al. 2001). In addition, fasting increases levels of endocannabinoids, specifically in limbic regions (Kirkham et al. 2002), and intra-AcbSh injection of 2-AG causes potent hyperphagia (Kirkham et al. 2002). These effects on feeding reward motivation appear to be mediated specifically via the central CB$_1$ receptor, as hyperphagia induced by anandamide in prefed rats can be greatly attenuated by pre-treatment with Rimonabant (Williams & Kirkham 1999). In addition, CB1$^{-/-}$ mice, which express a lean and hypophagic phenotype, show reduced sensitivity to the rewarding properties of sucrose in both fixed- and progressive-ratio schedules, indicating an integral role of the CB$_1$ receptor in food reward (Sanchis-Segura et al. 2004).
The binding of CB₁ receptors by inverse agonists, plays a modulatory role in the suppression of drug abuse relapse, as Rimonabant administration is capable of attenuating cue-induced reinstatement of drug-seeking behaviour (De Vries & Schoffelmeer 2005). In addition to drug reinforcers, such as heroin, cocaine and alcohol, this effect is also observed in response to natural reinforcers (De Vries and Schoffelmeer, 2005). For instance, using a reinstatement paradigm like the one used for drug reinforcers, Rimonabant attenuated cue-elicited sucrose seeking following long-term extinction (De Vries & Schoffelmeer 2005). Moreover, Rimonabant administration attenuates operant responding in rats for the acquisition of palatable food (Arnone et al. 1997, Ward & Dykstra 2005), and selectively decreases the intake of palatable food over regular chow (Arnone et al. 1997, Gessa et al. 2006).

An emerging body of evidence suggests that defects in the regulation of the endocannabinoid system may underlie, at least in part, eating disorders and obesity (Butler & Korbonits 2009). Plasma anandamide levels are significantly increased in subjects affected by anorexia nervosa or binge-eating disorder, but not in bulimic subjects (Monteleone et al. 2005). Moreover, a missense mutation of the gene encoding FAAH has been associated with a propensity toward drug abuse and obesity (Sipe et al. 2002, Sipe et al. 2005).

All these studies point to an attractive hypothesis that cannabinoids influence feeding behaviour by modulating brain reward pathways. Whilst this seems to be the case, at least in part, one can not overlook the localisation of the cannabinoid system in the hypothalamus.

1.4.5 Cannabinoids and the Hypothalamus

The hypothalamic nuclei involved in energy homeostasis are under the control of peripheral signals such as leptin and insulin, which can affect appetite, body weight and blood glucose levels (Morton et al. 2006). In this context, it important to note that leptin specifically reduces hypothalamic endocannabinoid levels, without changing expression in other brain regions (Di Marzo et al. 2001). In addition, genetically obese ob/ob mice (deficient in leptin), posses elevated hypothalamic endocannabinoid levels, and show a potent decrease in food intake when administered Rimonabant (Di Marzo et al. 2001). Therefore, the expression of CB₁ in the hypothalamus could provide another layer of control by which leptin signals are interpreted.
Within the hypothalamus itself, the CB\textsubscript{1} receptor protein is expressed within the pre-synaptic nerve terminals on several cell types found within the Arc, PVN, VMN and LH (Wittmann \textit{et al.} 2007). CB\textsubscript{1} mRNA has also been co-localised with several neuropeptides such as CART, CRH and MCH (Cota \textit{et al.} 2003). It therefore seems reasonable to hypothesise that the cannabinoid system not only modulates neurotransmitter release and subsequent signal flow-through within the hypothalamic circuits regulating feeding, but it may also influence the expression of feeding related neuropeptides.

Considering their established role in energy homeostasis and leptin sensing, neither NPY/AgRP nor POMC/CART neurones in the Arc express CB\textsubscript{1} mRNA (Cota \textit{et al.} 2003, Jelsing \textit{et al.} 2008). However, stimulation or blockade of CB\textsubscript{1} does increase and decrease Arc NPY levels, respectively (Allen \textit{et al.} 2003). Furthermore, administration of NPY to CB\textsubscript{1}\textsuperscript{-/-} mice has no effect on food intake (Poncelet M 2003, Ravinet Trillou \textit{et al.} 2004), whereas administration of Rimonabant to NPY\textsuperscript{-/-} mice still decreases food intake (Di Marzo V 2001). This suggests that while NPY is not essential for endocannabinoid modulation of food intake, endocannabinoids and CB\textsubscript{1} receptors are necessary for NPY’s orexigenic actions, and may therefore act downstream of NPY.

The majority of the NPY/AgRP and the POMC/CART projections from the Arc are to the PVN (Berthoud 2002). The PVN itself appears to be sensitive to cannabinoids; intra-PVN injection of \textDelta^9\textsubscript{-}THC produces marked hyperphagia, an effect reversible by Rimonabant (Verty \textit{et al.} 2005). This response may involve synergism between cannabinoids and glucocorticoids. Glucocorticoids in the PVN act via 2-AG to depress glutamatergic and elevate GABAergic neurotransmission onto CRH neurones, resulting in net inhibition of CRH release (Di \textit{et al.} 2005). There is also evidence that cannabinoids may negatively influence CRH expression directly in the PVN, given that CRH neurones in the PVN express CB\textsubscript{1} mRNA (Hermann \textit{et al.} 2002), and CB\textsubscript{1}\textsuperscript{-/-} mice have elevated CRH expression (Cota \textit{et al.} 2003).

Orexin- and MCH-containing neurones in the posterior LH project to the PVN, and may also modulate the inputs from the Arc (Berthoud 2002). Although there is no data as to whether CB\textsubscript{1} and orexin co-localise in the LH, CB\textsubscript{1} is co-expressed with the orexin receptor in the LH, Arc and PVN, and within MCH-expressing neurones at low levels (Matias \textit{et al.} 2008). CB\textsubscript{1} is also expressed on GABAergic neurones in the LH (Cota \textit{et al.} 2003, Matias \textit{et al.} 2008). Therefore, GABAergic inputs onto orexin and MCH neurones may be suppressed upon CB\textsubscript{1}
activation, facilitating orexin and MCH release in response to CB₁ activation. Further relevance of the LH is highlighted since retrograde tracing highlights the Acb as a major source of input (Marchant et al. 2009). As the Acb is highly implicated in the rewarding aspect of feeding, it is plausible to propose that this connection may serve as a bridge between the homeostatic and hedonistic mechanism of food intake.

1.4.6 Cannabinoid Facilitated Cross-talk between Homeostatic and Hedonic Feeding

A potential neural substrate to link reward/motivation with homeostatic mechanisms could involve the actions of cannabinoids on the cortico-striatal-hypothalmic pathway. Injection of cannabinoids into the AcbSh of free-feeding rats results in a marked hyperphagic response, which is open to antagonism by Rimonabant or AM251 (Kirkham et al. 2002, Soria-Gomez et al. 2007). In addition, intra-AcbSh administration of cannabinoids increases c-Fos immunoreactivity in the LH, suggesting the existence of an endocannabinoid/CB₁-mediated functional relationship between the hypothalamus feeding centres and the AcbSh (Soria-Gomez et al. 2007).

GABAergic neurones in the AcbSh receive glutamatergic afferents from the prefrontal cortex, representing a critical link between decision-making parts of the brain and the striatum (Christie et al. 1985, Bennett & Bolam 1994). It is not clear whether glutamatergic input into the AcbSh is predominantly direct onto the medium spiny neurones (MSN) which are the major output projection, or to local GABAergic interneurones, which do co-express mRNAs for glutamic acid decarboxylase and the CB₁ receptor (Hohmann & Herkenham 2000). GABAergic interneurones have a significant inhibitory effect on MSNs (Jaeger et al. 1994, Kawaguchi et al. 1995, Koos & Tepper 1999). Since both glutamatergic and GABAergic synapses within the AcbSh possess CB₁ receptors that can inhibit synaptic transmission (Manzoni & Bockaert 2001, Robbe et al. 2001, Lopez-Moreno et al. 2008), it is possible that the local action of cannabinoids is to reduce activity of local interneurones, leading to disinhibition of MSNs (Kelley et al. 2005).

Cannabinoids may also interact with dopaminergic input to the AcbSh. Administration of CB₁ agonists, both centrally and peripherally, increases extracellular dopamine release in the AcbSh (Lupica & Riegel 2005). The role of dopamine transmission in the AcbSh is well established as a mediator of appetitive motivation (Beninger & Miller 1998, Berridge & Robinson 1998, Horvitz 2002, Salamone & Correa 2002, Wise 2004). Increased dopaminergic
transmission in the AcbSh could therefore lead to an augmentation in “wanting” or motivational salience towards food (Wyvell & Berridge 2000, Wyvell & Berridge 2001, Berridge & Robinson 2003, Berridge 2004).

The AcbSh is the only striatal region to send a direct projection to the LH, suggesting that this region has unique access to hypothalamic feeding centres and may provide a link by which reward and motivational circuits could impinge on hypothalamic control of feeding (Groenewegen & Russchen 1984, Heimer et al. 1991, Kelley et al. 2005). An electrophysiological study has demonstrated that the orexigenic effects of cannabinoids include a presynaptic CB₁ receptor-mediated suppression of GABAergic transmission to postsynaptic LH neurones (Jo et al. 2005). Together, these studies suggest that cannabinoids could exert their orexigenic effects by disinhibiting LH neurones, which impart an orexigenic drive to other regions of the feeding BCC.

1.5 Experimental Approach

A significant part of this PhD thesis investigates the functional sites of action of specific pharmacological agents within the brain. The reliable characterisation and interpretation of “whole-brain” responses requires the complementation of functional brain techniques. In order to achieve this, this PhD complements functional immunohistochemistry with blood oxygen level dependent (BOLD) fMRI. As these techniques act only as indirect measures of neuronal activity, it is therefore important to describe the surrogate nature of each of these techniques.

1.5.1 Functional Immunohistochemistry

Immunohistochemistry is a technique that allows the localisation of specific protein(s) within a cell or tissue sample. The technique exploits the specific binding of antibodies to antigens or specific proteins. This PhD project uses immunohistochemistry to detect the presence of the c-Fos protein (the protein product of the immediate early gene, c-fos), as it is commonly used as a generic marker of neuronal activation (Luckman et al. 1993, St Andre et al. 2007, Kovacs 2008). C-fos expression has become one of the most widely used functional anatomical markers of activated neurones as it is endogenously expressed at low levels under basal conditions, its expression is relatively transient, and it is reliably induced in response to several extracellular signals, including ionic changes, neurotransmitters and drugs (Elias et al. 1999, Rajendren 2002, Zhang & Kelley 2002, Munzberg et al. 2007).
It is important to note that if \( c-fos \) is indeed a simple marker of neuronal activity, then under basal conditions, \( c-fos \) expression would be detected in millions of neurones throughout the brain. Instead, it is thought that \( c-fos \) is expressed in response to changes in afferent inputs or significant changes in neuronal stimulation (Luckman et al. 1994). Therefore, \( c-fos \) expression does not automatically follow neuronal activity, and it is seldom transcribed in response to inhibition. Even if it is, then neuronal inhibition and activation could not be discerned (Luckman et al. 1994, Hughes & Dragunow 1995). Although the kinetics of the \( c-fos \) response to acute stimuli is transient, with a peak of \( c-fos \) mRNA at approximately 30 min and \( c\)-Fos protein between 90–120 min (Fig 1.7) (Kovacs 1998), it lacks the temporal resolution to decipher the time course changes in neuronal activation seen over the period following pharmacological challenge.

![figure 1.7 general temporal profile of c-fos induction](image)

**Figure 1.7** General temporal profile of \( c-fos \) induction, with mRNA expression at 0.5 h and maximal \( c\)-Fos protein expression at 2 h after challenge (Kovacs 1998).

Although \( c\)-Fos immunohistochemistry gives very good spatial resolution and whole-brain coverage, it is important to complement it with other functional techniques in order to aid interpretation and characterisation of whole-brain responses to acute pharmacological stimuli.

### 1.5.2 Functional magnetic imaging

Functional magnetic imaging (fMRI) is a neuroimaging technique that provides neuroscientists a unique window into the brain, allowing visual representation of brain activity changes in response to a vast array of stimuli. fMRI is entirely non-invasive, and offers a spatial resolution within the region of 3-6 millimetres, allowing neuroscientists to observe patterns of neuronal activity across the whole brain with acquisition times of less than one minute.
To understand how fMRI detects neuronal activation, an understanding of the basics of the magnetic resonance (MR) signal is required. The primary sources of the MR signal are hydrogen nuclei (protons) found in water and fat. Each of these nuclei acts like a “spinning top”, with a random orientation in a non-magnetic environment (Fig 1.8a). When the nuclei are placed in a magnetic field they align in parallel formation relative to the direction of the magnetic field. The net magnetism from the aligned nuclei is depicted as a parallel vector (B₀ in Fig 1.8b) to the main external magnetic field. Increasing the strength of the magnetic field in this direction recruits more hydrogen nuclei to conform to the B₀ position giving the image better contrast. As the density of hydrogen nuclei varies between different tissues in the brain, e.g. cerebrospinal fluid, grey and white matter, it gives rise to difference in contrast.

Figure 1.8 Schematic showing the behaviour of hydrogen nuclei in magnetic and non-magnetic environments. a) In non-magnetic field nuclei exist in random spin orientations. b) When subjected to a uniform magnetic field, nuclei align parallel to magnetic field (B₀ depicts the vector of the main magnetic field), in a “low” energy state. c) The nuclei are pulsed with a magnetic field (B₁) perpendicular to that of B₀, which forces the nuclei to align in a temporary “high” energy state.

These aligned hydrogen nuclei exist in a low energy “spin” state. During MR, brief electromagnetic pulses are applied to create a magnetic field B₁, which is perpendicular to the main external magnetic field B₀. The hydrogen nuclei absorb the radiation, elevating them to a higher energy “spin” state, and consequently they flip into the transverse x-y plane. Initially when the nuclei are flipped (Fig 1.8c), all the hydrogen nuclei spin at the same frequency and are thus described to be “in-phase” (Fig 1.9a). This “in-phase” spin initiates an oscillating MR signal than can be detected by the magnet’s radiofrequency probe. The “in phase” spin is short lived (20-50 milliseconds), as interaction between the nuclei and their environment slows some nuclei more than others. This results in “de-phasing” and attenuation of signal strength (Fig 1.9b). Finally, all the nuclei become sufficiently slowed and “relax” back to their original low-energy state (equilibrium), parallel to B₀(Ferris et al. 2006).
There are 3 main constants that can be used to calculate an MR signal, each giving different weightings contrasts. Firstly, the time taken for the nuclei to relax back to their low energy state can be calculated, named T1-weighted imaging (measured in seconds). A second constant, named T2-weighted imaging (measured in milliseconds), relies upon local dephasing of spins following the application of the electromagnetic pulse. The third constant is a subtle, but important variant of the T2 technique, named T2*-weighted imaging. T2*-imaging employs a spin-echo technique, in which hydrogen nuclei are refocused to compensate for local magnetic field inhomogeneities. For this reason, T2*-weighted imaging is used in live-animal imaging as it reduces the possibilities of artefacts caused by physiological motion (i.e. cerebrospinal fluid movement).

There are number of fMRI techniques that measure different responses, including cerebral blood volume and cerebral blood flow. However, this PhD focuses on the BOLD technique. The analogy behind the BOLD technique is that when the brain increases synaptic activity and subsequent neuronal activity, it requires increased oxygen in order to sustain increased metabolic activity (Fig 1.10). The increase in brain activity and metabolic activity is followed by a subsequent increase in blood flow (reactive hyperaemia, Fig 1.10c) (Fox PT 1986). As there is a slight delay between these two events, the enhanced blood flow usually exceeds the neuronal metabolic demand, resulting in the active brain area to be exposed to high levels of oxygenated haemoglobin.

As neuronal activity initially acquires oxygen from haemoglobin, it renders blood haemoglobin de-oxygenated (Fig 1.10b). De-oxygenated haemoglobin is paramagnetic (i.e. creates its own magnetic field), and thus promotes de-phasing of the hydrogen nuclei in the
B₁ plane, resulting in the attenuation of the MR signal (Fig 1.10b). In contrast, when the active brain area is subjected to the subsequent increase in oxygenated blood flow, the nuclei are encouraged to align “in-phase”, which heightens the MR signal (Fig 1.10c). This increase in MR signal is because oxygenated haemoglobin is diamagnetic (i.e. only magnetic in the presence of a magnetic field). In response to decreased neuronal activity, the response is reversed, enabling BOLD fMRI to detect qualitative increases or decreases in regional neuronal activity.

As changes in BOLD response happen rapidly, a gradient spin echo-T₂* weighted image is usually acquired (Ferris CF 2006). In a gradient spin echo sequence, the initial electromagnetic pulse is applied so quickly (∼50ms) that the hydrogen nuclei are only partially flipped into the B₁ plane (∼30°). This results in the nuclei rapidly losing their high-energy magnetism and returning back to equilibrium state. For this reason, a number of short electromagnetic pulses are applied, spaced approximately 50 milliseconds apart. This results in a relatively poor image resolution but high speed image acquisition; an essential feature in order to capture the rapid changes in BOLD activity. This PhD attempts to compensate for the relatively poor spatial resolution of fMRI through complementation with c-Fos immunohistochemistry, described in Chapter 1.5.1.

Figure 1.10 Schematic diagram showing conditions contributing to BOLD signal following neuronal activation. a) Depicts the condition under basal neuronal activity. b) Following neuronal activation the initial “steal” of oxygen from the vasculature results in initial de-phasing and a reduced BOLD signal. c) Neuronal activation then results in reactive hyperaemia (enhanced blood flow and blood volume) leading to enhanced “in-phase” behaviour of the nuclei, and a subsequent increase in BOLD signal. The opposite is true in a decrease neuronal activity and negative bold situation.
1.6 PhD Aims and Objectives

- **To investigate brain areas underlying energy homeostasis, through characterisation of whole-brain responses following glucoprivic doses of a glucose anti-metabolite, in order to identify components of the behavioural control column.**

The glucose analogue, 2-deoxy-D-glucose (2-DG), is an inhibitor of glycolysis and, when administered systemically or centrally, induces glucoprivation leading to counter-regulatory responses, including an increase in feeding. I aim to further define glucostatic brain circuitry through the combination of blood-oxygen level dependent (BOLD) pharmacological-challenge magnetic resonance imaging (phMRI) and whole brain c-Fos functional activity mapping in response to an orexigenic dose of 2-DG. This enables identification of brain areas that show functional responses to a primarily homeostatic stimulus.

- **To investigate endocannabinoid action sites within the brain, using behaviourally relevant doses of CB₁ ligands, in order to define the functional brain response to a primarily hedonic stimulus.**

CB₁ receptors are located primarily presynaptically and, due to the varied strength of downstream receptor coupling in different brain regions, expression studies alone do not provide a firm basis for interpreting endocannabinoid sites of action. Most previous functional studies have used exceptionally high drug dosages which can bias results toward non-relevant adverse or off-target effects, and mask behaviourally-relevant actions. I aim to use doses of a full CB₁ agonist, CP55940, and CB₁ inverse agonist, Rimonabant, that are relevant to feeding behaviour to map responsive brain regions by the complementary techniques of phMRI and immediate-early gene immunohistochemistry.

- **To investigate the influence of the putative novel endogenous CB₁ inverse agonist, hemopressin, on feeding behaviour.**

Hemopressin is a short, nine-amino acid peptide isolated from rat brain that has been shown previously in vitro to behave as a CB₁ inverse agonist, making it the first peptidergic CB₁ receptor ligand to be described. Since this peptide was discovered in the rodent brain, I aim to assess its effects at the CB₁ receptor both in vitro, and in in vivo feeding experiments. Having shown a CB₁-selective reduction in feeding, I will go on to further describe the biology of hemopressin and closely-related peptides.
1.7 Thesis Structure

In accordance with guidance issued by the University of Manchester, this thesis is presented in the alternative format, whereby peer-reviewed research constitutes the main thesis chapters. All experiments were undertaken by Garron T. Dodd under the supervision of Professor Simon M. Luckman, with the exception of the experiment providing results for Figure 3b in Chapter 4, which was undertaken jointly by Garron T. Dodd and Giacomo Mancini under the supervision of Professor Simon M. Luckman and Professor Beat Lutz. The remainder of this thesis is structured as follows:

Chapter 2 - Functional magnetic resonance imaging and c-Fos mapping in rats following a glucoprivic dose of 2-deoxy-D-glucose
Garron T. Dodd, Steve R. Williams, and Simon M. Luckman
Published in Journal of Neurochemistry 2010 Mar 17; 113: 1123-32, PMID 20236391
This study addresses the first aim of the thesis. It provides evidence for an integrated circuit that may be commonly involved in feeding induction, and also illustrates the valuable potential of rodent phMRI in investigating central pharmacological actions.

Chapter 3 - Central cannabinoid signaling mediating food intake: a pharmacological-challenge MRI and functional histology study in rat
Garron T. Dodd, Jennifer A. Stark, Shane McKie, Steve R. Williams and Simon M. Luckman
This study addresses the second aim of the thesis. It provides evidence of a functional cortico-striatal-hypothalamic pathway central to the cannabinoid mediated effects on feeding.

Chapter 4 - The peptide hemopressin acts through CB₁ cannabinoid receptors to reduce food intake in rats and mice
Garron T. Dodd, Giacomo Mancini, Beat Lutz and Simon M. Luckman
Published in Journal of Neuroscience 2010 May 26: 30 (21): 7369-76, PMID 20505104
This study addresses the third aim of the PhD thesis. It provides evidence that hemopressin acts as a functional antagonist at central CB₁ receptors to modulate appetite pathways.

Chapter 5 – Discussion
This chapter provides a final concluding discussion of the research contained within Chapters 2-4; introduces an extended form of hemopressin which acts as a CB₁ receptor agonist to have the opposite biological effect; provides evidence to show that hemopressin is an endogenous peptide; and suggests ideas for future work.
Chapter 6 – Manuscript off-prints
This chapter provides a complete set of off-prints for each of the published articles.

Chapter 7 – References
This chapter provides a complete reference list for all chapters contained within the thesis.
For ease of viewing, individual chapters also contain chapter-specific reference lists.
1.8 References


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Chapter 2

Functional magnetic resonance imaging and c-Fos mapping in rats following a glucoprivic dose of 2-deoxy-D-glucose

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Chapter 2 – Functional magnetic resonance imaging and c-Fos mapping in rats following a glucoprivic dose of 2-deoxy-D-glucose

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2.1 Abstract

The glucose analogue, 2-deoxy-D-glucose (2-DG) is an inhibitor of glycolysis and, when administered systemically or centrally, induces glucoprivation leading to counter-regulatory responses, including increased feeding behaviour. Investigations into how the brain responds to glucoprivation could have important therapeutic potential, as disruptions or defects in the defence of the brain’s “glucostatic” circuitry may be partly responsible for pathological conditions resulting from diabetes and obesity. To define the “glucostat” brain circuitry further we have combined blood-oxygen-level-dependent (BOLD) pharmacological-challenge magnetic resonance imaging (phMRI) with whole brain c-Fos functional activity mapping to characterise brain regions responsive to an orexigenic dose of 2-DG (200 mg/kg; s.c.). For phMRI, rats were imaged using a T2*-weighted gradient echo in a 7T magnet for 60 minutes under α-chloralose anaesthesia, while animals for immunohistochemistry were unanaesthetised and freely behaving. These complementary methods demonstrated functional brain activity in a number of previously characterised glucose-sensing brain regions such as those in the hypothalamus and brainstem following administration of 2-DG compared with vehicle. As the study mapped whole brain functional responses, it also identified the orbitofrontal cortex and striatum (nucleus accumbens and ventral pallidum) as novel 2-DG-responsive brain regions. These regions make up a corticostriatal connection with the hypothalamus, by which aspects of motivation, salience and reward can impinge on the hypothalamic control of feeding behaviour. This study, therefore, provides further evidence for a common integrated circuit involved in the induction of feeding behaviour, and illustrates the valuable potential of phMRI in investigating central pharmacological actions.
2.2 Introduction

Glucose is a vital component of cellular function, and the maintenance of an adequate supply of glucose is of major importance to both central and peripheral tissues in order to support life (Levin et al. 1999, Williams et al. 2001). The constant level of glucose in the blood is regulated homeostatically by the neural and endocrine interplay between the brain and the regulatory mechanisms it controls in peripheral organs, notably the liver and the pancreas (Anand et al. 1964, Oomura et al. 1964, Donovan et al. 1991, Hevener et al. 1997, Cryer et al. 2003). It is likely that disruption or defects in the defence of a brain’s glucostatic “set point” may contribute to a number of pathological conditions, including diabetes and obesity (Levin & Sullivan 1987, Levin et al. 1999). Further understanding of how the brain regulates this glucose set-point could, therefore, have important therapeutic potential.

Research over the past four decades has identified a subset of neurones within the brain that are able to modulate their firing activity in response to changes in extracellular glucose levels (Anand et al. 1964, Oomura et al. 1969, Oomura & Yoshimatsu 1984, Dunn-Meynell et al. 2002, Kang et al. 2004, Levin et al. 2004). These neurones can be split broadly into two populations: those that increase their firing rate in response to elevations in extracellular glucose concentration (termed glucose excited); and those which are activated by a decrease in extracellular glucose concentration or cellular glucoprivation (termed glucose inhibited) (Song et al. 2001, Routh 2002, Thorens 2003, Yang et al. 2004, Burdakov et al. 2005). Both types of neurone are widely distributed in the brain, but are particularly represented in the hypothalamus and the brainstem, regions involved in the control of energy homeostasis and food intake (Oomura et al. 1974, Mizuno & Oomura 1984, Kow & Pfaff 1985, Silver & Erecinska 1998).

Glucose-sensing neurones are thought to act as sentinels of the body’s glucostatic “set point,” so that in times of hypo- or hyperglycaemia, they initiate a constellation of integrated counter-regulatory responses, the most notable of which are modulation of the hypothalamo-pituitary-adrenal axis, glucagon/insulin secretion, and energy intake (DiRocco & Grill 1979, Borg et al. 1995, Hevener et al. 1997, Ritter et al. 2000). The physiological importance of central glucose sensing has been further highlighted in a number of studies whereby peripheral injection of gold thioglucose, which selectively destroys glucose-responsive neurones in brain regions with a restricted blood-brain barrier, results in
impaired feeding regulation and the subsequent onset of obesity (Bergen et al. 1996, Homma et al. 2006).

To understand the brain mechanisms underlying glucose homeostasis, particularly in the context of glucoprivation, many studies have used the glucose anti-metabolite, 2-deoxy-D-glucose (2-DG). 2-DG is an inhibitor of glycolysis and, when administered systemically or centrally, induces glucoprivation leading to counter-regulatory responses, including increased feeding behaviour (Brown 1962, Novin et al. 1973, Miselis & Epstein 1975, Berthoud & Mogenson 1977, Marty et al. 2007). Importantly, the increase in feeding behaviour can be blocked by central, but not peripheral administration of glucose, suggesting that the metabolic receptors responsible for glucoprivation-induced feeding are located primarily within the brain (Singer & Ritter 1996, Burdakov et al. 2005).

Studies with cellular resolution (electrophysiology and c-Fos immunohistochemistry) have demonstrated the existence of 2-DG-responsive neurones in the arcuate (Arc), ventromedial (VMN), paraventricular (PVN), and lateral hypothalamic (LH) nuclei, the nucleus of the tractus solitarius (NTS), parabrachial nucleus (PBN), and regions of the basolateral medulla containing A1/C1 noradrenergic and adrenergic neurones (Ritter & Dinh 1994, Dallaporta et al. 1999, Briski & Sylvester 2001, Yang et al. 2004). However, these studies are restricted to focusing on the above-mentioned nuclei. In an attempt to characterise the whole-brain response to 2-DG-induced glucoprivation and potentially identify connections with the hypothalamus and the brainstem, this study combines blood-oxygen level-dependent (BOLD) pharmacological-challenge magnetic resonance imaging (phMRI) with c-Fos protein functional activity mapping, providing an insight into the whole-brain response to systemic 2-DG administration and the co-ordination of counter-regulatory responses. The study also illustrates the valuable potential of BOLD phMRI in investigating central drug action.

2.3 Materials and Methods

2.3.1 Animals

All experiments were carried out using adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Sandwich, UK). Animals were group housed in The University of Manchester animal unit in a constant environment of 21 ± 2°C and 45 ± 10% humidity, on a 12:12h light-dark cycle with the dark phase commencing at 20:00. Rat chow (Beekay
International, Hull, UK) and tap water were available ad libitum unless stated otherwise. All procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act, 1986 and local ethical review.

2.3.2 Feeding and c-Fos protein immunohistochemistry

Rats (275 ± 20 g; n = 5/6 per group) were housed singly five days prior to the experiment and received food and water ad libitum. During this acclimatisation period, rats were handled daily and food intake monitored. Rats were assigned randomly to receive subcutaneous (s.c.) injections of either vehicle (0.9 % NaCl) or 200 mg/kg body weight (b.w.) 2-DG (Sigma-Aldrich Corp. Ltd., Poole, UK) between the hours of 09:00 and 14:00. All injections were given in a volume of 1 ml/kg body weight. The dose of 2-DG was based on literature (Nonavinakere & Ritter 1983, Tepper & Kanarek 1984, Giraudo et al. 1998) and previous in-house experiments. Food was weighed just before injection and again 90 minutes later before the animals were culled. The animals were deeply anaesthetized with 5 % isoflurane (Concord Pharmaceuticals Ltd., Essex, UK) in oxygen (1 l/min) and perfused transcardially with heparinised saline (10,000 units/l heparin in 0.9 % NaCl), followed by 4 % paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.3). The brains were post-fixed overnight and then kept for two days in 30% sucrose in 0.1 M PB to cryoprotect the tissue, before freezing on dry ice. 30 μm sections (120 μm apart) were cut in the coronal plane throughout the entire rostrocaudal extent of the brain and incubated in 20 % methanol, 0.2 % Triton X-100, 1.5 % hydrogen peroxide for 30 min to deactivate endogenous peroxidases. Sections were then incubated at room temperature for 1 hour in the blocking buffer: 0.1 M PB, 0.3 % Triton X-100, 1 % normal sheep serum, and then overnight at 4ºC in rabbit anti-c-Fos antibody (Oncogene Science Inc., Bayer Healthcare, MA, USA) diluted to 1:10000 in blocking buffer. After washing, the sections were incubated sequentially at room temperature for 1 hour in goat anti-rabbit IgG-biotin complex (Vector Laboratories, CA, USA) diluted 1:500 in blocking buffer followed by avidin-biotin–peroxidase complex (GE Healthcare, UK) diluted 1:500 in PB and, finally, visualized with nickel-intensified diaminobenzidine (Vector Laboratories, UK).

C-Fos immunoreactivity was first examined qualitatively in order to determine areas expressing c-Fos-positive neurones. Areas that showed qualitative changes in immunoreactivity were then analysed blind by counting c-Fos expressing nuclei in the areas
of interest. The areas that showed qualitative changes were the nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), thalamic paraventricular nucleus (PVA), hypothalamic paraventricular nucleus (PVN), arcuate nucleus (Arc), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamus (LH), central amygdala (CeA), periaqueductal grey (PAG), and the nucleus of the solitary tract (NTS). Brain areas were photographed using an Axiovison upright microscope (Zeiss, UK) and an Axiocam colour CCD camera. The number of c-Fos-expressing cell nuclei was quantified in areas defined according to a standard atlas (Paxinos and Watson, 1986). Results are presented as mean and S.E.M. for food intake at 90 min and the number of c-Fos-immunoreactive cells per section in each brain area. Treatments were compared using the two-way unpaired t-test using the Prism statistical package (GraphPad Software Inc, San Diego, CA, USA).

2.3.3 BOLD fMRI

Fourteen rats (260 ± 25 g, n = 7) were assigned randomly to receive subcutaneous injections of vehicle (0.9 % w/v NaCl) or 2-DG (200 mg/kg) (Sigma-Aldrich Corp. Ltd, Poole, UK). Animals were anaesthetized with 2.5% isoflurane (Concord Pharmaceuticals Ltd, Dunmow, Essex, UK) in oxygen (2 l/min) to allow cannulation of a tail vein and subsequent anaesthetic maintenance by intravenous (i.v.) α-chloralose-HBC (Sigma-Aldrich Corp. Ltd., Poole, UK). A bolus of α-chloralose (60 mg/kg body weight; i.v.) was injected manually over a period of five minutes whilst the isoflurane and oxygen were turned off. Then α-chloralose was infused continuously at a rate of 30 mg/kg/hr i.v. by infusion pump for the remainder of the experiment. For imaging, rats were secured into an in-house-built cradle with a nose cone to minimize movement. Temperature (RS 51 K-type thermometer; RS Components Ltd, UK), respiration rate (MR10 respiration monitor; Graseby Medical Ltd, UK) and transcutaneous pCO₂ (tcpCO₂) and pO₂ (tcpO₂) were monitored (see supplementary methods), while the rats were allowed to breathe spontaneously. Imaging was carried out using a 7-Tesla, horizontal-bore magnet (Magnex Scientific Ltd., Abingdon, UK) with a transmit/receive birdcage volume coil connected to a SMIS computer console (Surrey Medical Imaging Systems Limited, Guildford, UK). For anatomical reference images, a T₂-weighted fast spin echo was used (repetition time = 2 seconds, flip angle = 90°, base echo time = 30 ms, effective echo time = 60 ms, number of samples = 256, number of views = 128, number of averages = 16). For functional images, a T₂*-weighted gradient echo was used to measure BOLD signal...
(repetition time = 172 ms, echo time = 15 ms, number of samples = 128, number of views = 64, number of averages = 4, voxel size = 0.313mm X 1mm X 0.313mm, each volume took 70 seconds to acquire). Eleven contiguous slices, each of 1 mm thickness were aligned horizontally through the brain (Paxinos and Watson, 1986). A total of 60 brain volumes over a period of 70 minutes were acquired in all. 2-DG or vehicle was administered during volume 12.

Data was analysed with Statistical Parametric Mapping (SPM5) programme using a random effects model (The Wellcome Trust Centre for Neuroimaging, London, UK; http://www.fil.ion.ucl.ac.uk/spm/software/spm5/). Individual brains were realigned and co-registered to the first volume, spatially normalized and smoothed to a full width half maximum of 0.939 mm isotropic Gaussian kernel. In a first-level analysis, a series of contrasts were constructed between 5 successive time blocks each consisting of 12 consecutive volumes (14 minutes each). The contrasts compared time blocks following injection (4 time blocks, each 12 volumes) to that of the pre-infusion period (1 time block, 12 volumes). These images were combined in a second-level random effects analysis using a two sample t-test. T contrasts were then constructed to discern the positive and negative effects of 2-DG compared with vehicle. The resulting \( T_2 \) contrast statistical parametric maps were overlaid onto a \( T_2 \)-weighted anatomical template image (Schwarz et al. 2006), with a threshold level of \( P < 0.05 \) uncorrected. For the unbiased (operator-independent) identification of the BOLD MRI data, regions of interest were delineated using a 3D digital reconstruction of the Paxinos and Watson rat brain atlas (Paxinos & Watson 1998), co-registered with the rat brain template (Pic atlas) (Schwarz et al. 2006). Only clusters within regions containing \( \geq 3 \) voxels were considered for further analysis. To provide a measure of response in these areas, Z scores and mean percentage BOLD contrast changes for the maximally responding voxel in each cluster were obtained using SPM5.

2.4 Results

2.4.1 Effects 2-DG on food intake and c-Fos immunoreactivity in freely-behaving rats

An unpaired, two tailed t-test revealed a significant increase in food intake in 2-DG -treated animals compared with controls, 90 minutes post injection (\( P = 0.0024 \), Fig. 2.1). As this dose give a robust orexigenic response, it was also used in the subsequent phMRI experiment.
Quantitative analysis of the number of c-Fos-positive neurones in each of the brain areas of interest showing qualitative activity (Fig. 2.2) revealed a significant increase in counts following 2-DG administration compared with vehicle. Increased numbers of c-Fos-positive cells were seen in the AcbC ($P = 0.0098$), AcbSh ($P < 0.0098$, Fig. 2.3a and d), VP ($P < 0.0001$), PVA ($P < 0.0001$, Fig. 2.3b and e), PVN ($P < 0.0001$, Fig. 2.3c and f), Arc ($P = 0.0134$, Fig. 2.4a and d), DMN ($P < 0.0001$, Fig. 2.4b and e), LH ($P < 0.0001$), CeA ($P = 0.0019$, Fig. 2.4c and f), PAG ($P < 0.0001$) and NTS ($P < 0.0001$). None of the brain areas analysed showed a significant decrease in c-Fos immunoreactivity following 2-DG administration. No statistically significant difference between groups was seen in the VMN ($P = 0.1681$).

2.4.2 phMRI: Effects of 2-DG on BOLD signal in α-chloralose-anaesthetized rats

In this study we show that administration of 2-DG induced significant positive BOLD signal in the agranular insular cortex, somatosensory cortex, hippocampus, inferior colliculus (IC), olfactory tubercle, caudate putamen, septum, substantia innominata, Arc, VMN, mesencephalic region, locus coeruleus (LC), parabrachial nucleus (PBN), pontine reticular nucleus (PRN), and the superior olive (SO) (Table. 2.1, Fig. 2.5).

In addition, administration of 2-DG induced significant negative BOLD signal in the cingulate cortex, entorhinal cortex, medial prefrontal cortex, ventral orbitofrontal cortex, ventral hippocampus, olfactory nucleus, AcbC, AcbSh, caudate putamen, bed nucleus of the stria terminalis (BNST), diagonal band, nucleus of posterior limb of the anterior commissure, VP, CeA, thalamus, zona incerta, DMN, LH, PVN, PAG and raphé nucleus (Table. 2.1, Fig 2.5).
2.4.3 phMRI: blood gases

As detailed in Supplementary Information, blood O$_2$ and CO$_2$ were measured transcutaneously throughout the imaging experiments. CO$_2$ was very stable in all animals throughout the experiment (before and after infusion of 2-DG or vehicle, Supplementary Fig. 2.1a). O$_2$ was more variable, but there was no evidence for systematic effects of time or infusion of either vehicle or 2-DG (Supplementary Fig. 2.1b). Further details are given in the Supplementary Information.

![Graph illustrating the number of c-Fos-positive neurons per section in brain regions after subcutaneous administration of vehicle or 2-DG (200 mg/kg b.w.). Bars represent mean and S.E.M (n = 5/group). Data analysed using an unpaired two-tailed t-test. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle. Abbreviations; nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), thalamic paraventricular nucleus (PVA), hypothalamic paraventricular nucleus (PVN), arcuate nucleus (Arc), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamus (LH), central amygdala (CeA), periaqueductal grey (PAG), and the nucleus of the solitary tract (NTS).]
Figure 2.3 Photomicrographs showing c-Fos-labelled nuclei in selected brain areas following administration of vehicle (A, B, C), or 2-DG (200 mg/kg b.w., D, E, F). Abbreviations: anterior commissure (ac), nucleus accumbens shell (AcbSh), hypothalamic paraventricular nuclei (PVN), and thalamic paraventricular nuclei (PVA). Scale bars = 200μm.
Figure 2.4 Photomicrographs showing c-Fos-labelled nuclei in selected brain areas following administration of vehicle (A, B, C), or 2-DG (200 mg/kg b.w., D, E, F). Abbreviations: third ventricle (3V), central amygdala (CeA), hypothalamic arcuate (Arc), dorsomedial (DMN), and ventromedial (VMN) nuclei. Scale bars = 200μm.
Figure 2.5 Group (n = 7) statistical parametric maps showing changes in BOLD contrast, with a significance threshold set to \( P < 0.01 \) uncorrected, following acute administration of 2-DG (200 mg/kg, s.c.). BOLD blobs in red indicate regions of increased activity compared with vehicle, whereas blobs in blue are regions of decreased activity. The colour bar represents \( t \)-values. Values above images represent approximate distances from bregma (mm). The histogram represents the total area under the BOLD signal percentage change curve produced following treatment with vehicle or 2-DG for each of the corresponding brain regions. Bars represent mean and S.E.M. Abbreviations: arcuate nucleus (Arc), central amygdala (CeA), dorsomedial hypothalamic nucleus (DMN), inferior colliculus (IC), locus coeruleus (LC) lateral hypothalamus (LH), nucleus accumbens shell (Acb Shell), orbitofrontal cortex (Orb), periaqueductal grey (PAG), parabrachial nucleus (PBN), paraventricular hypothalamic nucleus (PVN), superior olive (SO), ventral pallidum (VP), ventromedial nucleus (VMN).
Chapter 2 – Functional magnetic resonance imaging and c-Fos mapping in rats following a glucoprivic dose of 2-deoxy-D-glucose

Table 2.1 Regions of significant BOLD activation relative to vehicle following administration of 2-DG (200 mg/kg, s.c.) detected by pharmacological-challenge magnetic resonance imaging (n = 7). Columns show Z scores, for the peak-responding voxel in brain areas showing significant changes in BOLD signal following 2-DG treatment (analyzed using a two-sample t-test). Abbreviations: bed nucleus of the stria terminalis (BNST), interstitial nucleus of posterior limb of the anterior commissure (IPAC).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>phMRI Z - Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
</tr>
<tr>
<td>Agranular Insular</td>
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</tr>
<tr>
<td>Cingulate Cortex</td>
<td>-</td>
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<tr>
<td>Entorhinal Cortex</td>
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</tr>
<tr>
<td>Medial Prefrontal Cortex</td>
<td>-</td>
</tr>
<tr>
<td>Somatosensory Cortex</td>
<td>2.39</td>
</tr>
<tr>
<td>Ventral Orbitofrontal Cortex</td>
<td>-</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
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<tr>
<td>Hippocampus Medial</td>
<td>3.15</td>
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<tr>
<td>Hippocampus Posterior-dorsal</td>
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</tr>
<tr>
<td>Hippocampus Subiculum</td>
<td>3.4</td>
</tr>
<tr>
<td>Hippocampus Ventral</td>
<td>-</td>
</tr>
<tr>
<td>Hippocampus Anterior-dorsal</td>
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</tr>
<tr>
<td>Olfactory Region</td>
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<tr>
<td>Olfactory Nuclei</td>
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</tr>
<tr>
<td>Olfactory Tubicule</td>
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<tr>
<td>Basal Ganglia</td>
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<tr>
<td>Accumbens Core</td>
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<td>Accumbens Shell</td>
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</tr>
<tr>
<td>BNST</td>
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<tr>
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<tr>
<td>Diagonal Band</td>
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<td>IPAC</td>
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<td>Central Amygdala</td>
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<td>Zona incerta</td>
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<td>Arcuate Nucleus</td>
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<td>Mesencephalic Region</td>
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<td>Periaqueductal grey</td>
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<tr>
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<td>-</td>
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<tr>
<td>Superior Olive</td>
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</tr>
</tbody>
</table>
2.5 Discussion

The central sites mediating 2-DG-induced responses are poorly understood outside the realm of the classic homeostatic centres of the hypothalamus and caudal brainstem. In this study, we have shown “whole-brain” responses to a behaviourally relevant glucoprivic dose of 2-DG, using the complementary techniques of BOLD phMRI and c-Fos protein functional activity mapping.

Concurring with previous studies, intraperitoneal administration of 200 mg/kg 2-DG resulted in acute hyperphagia (King et al. 1978, Tepper & Kanarek 1984, Ritter & Taylor 1989, Ritter & Taylor 1990, Giraud et al. 1998). Functional brain activity following 2-DG administration was compared with vehicle injections, using the neuronal activity marker protein, c-Fos. 2-DG increased c-Fos expression in the PVA, PVN, Arc, VMN, DMN, LH, CeA, and the NTS, supporting previous studies investigating 2-DG-induced c-Fos immunoreactivity (Ritter & Dinh 1994, Solomon et al. 2006). Previous studies, however, limit reporting to selected brain areas and do not provide whole-brain coverage. The current study provides a complete whole-brain analysis and demonstrates, additionally, increased c-Fos immunoreactivity in the striatum (VP, AcbC, AcbSh) and PAG following treatment with 2-DG. A clear deviation between this and previous studies is the lack of c-Fos induction in some regions of the pons and medulla oblongata. Other, similar studies have reported robust c-Fos immunoreactivity in the PBN, LC, SO and ventrolateral medulla (Ritter & Dinh 1994, Ritter et al. 1998). The latter have tended to use higher doses of 2-DG and to remove food from the animals after injection, which might affect the response. However, interestingly, although these areas were not detected in the present study using c-Fos immunohistochemistry, they were identified using phMRI, highlighting complementarity between the two techniques.

c-Fos immunohistochemistry provides high spatial resolution and whole-brain coverage, although c-Fos expression does not automatically follow neuronal activity (Luckman et al. 1994), which can lead to false negative results. In addition, c-Fos is seldom translated in response to reduced activity so, in these instances, neuronal inhibition cannot be discerned (Hughes & Dragunow 1995). c-Fos protein induction occurs over a period of 30-90 minutes, thus limiting temporal resolution. For these reasons, it is essential to complement functional immunohistochemistry with other functional imaging techniques in order to provide additional data and to aid interpretation. Systemic administration of 2-DG produced
significantly enhanced BOLD activity in several brain areas including the frontal cortices (orbitofrontal, cingulate, insular cortex), mesolimbic system (AcbC, AcbSh, VP, BNST), striatum (caudate putamen, globus pallidus), amygdala (CeA), thalamus (PVA, IC), midbrain (PAG), hypothalamus (Arc, DMN, LH, PVN, VMN) and pons (LC, PBN, PRN, SO).

In terms of functionality, the combination of BOLD and c-Fos immunohistochemistry highlights a number of regions that show changes in both measures following 2-DG. Positive BOLD signals are triggered by oxygen depletion in response to increased metabolic demand of neuronal firing and synaptic activity (for review see Logothetis & Wandell 2004, Nair 2005). In fact a linear relationship between positive BOLD and neuronal activity has been described (Heeger et al. 2000, Rees et al. 2000). Therefore areas showing increased signal using both techniques following 2-DG administration in this study (Arc and VMN) potentially reflect increased synaptic and cellular electrical activity.

The functional origins underlying the negative BOLD signal are far more speculative, as they are thought to arise from a complex interplay between decreased neuronal metabolic demand (Shmuel et al. 2002, Shmuel et al. 2006, Devor et al. 2007) and the “vascular-steel” effect (Harel et al. 2002, Shmuel et al. 2002). Despite this, numerous studies show strong evidence to suggest that negative BOLD is indicative of a true suppression of neuronal activation (Shmuel et al. 2002, Stefanovic et al. 2004, Devor et al. 2007). Taken in this context, the brain areas depicted in this study showing a decrease in BOLD signal and an increase in c-Fos immunoreactivity following 2-DG administration (AcbC, AcbSh, VP, DMN, PVN, LH, CeA, PVA, PAG) potentially reflect decreased synaptic activity and increased cellular activity; an effect which could be occurring due to disinhibition in these regions.

This study does, however, illustrate clear evidence of dissociation between BOLD fMRI and c-Fos immunoreactivity in a number of brain regions. For example, the IC showed a robust change in BOLD activity but failed to show any changes in c-Fos immunoreactivity. The possible reasons for this dissociation are discussed in detail elsewhere (Stark et al. 2006, Preece et al. 2009).

In light of the widespread effects of 2-DG on metabolism, it is interesting to note the relatively small number of brain sites expressing significant c-Fos immunoreactivity and BOLD activity in response to 2-DG. In addition, previous studies have found that both 2-DG-
induced hyperphagia and c-Fos immunoreactivity persist in many of the brain areas described above following subdiaphragmatic vagotomy, implying that these effects are a consequence of the direct central metabolic effect of 2-DG (Miselis & Epstein 1975, Ritter & Dinh 1994). The functional responses to 2-DG observed in the current study may represent direct actions of 2-DG on neurones in each of these brain areas, or may represent transynaptic activation of these sites following more selective activation of specific populations of specialized glucoreceptive neurones within the brain.

Interpretation of the current results in the context of feeding centres of the brain, highlights the hypothalamus and hindbrain as potential sites of action underlying the hyperphagia. Studies attempting to establish the primary site coordinating the counter-regulatory hyperphagic response initiated by 2-DG-induced hypoglycaemia, have resulted in the emergence of two separate views. A substantial body of evidence points to an integral role of the ventromedial hypothalamus (VMH, comprising both the Arc and the VMN). Breakthrough studies demonstrated that perfusion of glucose directly into the VMH of systemically-induced hypoglycaemic animals abolishes the normal counter-regulatory responses, while infusion of 2-DG into the VMH caused a prompt increase in plasma glucose, glucagon, and catecholamines (Borg et al. 1994, Borg et al. 1995), suggesting that the neurones sensing glucopenia may be localised in the VMH. A recent study found that manipulation of VMH glucosensing by blocking VMH glucokinase mRNA expression reduced glucoprivic feeding, but exerted no effect on spontaneous feeding behaviour, implying behavioural specificity in the role of the VMH in food intake (Dunn-Meynell et al. 2009). In light of this evidence, the significant functional activation of the VMH by 2-DG seen in this study further illustrates that VMH glucosensing plays an important role in the counter-regulatory behavioural and neuroendocrine responses to glucoprivation.

A second body of evidence points to a primary role for the hindbrain (DiRocco & Grill 1979). Direct injection of 2-DG into the LH, VMH, amygdala or striatum has no effect on feeding behaviour, despite a marked response to intracerebroventricular administration (Berthoud & Mogenson 1977). This intracerebroventricular injection of 2-DG failed to activate feeding in the presence of an obstruction to the cerebral aqueduct (Ritter et al. 1981), suggesting that 2-DG-induced glucopenia may be sensed predominantly by neuronal populations in the hindbrain. Food intake can be stimulated by direct injections of the glucose anti-metabolite
5-thioglucose into areas of the hindbrain, such as the ventrolateral and dorsomedial medulla (Ritter et al. 2000). These regions, along with other hindbrain nuclei highlighted by phMRI in this study (LC, PBN, PRN), contain neurones that respond either directly or indirectly to a glucoprivic dose of 2-DG (Ritter & Dinh 1994, Ritter et al. 1998). Neurones in the NTS sense glucose both directly, and indirectly via vagal afferents from peripheral glucosensors in the hepatic portal vein. Confirming previous reports (Ritter & Dinh 1994, Moriyama et al. 2003), the current study found a marked increase in c-Fos immunoreactivity in the NTS, but no significant change in BOLD signal. There are various explanations for this. First, there may be a drug/anaesthetic interaction in this area not seen elsewhere in the brain; second, there may only be a delayed response in these regions which were not detected maximally in the time frame examined; and third, the response in NTS neurones is transcriptional but not electrical. Either way, counter regulation in response to glucoprivation is likely to require an integrated brain response.

As this study has mapped functional responses of the whole brain, it has also identified a number of novel 2-DG-responsive brain regions. Areas of particular interest are the Orb (cortex), AcbShell, and VP (mesolimbic system). These regions make up a corticostriatal connection with the hypothalamus, by which aspects of motivation, salience and reward can impinge on the hypothalamic control of feeding behaviour (Swanson 2000, Fulton 2009). Furthermore, the hypothalamus can be viewed as the head of a brainstem control column which implements the feeding behaviour. A recent study investigating functional brain responses to an orexigenic dose of a cannabinoid CB$_1$ receptor agonist, also highlights this corticostriatal-hypothalamic pathway (Dodd et al. 2009). Thus, we have provided evidence for a common integrated circuit involved in the induction of feeding behaviour to different types of stimulus.

In summary, we have characterised the whole-brain response to 2-DG-induced glucoprivation using c-Fos protein functional activity mapping and BOLD phMRI. By using the complementarity of two functional techniques, we have identified the well-characterized connections of the hypothalamus and brainstem, whilst highlighting areas of the frontal cortices (orbitofrontal, cingulate, insular cortex), mesolimbic system (AcbC, AcbSh, VP, BNST), striatum (caudate putamen, globus pallidus), amygdala (CeA) and thalamus (PVA, IC) as additional brain regions responding to 2-DG-induced glucoprivation. This study, therefore, provides an insight into how the whole-brain responds to 2-DG in order to co-ordinate...
complex counter-regulatory responses, whilst further illustrating the accuracy and valuable potential of phMRI in investigating central pharmacological activity.

2.6 Supplementary Data

2.6.1 Blood gas measurement

In parallel to the fMRI all rats were continuously monitored for transcutaneous blood pCO$_2$ and pO$_2$ ($n = 7$) throughout the duration of the scan. Prior to scanning the fur above the xiphoid process was shaved to 5 cm diameter with an electric razor and depilatory cream (Veet, Clevedon, UK) was applied for 5 min and, after removal of the cream, the skin was carefully cleaned with alcohol wipes. The blood gas electrode was fixed to the dry skin using an adhesive fixation ring (15 mm outer diameter), and filled with contact fluid containing 1,2-propanediol and de-ionised water. Blood gases were continuously monitored using a transcutaneous blood gas analysing system (model TINA TCM4; Radiometer Copenhagen, Willich, Germany). This system uses a combined Stowe–Severinghaus (for pCO$_2$ measurement) and Clark-type polarographic (for pO$_2$ measurement) electrode. The electrode working temperature is 44 °C and the system was calibrated each time prior to positioning, using a standard calibration gas containing 7.5 % CO$_2$, 20.9 % O$_2$ and 71.6 % N$_2$ (Radiometer Copenhagen). The SmartHeat function of the system was used to add 1°C to the selected electrode temperature in the first 5 min after placement to ameliorate CO$_2$ and O$_2$ diffusion at the beginning of the measurement. For each animal, once a stable plateau of the transcutaneous partial pressure (tcp) O$_2$ and tcpCO$_2$ was reached (approximately 10 min from electrode placement), a 15 minute baseline period (100 %) was acquired. tcpO$_2$ and tcpCO$_2$ values were taken every 10 sec for a further 35 min and normalised to the baseline period. Results are expressed as a mean and S.E.M. percentage change from baseline for each time point.

2.6.2 Results and Discussion

One of the major physiological parameters that has a substantial detrimental effect on the neurovascular and neurometabolic basis of the BOLD response is hypo- and hypercapnia (Ogawa et al. 1990, Ogawa et al. 1993, Mandeville et al. 1998, Silva et al. 1999, Cohen et al. 2002). A key study by Ramos-Cabrer et al (2005) demonstrated that BOLD contrast in the somatosensory cortex of anaesthetised rats following stimulation is masked if tcpCO$_2$ levels exceed 20% of the baseline (Ramos-Cabrer et al. 2005). Although slight variations between
the treatment groups are evident over the time course of this experiment, no changes in tcpCO₂ (Sup Fig. 2.1a.) or tcpO₂ (Sup Fig. 2.1b.) following treatment exceed beyond 20% of baseline levels.

Supplementary Figure. 2.1. Line graph illustrating time course changes of (a) tcpCO₂ and (b) tcpO₂ following subcutaneous administration of vehicle, or 2-DG (200 mg/kg) in α-chloralose anaesthetized rats (n = 7). An initial fifteen minute baseline period was acquired before drugs were injected (vertical dotted line). Results are presented as percentage change relative to baseline values (100 %). Horizontal dotted line represents threshold level as described by Cabrer et al (2005) by which BOLD signal is lost if tcpCO₂ levels exceed 20 % above baseline (Ramos-Cabrer et al. 2005). Error bars show SEM.
Chapter 2 – Functional magnetic resonance imaging and c-Fos mapping in rats following a glucoprivic dose of 2-deoxy-D-glucose

2.7 References


Chapter 3

Central cannabinoid signaling mediating food intake: a pharmacological-challenge MRI and functional histology study in rat


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3.1 Abstract

Endocannabinoids have a variety of effects by acting through CB₁ receptors located throughout the brain. However, since CB₁ receptors are located presynaptically, and because the strength of downstream coupling varies with brain region, expression studies alone do not provide a firm basis for interpreting sites of action. Likewise, to date most functional studies have used high doses of drugs which can bias results toward non-relevant adverse effects, and which mask more behaviourally-relevant actions. Here we use a low, orexigenic dose of the full CB₁ agonist, CP55940, to map responsive brain regions using the complementary techniques of pharmacological-challenge functional magnetic resonance imaging (phMRI) and immediate-early gene activity. Areas of interest demonstrate a drug interaction when the CB₁ receptor inverse agonist, Rimonabant, is co-administered. This analysis highlights the cortico-striatal-hypothalamic pathway which is central to the motivational drive to eat.
3.2 Introduction

Endogenous cannabinoids are released widely in the brain and act primarily as retrograde transmitters on presynaptically located receptors (Freund et al. 2003, Di Marzo et al. 2004, Kawamura et al. 2006). Endocannabinoids are synthesised and released by the postsynaptic neurone “on demand”, and impart an inhibitory action on further synaptic transmission (Di Marzo et al. 1998, Schlicker & Kathmann 2001, Wilson & Nicoll 2002, Di Marzo et al. 2004). Receptor CB₁ is expressed throughout the brain (Herkenham et al. 1991, Matsuda et al. 1993, Egertova & Elphick 2000), while receptor CB₂ is much less represented, instead being found mostly in the periphery (Munro et al. 1993, Van Sickle et al. 2005). Cannabinoids have been used to target pain relief and to treat the symptoms of multiple sclerosis and glaucoma, through their actions on peripheral nervous tissues (Robson 2005). However, the diverse central actions of endocannabinoids (implicated in the modulation of nociception, locomotion, body temperature, memory and appetite) and the relative paucity of information regarding their sites of action have hindered their development as pharmaceutical agents. Recently, Rimonabant (Acomplia; SR141716) did not receive approval for the treatment of obesity in the USA, and was withdrawn in 2008 from the European market due to some concerns that prolonged use may occasionally lead to depression, anxiety or suicidality (Christensen et al. 2007). Rimonabant is an inverse agonist at CB₁ (Bouaboula et al. 1997) and is capable of producing weight-reducing effects over extended periods, notably when used in conjunction with life-style changes (Van Gaal et al. 2005). However, its action to specifically reduce appetite is relatively short lived, and any continued weight loss is via peripheral interaction with lipid mobilisation in adipose tissue and cellular glucose uptake (Nogueiras et al. 2008). Further validation of the CB₁ receptor as a target for pharmaceutical intervention is concentrating on the development of inverse agonists or neutral antagonists which may not induce undesirable psychotic side effects, including those which do not pass the blood-brain barrier. An integral step in order to aid future development of therapeutically viable cannabinoid-based drugs is to gain further understanding of how cannabinoids act in the brain.

A number of histological techniques demonstrate high levels of CB₁ expression in the cortex, cerebellum, basal ganglia and striatum, and particularly low levels of expression in the hypothalamus (Herkenham et al. 1991, Matsuda et al. 1993, Egertova & Elphick 2000). However, these studies do not discern the functionality of cannabinoids or how they mediate particular behavioural outputs. Functional studies using activity markers, such as
the protein product of the c-fos gene, have highlighted a number of cannabinoid-responsive areas central to motivation and reward processes, with particular emphasis on the nucleus accumbens (Miyamoto et al. 1997, McGregor et al. 1998, Arnold et al. 2001, Allen et al. 2003). High doses of cannabinoids also induce c-Fos protein in the hypothalamic paraventricular nucleus and the central amygdala, two areas often associated with stress responses. Furthermore, phMRI studies also have tended to use exceptionally high doses, often orders of magnitude higher than that required to produce behavioural responses (Shah et al. 2004, Chin et al. 2008). Here, using behaviourally relevant doses and complementary functional techniques, we are able to provide strong evidence for target sites of cannabinoid signaling with whole-brain coverage.

### 3.3 Methods

#### 3.3.1 Animals and drugs

All experiments were carried out using male Sprague-Dawley rats (250 ± 22g; Charles River Laboratories, Inc., Sandwich, UK). Animals were group housed in The University of Manchester animal unit in a constant environment of 21 ± 2°C and 45 ± 10 % humidity, on a 12:12 hour light-dark cycle. Rat chow (Beekay International, Hull, UK) and tap water were available ad libitum unless stated otherwise. All procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act, 1986 and local ethical review.

Rimonabant was a kind gift from Sanofi-Avensis, (Longjumeau, France), and CP55940 was purchased from Tocris Bioscience Ltd (Brighton, UK). The vehicle for Rimonabant was a saline solution containing 1 drop of Tween 80 per (Sigma-Aldrich Corp. Ltd., Poole, UK) 2ml, and the vehicle for CP55940 was the same containing 2.5 % ethanol.

#### 3.3.2 Effects of CB₁ agonist and inverse agonist on food intake in pre-satiated rats

Rats were housed singly for one day and food was restricted 6 hours before the experiment was due to start. At lights out (20:00) animals were fed pre-weighed chow ad libitum for thirty minutes to partially satiate them. In the first experiment, rats were assigned randomly to receive a single intraperitoneal (i.p.) dose of CP55940 (0, 0.01, 0.03, 0.06, 0.1 mg/kg body weight) in a volume of 1 ml/kg of vehicle at 20:30. In the second experiment, rats were assigned randomly to receive two i.p. injections at 20:30 and 21:00. The first injection was either 1 mg/kg Rimonabant, or its vehicle. The second injection consisted of 0.06 mg/kg
CP55940 or its vehicle. Injections were made in a volume of 1 ml/kg body weight. The dose of Rimonabant was based on the literature (Arnone et al. 1997, Solinas & Goldberg 2005, Thornton-Jones et al. 2005), while that of CP55940 was determined from the first experiment. Food intake was determined 1, 2, and 12 hours following CP55940. Results are presented as mean and S.E.M. for food intake at each time point (n = 6 per group). Treatments were compared using a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison post hoc test using the GraphPad Prism statistical package (GraphPad Software, Inc. San Diego, CA, USA).

3.3.3 c-Fos protein immunohistochemistry

Eighteen rats were assigned randomly to receive i.p. injections of either vehicle, 0.06 mg/kg CP55940, or 1 mg/kg Rimonabant between the hours of 09:00 and 11:00. Ninety minutes post injection the animals were deeply anaesthetised with sodium pentobarbital (100 mg/kg body weight i.p.; Rhône-Poulenc, Lyon, France) and perfused transcardially with heparinised saline (10,000 units/l heparin in 0.9% NaCl) followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.3). The brains were post fixed overnight and then kept for two days in 30% sucrose in 0.1 M PB to cryoprotect the tissue before freezing on dry ice. 30 μm sections (120 μm apart) were cut in the coronal plane throughout the entire rostrocaudal extent of the brain and incubated in 20 % methanol, 0.2 % Triton X-100, 1.5 % hydrogen peroxide for 30 min to deactivate endogenous peroxidases. Sections were then incubated at room temperature for 1 hour in the blocking buffer: 0.1 M PB, 0.3 % Triton X-100, 1 % normal sheep serum and then overnight at 4ºC in rabbit anti-c-Fos antibody (Oncogene Science Inc., Bayer Healthcare, Cambridge, MA, USA) diluted to 1:10000 in blocking buffer. After washing, the sections were incubated sequentially at room temperature for 1 hour in goat anti-rabbit IgG-biotin complex (Vector Laboratories, Peterborough, UK) diluted 1:500 in blocking buffer followed by avidin-biotin–peroxidase complex (GE Healthcare, Bucks, UK) diluted 1:500 in PB and, finally visualized with nickel-intensified diaminobenzidine (Vector Laboratories, Peterborough, UK). c-Fos immunoreactive neurones were examined with the experimenter blinded to the treatments. Images were collected on an Axiovison upright microscope (Zeiss, Hertfordshire, UK) using an Axiocam colour CCD camera. Images were then processed using Axiovision software to quantify the number of c-Fos expressing nuclei in areas defined according to a standard atlas (Paxinos & Watson 1986). The results are presented as mean and S.E.M. for the number of c-Fos immunoreactive cells in each brain area per section.
Treatments were compared using a one-way ANOVA followed by Bonferroni’s multiple comparison post hoc test.

3.3.4 Blood oxygen level-dependent (BOLD) phMRI

Twenty four rats were assigned randomly to receive pre-treatment of vehicle or Rimonabant (1 mg/kg body weight, i.p.) followed by subsequent administration of either vehicle or CP55940 (0.06 mg/kg body weight, i.p., n = 6 per group). Animals were anaesthetized with 2.0 - 2.5% isoflurane (Concord Pharmaceuticals Ltd, Dunmow, Essex, UK) in oxygen (2 l/min) to allow cannulation of a tail vein and subsequent anaesthetic maintenance by intravenous (i.v.) α-chloralose-HBC (Sigma-Aldrich Corp. Ltd., Poole, UK). A bolus of α-chloralose (60 mg/kg body weight; i.v.) was injected manually over a period of five minutes whilst the isoflurane and oxygen were turned off. Then α-chloralose was infused continuously at a rate of 30 mg/kg/hr i.v. by infusion pump for the remainder of the experiment. To confirm that neither treatment interacted with the anaestheia to produce confounding actions on circulating blood gas levels, the effects of CP55940 and Rimonabant on the transcutaneous partial pressure of blood CO$_2$ and O$_2$ were tested in separate anaesthetized rats (see Supplemental data). For imaging, rats were secured into an in-house built cradle with a nose cone to minimize movement. Rectal temperature (RS 51 K-type thermometer; RS Components Ltd, Northants, UK) and respiration rate (MR10 respiration monitor; Graseby Medical Ltd, Hertfordshire, UK) were monitored, while the rats were allowed to breathe spontaneously. Imaging was carried out using a 7-Tesla, horizontal-bore magnet (Magnex Scientific Ltd., Abingdon, UK) connected to a SMIS computer console (Surrey Medical Imaging Systems Limited, Guildford, UK) with a transmit/receive birdcage volume coil. For anatomical reference images, a T$_2$-weighted fast spin echo was used (repetition time = 2 seconds, flip angle = 90$^\circ$, base echo time = 30 ms, effective echo time = 60 ms, number of samples = 256, number of views = 128, number of averages = 16). For functional images, a T$_2$*-weighted gradient echo was used to measure BOLD signal (repetition time = 172 seconds, base echo time = 15 ms, number of samples = 128, number of views = 64, number of averages = 4, voxel size = 0.313mm X 1mm X 0.313mm, each volume took 70 seconds to acquire). Eleven contiguous slices, each of 1 mm thickness were aligned horizontally through the brain (Paxinos & Watson 1986). A total of 72 brain volumes over a period of 84 minutes were acquired in all. Rimonabant or its vehicle was administered during volume 12 and CP55940 or its vehicle was administered during volume 24.
Data were analysed with Statistical Parametric Mapping (SPM5) using a random effects model (The Wellcome Trust Centre of Neuroimaging, London, UK; http://www.fil.ion.ucl.ac.uk/spm/software/spm5/). Individual brains were realigned and coregistered to the first volume, spatially normalized and coronally oriented to a T₂-weighted anatomical template image (Schwarz et al. 2006), and spatially smoothed to a full width half maximum of 0.939 mm isotropic Gaussian kernel.

In a first-level analysis, a series of contrasts were constructed between 6 successive time blocks each consisting of 12 consecutive volumes (14 minutes each). The contrasts compared the 5 time blocks following injection to that of the pre-infusion period. These images were combined in a second-level random effects analysis using a full factorial ANOVA. T-contrasts were then constructed to discern areas of positive and negative BOLD induced by CP55940 treatment, and the positive and negative effects of Rimonabant on this signal. By looking at the interaction of the two drugs in this way, we are able to specifically highlight regions responsive to CP55940 that are significantly modulated by Rimonabant. For a fuller explanation of types of interaction with examples, see results section (Fig. 3.5). The resulting T-contrast statistical parametric maps were overlaid onto a T₂-weighted anatomical template image (Schwarz et al. 2006), with a threshold level of p < 0.05 uncorrected.

For the unbiased (operator-independent) identification of the BOLD MRI data, regions of interest were delineated using a 3D digital reconstruction of the Paxinos and Watson rat brain atlas (Paxinos & Watson 1998), coregistered with the rat brain template (Schwarz et al. 2006). Only clusters within regions containing ≥3 voxels and in which the two drugs had a statistically significant interaction were considered for further analysis. To provide a measure of response in these areas, Z scores and mean percentage BOLD contrast changes for the maximally responding voxel in each cluster were obtained using SPM5.

## 3.4 Results

### 3.4.1 Effects of CB₁ agonist CP55940 on food intake in pre-satiated rats

An effect of treatment was detected at the 1-hour post-injection time point, revealed by one-way ANOVA (F₄,₂₅ = 11.77, p < 0.001; Fig. 3.1). CP55940 showed a dose dependant increase in food intake, though the highest dose caused hypolocomotion. Bonferroni’s multiple comparison post hoc analysis identified a significant increase in food intake...
following 0.06mg/kg CP55940 (p < 0.001). As this dose showed a robust orexigenic response, it was used in subsequent experiments. At the 2-hour time point (data not shown) the hyperphagic response to 0.06 mg/kg was no longer statistically significant (F_{4,25} = 4.649, p > 0.05). At the 12-hour time point (data not shown) there was no longer any differences between groups (F_{4,25} = 1.905, p = 0.1409).

![Figure 3.1](image)

**Figure 3.1.** Graph illustrating total food intake at 1 hour post injection of animals treated with vehicle, 0.01 mg/kg, 0.03 mg/kg, 0.06 mg/kg, and 0.1 mg/kg body weight CP55940. All treatments were administered intraperitoneally. Bars represent mean and S.E.M. One-way ANOVA followed by Bonferroni’s multiple comparison post hoc test. ***p < 0.001 compared with vehicle (n = 6/group).

### 3.4.2 Effect of CB₁ inverse agonist Rimonabant on CP55940-induced hyperphagia in pre-satiated rats

An effect of treatment was detected at the 1-hour time point, revealed by one-way ANOVA (F_{3,20}=10.92, p < 0.0002; **Fig. 3.2**), and not at the 2-or 12-hour (F_{3,20}=1.732, p < 0.1928, and F_{3,20}= 0.9144, P < 0.4517, respectively; data not shown) post-injection time points. At the 1-h time point, Bonferroni’s multiple comparison post hoc analysis identified a significant increase in food intake following CP55940 alone, and a significant decrease in food intake following Rimonabant alone (1 mg/kg body weight) compared with both the vehicle/vehicle control group and the Rimonabant/CP55940 co-administration group (both p < 0.01). The effect of either drug was effectively cancelled out when they were co-administered, resulting in food intake being comparable to that of the control group.
3.4.3 Effect CB₁ agonist, CP55940, and CB₁ inverse agonist, Rimonabant on c-Fos immunoreactivity in freely behaving rats

All brain sections for each treatment were initially analysed qualitatively to ascertain brain regions showing c-Fos immunoreactivity. Qualitative analysis of the whole brain highlighted the following brain areas of interest: medial orbitofrontal cortex (MO), ventral orbitofrontal cortex (VO), lateral orbitofrontal cortex (LO), agranular insular cortex (AI), cingulate cortex (Cg), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), medial amygdala (MeA), central amygdala (CeA), lateral hypothalamus (LH), and the hypothalamic arcuate (Arc), ventromedial (VMN), dorsomedial (DMN) and paraventricular (PVN) nuclei.

Quantitative analysis of the number of c-Fos-positive neurones in each of these brain area of interest (Fig. 3.3) revealed a significant increase in counts following CP55940 administration compared with vehicle in the following regions: LO, VO, MO, Cg, AI (Fig. 3.4 A - C), VP, AcbC, AcbSh (Fig. 3.4 D - F), CeA (Fig. 3.4 G - I), LH and PVN. None of the brain areas analysed showed a significant decrease in c-Fos immunoreactivity following CP55940 administration. The only significant increase in immunoreactivity following Rimonabant administration was in the PVN; however, significant decreases in immunoreactivity were seen in the CeA (Fig. 3.4 G - I) and VMN (Fig. 3.4 J - L). No significant differences for either treatment were seen in the Arc or DMN. N.B. All rats for immunostaining were killed 90 minutes post drug treatment.
Figure 3.3. Graph illustrating the number of c-fos-positive neurones per section in brain regions after intraperitoneal administration of vehicle, Rimonabant (1 mg/kg body weight), or CP55940 (0.06 mg/kg body weight). Bars represent mean and S.E.M. One-way ANOVA followed by Bonferroni’s multiple comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 compared with vehicle. ****p < 0.001 compared with Rimonabant (n = 6/group). Abbreviations: medial orbitofrontal cortex (MO), ventral orbitofrontal cortex (VO), lateral orbitofrontal cortex (LO), agranular insular cortex (AI), cingulate cortex (Cg), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), medial amygdala (MeA), central amygdala (CeA), lateral hypothalamus (LH), arcuate nucleus (Arc), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and the paraventricular nucleus (PVN).
Figure 3.4. Photomicrographs showing c-Fos-labelled nuclei in selected brain areas following administration of vehicle (A, D, G, J), Rimonabant (1 mg/kg body weight, B, E, H, K) or CP55940 (0.06 mg/kg body weight, C, F, I, L). Abbreviations; third ventricle (3V), anterior commissure (ac), agranular insular cortex (AI), central amygdala (CeA), nucleus accumbens shell (AcbSh), hypothalamic arcuate (Arc) and ventromedial (VMN) nuclei. Scale bars = 200μm.
3.4.4 phMRI: interaction of the CB₁ inverse agonist Rimonabant with the CB₁ agonist CP55940

Using statistical analysis and the PickAtlas (Schwarz et al. 2006) it was possible to identify regions of the brain in which an interaction had occurred between the two CB₁ acting drugs. Only regions responding to CP55940 that also were modulated by Rimonabant (an interaction, see Fig. 3.5 for explanation) were considered for analysis, illustrating more accurately where cannabinoids act within the brain. Brain areas responding to CP55940 that show an interaction with Rimonabant are described in Table 3.1, with Z-scores and BOLD percentage change of the peak-responding voxel in a cluster for each area of interest.

Figure. 3.5. Examples of negative and positive BOLD interactions. a) A negative interaction in the highest responding voxel of the accumbens shell (AcbSh). Here CP55940 significantly increases BOLD signal compared with controls. However, when the two drugs are co-administered, Rimonabant significantly attenuates the CP55940-induced BOLD increase. b) A positive interaction in the highest responding voxel of the bed nucleus of the stria terminalis (BNST). Here CP55940 significantly decreases BOLD signal compared with controls. However, when the two drugs are co-administered, Rimonabant significantly attenuates the CP55940-induced BOLD decrease.

Administration of CP55940 induced positive BOLD signal in the piriform cortex, somatosensory cortex, rostral olfactory nucleus, olfactory tubercle, AcbC, AcbSh, ventrolateral caudate putamen, diagonal band, interstitial nucleus of posterior limb of the anterior commissure, VP, MeA, Arc, VMN and caudal substantia innominata (Fig. 3.6a and Table. 3.1). In addition, administration of CP55940 induced negative BOLD signal in the AI, LO, entorhinal cortex, AI, caudal olfactory nucleus, bed nucleus of the stria terminalis, dorsolateral caudate putamen, globus pallidus, septum, CeA, dorsolateral thalamus, ventrolateral thalamus, LH, PVN, mesencephalic region, rostral substantia innominata, zona incerta and superior olive (Fig. 6a and Table. 1). Rimonabant showed a significant interaction in all of the above areas; that is, Rimonabant administration resulted in a significant attenuation of the CP55940-induced positive and negative BOLD signals (Fig. 3.6b).
Figure 3.6. a) Group (n = 6/group) statistical parametric maps showing changes in BOLD contrast with a significance threshold set to p < 0.05 (uncorrected) following acute administration of CP55940 (0.06 mg/kg body weight, i.p.). Blobs in red indicate regions of increased BOLD signal compared with vehicle, whereas blobs in blue are regions of decreased BOLD signal. b) Group statistical parametric maps showing the interaction of Rimonabant (1 mg/kg body weight, i.p.) on CP55940-induced BOLD signal. Significance threshold set to p < 0.05 (uncorrected). Blobs in green indicate regions where Rimonabant significantly attenuated the CP55940-induced negative BOLD signal. Blobs in magenta indicate regions where Rimonabant significantly attenuated the CP55940-induced positive BOLD signal. The colour bars represent t values. Abbreviations: arcuate nucleus (Arc), central amygdala (CeA), lateral hypothalamus (LH), medial amygdala (MeA), nucleus accumbens shell (AcbSh), ventral pallidum (VP), ventromedial nucleus (VMN). Values above images represent distance from bregma (mm).
### Table. 3.1. Regions of significant activation relative to vehicle associated with the main effect of CP55940 (0.06 mg/kg body weight, i.p.), and their interaction with Rimonabant (1 mg/kg body weight, i.p.) detected by pharmacological-challenge magnetic resonance imaging (n = 6/group). Columns show Z scores and mean BOLD percentage changes, for the peak-responding voxel in brain areas showing significant changes in BOLD signal following CP55940 treatment (analyzed using a full factorial ANOVA). The direction of Rimonabant’s interaction with these brain areas is depicted by ↑ = positive interaction (Rimonabant significantly attenuates the CP55940-induced negative BOLD signal), and ↓ = negative interaction (Rimonabant significant attenuates the CP55940-induced positive BOLD signal). Abbreviations; bed nucleus of the stria terminalis (BNST), interstitial nucleus of posterior limb of the anterior commissure (IPAC).

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Z Score</th>
<th>Change (%)</th>
<th>Interaction with Rimonabant</th>
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<tr>
<td><strong>Cortex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agranular Insular Cortex</td>
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<tr>
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<td>-4.69</td>
<td>↑</td>
</tr>
<tr>
<td>Piriform Cortex</td>
<td>2.61</td>
<td>2.93</td>
<td>↓</td>
</tr>
<tr>
<td>Somatosensory Cortex</td>
<td>2.01</td>
<td>3.27</td>
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<tr>
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<td>-7.54</td>
<td>↑</td>
</tr>
<tr>
<td>Olfactory Nucleus, Rostral</td>
<td>2.61</td>
<td>2.93</td>
<td>↓</td>
</tr>
<tr>
<td>Olfactory Tubercle</td>
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<td>4.14</td>
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<td>Accumbens, Shell</td>
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<td>IPAC</td>
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<td>Arcuate Nucleus</td>
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<td>↓</td>
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<td>Ventromedial Nucleus</td>
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<td>5.43</td>
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<tr>
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<td>Mesencephalic Region</td>
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<tr>
<td>Superior Olive</td>
<td>-2.93</td>
<td>-7.86</td>
<td>↑</td>
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</tbody>
</table>
3.5 Discussion

High doses of cannabinoids cause hypokinesia, catalepsy and hypothermia, and have strong effects on cognition and memory (Fitton & Pertwee 1982, Pertwee et al. 1993, Rubino et al. 1994, McGregor et al. 1996, McGregor et al. 1998, Howlett et al. 2002). We have used CP55940 at a dose sufficient to cause acute hyperphagia, but at which further effects are minimised. Experiments were carried out in satiated animals to diminish homeostatic drive, allowing assessment of the rewarding motivation to eat. CP55940 produced dose-dependent hyperphagia, with significance at 0.06 mg/kg body weight. This dose was used for the subsequent mapping studies. Higher doses resulted in marked hypophagia due to hypolocomotion, which has been noted previously (McGregor et al. 1996, Gallate et al. 1999). 1 mg/kg body weight Rimonabant attenuated food intake, confirming earlier studies that also have implicated it in reward/motivation (Arnone et al. 1997, Ward & Dykstra 2005). When given together the actions of the drugs were cancelled out, confirming Rimonabant’s ability to attenuate anandamide- or THC-induced hyperphagia (Williams & Kirkham 2002), as well as attenuating increased motivational “break point” for a sweet reward following CP55940 (Gallate et al. 1999).

As Rimonabant and CP55940 have high specificity and affinity for CB$_1$ receptors (Miller et al. 2004, Pertwee 2005, Xie et al. 2007), the simplest hypothesis is that Rimonabant is functionally antagonising CP55940 at the same receptors in the brain. CP55940 significantly increased or decreased BOLD signal in several key feeding reward/motivational areas, including the frontal cortices, limbic striatum, amygdala and hypothalamus. Furthermore, BOLD activity in all areas was reversed by Rimonabant, suggesting it is specific to CB$_1$ activation, and that Rimonabant functionally antagonises CP55940 at the same receptors. The regional activation by CP55940 contrasts with two previous studies that used phMRI and the non-selective cannabinoid agonists, HU210 and A-834735 (Shah et al. 2004, Chin et al. 2008), where a more global activation of the brain was noted, presumably due to non-selective actions.

CP55940 increased c-Fos in the AcbSh, AcbC, PVN, LH and CeA, supporting previous CB$_1$ studies (McGregor et al. 1998, Arnold et al. 2001, Allen et al. 2003, Singh et al. 2005, Soria-Gomez et al. 2007). Most previous publications, however, were limited to selected areas, and did not attempt whole-brain coverage. The present study provides a complete analysis
and, in addition, showed increases in cortical and pallidal regions (LO, VO, MO, Cg, AI and VP). However, in contrast, the current experiment produced a weak response to Rimonabant, increasing c-Fos in the PVN, and decreasing it in the CeA and VMN. Several studies demonstrate a robust induction of c-Fos following Rimonabant in the striatum, hypothalamus and frontal cortices (Alonso et al. 1999, Singh et al. 2004). However, these used doses up to ten times greater and could, therefore, be distinguishing non-specific effects of Rimonabant. In support of our observations, intra-AcbSh administration of AM251, which is similar in structure to Rimonabant, did not induce c-Fos in the striatum or hypothalamus at a low, hypophagic dose (Soria-Gomez et al. 2007).

BOLD phMRI and c-Fos immunohistochemistry produce complementary results, as discussed in detail elsewhere (Stark et al. 2006, Preece et al. 2009). Here we highlight a number of regions showing positive changes in both measures following CP55940, including the AcbC, AcbSh, VP, and VMN, which reflects increased neuronal activation. The Arc and MeA displayed increased BOLD signal, but no induction of c-Fos, which could reflect an increase in inhibitory inputs or a lack of a transcriptional response to the stimulus. BOLD signal is directly related to overall metabolic demand, which is strongly influenced by synaptic activity (Arthurs & Boniface 2002, Lauritzen & Gold 2003). Areas displaying a decrease in BOLD and an increase in c-Fos (AI, LO, LH, CeA, PVN), might reflect disinhibition by CP55940. Thus, reduced activity primarily in inhibitory synapses could lead to the observed reduction in BOLD, but with increased activity in post-synaptic neurones. Dissociations between c-Fos induction and BOLD activity are likely due to CB1 receptor negative coupling to adenylate cyclase in presynaptic terminals (Howlett 1985). Using radiolabelled [3H]CP55940, Herkenham and colleagues (1991) found CB1 heterogeneously expressed throughout the brain. Many areas activated following CP55940 (AcbC, AcbSh, VP, PVN, LH, VMN), contain only moderate to sparse densities of CB1 binding. Although expression is relatively low in these areas, functional studies using GTPαS binding suggest some CB1 receptors, particularly those in the hypothalamus, are more strongly coupled to G-proteins than in any other brain region (Breivogel et al. 1997). This offers an explanation why the relatively low doses of cannabinoid ligands used in this study can elicit significant functional responses in regions of low CB1 receptor expression.

Interpreting drug interactions in specific brain areas is difficult without further experimentation, but results can be put into the context of known circuitry. In “minimal
circuit analysis,” behavioural control columns (BCCs) for motivated behaviours, such as feeding, are regulated by cascading projections from the telencephalon (Swanson 2000). The BCC contains elements of the brainstem controlling feeding that are headed by the hypothalamus. Excitatory projections from the cortex to the BCC send collaterals to the striatum. The major output of the striatum, GABAergic medium spiny neurones (MSNs), send collaterals to the BCC and the pallidum. Finally, the pallidum sends an inhibitory input to the BCC, which is functionally a disinhibitory projection as it is inhibited by the striatum, which is in turn activated by the cortex. The basal ganglia and BCC also send feedback through thalamocortical projections. In this study, each of these areas showed significant changes in BOLD signal in response to CB₁ stimulation.

A cortico-striatal-hypothalamic pathway is relevant to feeding, since local injection of glutamate antagonist to block cortical input to the AcbSh produces a behaviourally selective hyperphagia (Maldonado-Irizarry et al. 1995, Kelley & Swanson 1997, Haberny et al. 2004). Injection of cannabinoids into the AcbSh also results in marked hyperphagia, which is antagonized by Rimonabant or AM251 (Kirkham et al. 2002, Soria-Gomez et al. 2007). In addition, intra-AcbSh cannabinoids increases c-Fos in the LH, suggesting the existence of a CB₁-mediated functional relationship between the AcbSh and feeding centres in the hypothalamus (Soria-Gomez et al. 2007). AcbSh GABAergic neurones receive glutamatergic afferents from the prefrontal cortex (Christie et al. 1985, Bennett & Bolam 1994), representing a critical link between cortex and striatum. However, it is not clear whether glutamatergic input is predominantly direct to MSNs or local GABAergic interneurones. Local interneurones do express mRNA for glutamic acid decarboxylase and CB₁ (Hohmann & Herkenham 2000). Furthermore, the interneurones have a significant inhibitory effect on MSNs (Jaeger et al. 1994, Kawaguchi et al. 1995, Koos & Tepper 1999). Since both glutamatergic and GABAergic synapses within the AcbSh possess CB₁ receptors that can inhibit synaptic transmission (Manzoni & Bockaert 2001, Robbe et al. 2001, Lopez-Moreno et al. 2008), it is possible that the local action of cannabinoids reduces the activity of interneurones leading to a disinhibition of MSNs. Since MSNs constitute approximately 90% of the AcbSh population (Tepper et al. 2004) this could result in the increases in BOLD activity and c-Fos noted here. Since a feature of CB1 receptors is that they are located on both glutamatergic and GABAergic terminals, one difficulty in interpreting function is that an agonist may have low-dose effects on one distinct population and high-dose effects on another.
The AcbSh is the only striatal region to send a direct projection to the LH, suggesting it has unique access to hypothalamic feeding centres (Groenewegen & Russchen 1984, Heimer et al. 1991, Kelley et al. 2005) and provides a link whereby reward/motivational circuits impinge on hypothalamic control of feeding. This study shows a decrease in BOLD signal and increase in c-Fos in the LH following CP55940. Furthermore, a recent electrophysiological study demonstrated that orexigenic effects of cannabinoids include a presynaptic CB₁-mediated suppression of GABAergic transmission to postsynaptic LH neurones (Jo et al. 2005). Taken together this suggests that cannabinoids could exert their orexigenic effects by disinhibiting LH neurones, which impart drive to other regions of the feeding BCC. If the LH neurones constitute the final pathway to the BCC, the effects of exogenous cannabinoids in upstream brain systems would have little bearing on the behavioural output.

The current study demonstrates CB₁ agonist-induced hyperphagia is reversed by pretreatment with a receptor inverse agonist. Using phMRI to identify specific areas where Rimonabant functionally antagonises the CP55940-induced BOLD signal, we have deciphered regions which are potentially responsible for cannabinoidergic effects on food intake. By complementing phMRI with c-Fos mapping, we highlight areas involved in the direct regulation of food intake (hypothalamus) and those concerned with reward and motivation (striatum, prefrontal cortex). We have demonstrated that phMRI can produce meaningful insights into behavioural function, and that it is a powerful tool for investigating both drug mechanisms and functional neural circuits at the whole-brain level.
3.6 Supplemental Data

3.6.1 Blood-gas measurement

Eighteen rats (255 ± 22g) were assigned randomly to receive injections of either vehicle, 0.06 mg/kg body weight CP55940, or 1 mg/kg body weight Rimonabant. Animals were prepared with α–chloralose anesthesia in the same way as that described in the phMRI experiment. For the placement of the blood-gas electrode, the fur around the xiphoid process was shaven to a 5 cm diameter with an electric razor. Depilatory cream (Veet, Clevedon, UK) was applied for 5 minutes and, after removal of the cream, the skin was carefully cleaned with alcohol wipes. The electrode was fixed to the dry skin using an adhesive fixation ring (15 mm outer diameter), and filled with contact fluid containing 1,2-propanediol and de-ionised water. Blood gases were continuously monitored using a transcutaneous blood-gas analysing system (model TINA TCM4; Radiometer Copenhagen, Willich, Germany). This system uses a combined Stowe–Severinghaus (for pCO$_2$ measurement) and Clark-type polarographic (for pO$_2$ measurement) electrode. The electrode working temperature was 44 °C and the system was calibrated each time prior to positioning, using a standard calibration gas containing 7.5 % CO$_2$, 20.9 % O$_2$ and 71.6 % N$_2$ (Radiometer Copenhagen). The SmartHeat function of the system was used to add 1 °C to the selected electrode temperature in the first 5 minutes after placement to ameliorate CO$_2$ and O$_2$ diffusion at the beginning of the measurement. A 15 minutes baseline period (100 %) was acquired once each value reached a stable plateau. pO$_2$ and pCO$_2$ values were acquired every 10 seconds for a further 45 minutes and normalised to the baseline period. Results are expressed as mean ± S.E.M. percentage change from baseline for each time point.

3.6.2 Results and Discussion

One of the major physiological parameters that has a substantial detrimental effect on the neurovascular and neurometabolic basis of the BOLD response is hypo- and hypercapnia (Ogawa et al. 1990, Ogawa et al. 1993, Mandeville et al. 1998, Silva et al. 1999, Cohen et al. 2002). A key study by Ramos-Cabrер et al (2005) demonstrated that BOLD contrast in the somatosensory cortex of anaesthetised rats following stimulation is masked if tcpCO$_2$ levels exceed 20% of the baseline (Ramos-Cabrер et al. 2005). Although slight variations between the treatment groups are evident over the time course of this experiment, no changes in
tcpCO₂ (Sup Fig. 1.) or tcpO₂ (Sup Fig. 1b.) following treatment exceed beyond 20% of baseline levels.

Supplementary Figure. 3.1. Line graph illustrating time course of tcpCO₂ (a) and tcpO₂ (b) following intraperitoneal administration of vehicle, CP55940 (0.06 mg/kg body weight), or Rimonabant (1 mg/kg body weight) in α-chloralose anaesthetized rats (n = 6). Vertical dotted line represents drug injection. Results are presented as mean percentage change relative to baseline values (100%). Horizontal dotted line represents threshold level at which BOLD signal is lost if tcpCO₂ levels exceed 20% above baseline (Ramos-Cabrer et al. 2005). Error bars show S.E.M.
3.7 References


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Chapter 3 – Central cannabinoid signaling mediating food intake: a pharmacological-challenge MRI and functional histology study in rat


Ward, S. J. and Dykstra, L. A. (2005) The role of CB1 receptors in sweet versus fat reinforcement: effect of CB1 receptor deletion, CB1 receptor antagonism (SR141716A) and CB1 receptor agonism (CP-55940). *Behav Pharmacol*, 16, 381-388.


Chapter 4

The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice

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The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice
4.1 Abstract

Hemopressin is a short, nine amino acid peptide (H-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-OH) isolated from rat brain that behaves as an inverse agonist at the cannabinoid receptor CB1, and is shown here to inhibit agonist-induced receptor internalization in a heterologous cell model. Since this peptide occurs naturally in the rodent brain, we determined its effect on appetite, an established central target of cannabinoid signalling. Hemopressin dose-dependently decreases night-time food intake in normal male rats and mice, as well as in obese ob/ob male mice, when administered centrally or systemically, without causing any obvious adverse side effects. The normal, behavioural satiety sequence is maintained in male mice fasted overnight, though re-feeding is attenuated. The anorectic effect is absent in CB1 receptor null mutant male mice, and hemopressin can block CB1 agonist-induced hyperphagia in male rats, providing strong evidence for antagonism of the CB1 receptor in vivo. We speculate that hemopressin may act as an endogenous functional antagonist at CB1 receptors and modulate the activity of appetite pathways in the brain.
4.2 Introduction

Hemopressin is a product of the hemoglobin α chain, discovered in rat brain using an enzyme-substrate capture technique and so named as it can cause small decreases in blood pressure (Rioli et al. 2003, Lippton et al. 2006). Subsequently, hemopressin was found also to have non-opioid antinociceptive effects (Dale et al. 2005). In vitro studies show that the peptide acts as a CB$_1$ receptor inverse agonist, and can interact with both peripheral and central pain pathways in vivo (Heimann et al. 2007). To date, all known endogenous cannabinoids, such as 2-arachidonoylglycerol and anandamide, are fatty acid derivatives (Bisogno 2008, Petrosino et al. 2009). These endocannabinoids are released by postsynaptic neurones “on demand”, following the Ca$^{2+}$ influx produced in response to postsynaptic depolarisation or activation of metabotropic receptors (Kano et al. 2009). When released into the synaptic cleft, endocannabinoids activate presynaptic CB$_1$ receptors, and impart an inhibitory action on further presynaptic transmission. The administration of exogenous CB$_1$ agonists, such as Δ$^9$-tetrahydrocannabinol (THC, the active ingredient of Cannabis sativa), or the synthetic compounds CP55940 and WIN 55212-2, increase food intake by increasing motivational reward (Cota et al. 2003a, Pagotto et al. 2006). By comparison, the synthetic compound, rimonabant (SR141716A), is an inverse agonist at the CB$_1$ receptor and is capable of producing weight-reducing effects over extended periods in rodents and humans (Van Gaal et al. 2005, Di Marzo 2008). The action of rimonabant to reduce specifically motivational appetite is relatively short lived, and any continued weight loss is thought to be mediated mainly via peripheral CB$_1$ interaction with lipid mobilisation pathways in adipose tissue and liver, energy expenditure and cellular glucose uptake (Di Marzo 2008, Nogueiras et al. 2008, Kunos et al. 2009).

We hypothesize that hemopressin may be a naturally-occurring inverse agonist of brain CB$_1$ receptors, capable of antagonising central orexigenic pathways.

4.3 Methods

4.3.1 Cell culture and transfection

COS-7 Monkey Kidney Fibroblasts cells (Invitrogen, Paisley, UK) were grown on coverslips in a 24-well plate, in a medium of DMEM containing 10 % foetal bovine serum and 1 % penicillin-streptomycin. Throughout the experiment cells were kept under 5 % CO$_2$ in air at 37 °C and passage numbers P1-P20 of undifferentiated cells were used for experiments. At
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. Cells were transfected with pEGFP-N1-CB1 plasmid (mouse CB1 cDNA was cloned into a pEGFP-N1 vector which encodes the GFPmut1 variant (Clontech Labs, CA, USA), leading to an eGFP fusion at the C-terminus of CB1) using Lipofectamine according to the manufacturer’s protocol (Invitrogen, Paisley, UK). Following an overnight transfection, the growth medium was changed, and cells were treated with vehicle (0.25 % DMSO), 100 nM AM251, 100 nM, 10 μM, and 100 μM hemopressin, in the absence, or presence of 100 nM WIN 55212-2 (all Tocris Bioscience Ltd., Brighton, UK). Cells were stimulated with drugs for 2 h, and then fixed in an ice-cold solution of 4 % paraformaldehyde, 4 % sucrose in 0.1 M phosphate buffer for 45 min. Slides were coverslipped using VectorShield hard set (Vector Labs, Peterborough, UK) containing 4’,6-diamidino-2-phenylindole (DAPI) to stain cell nuclei. Images of transfected cells were viewed by an experimenter blinded to treatment group using an Olympus BX51 upright microscope with a 60x/1.4 UPlanApo objective. Images were captured at random using a Cool snap ES camera (Photometrics, AZ, USA) through MetaVue Software (Molecular Devices, PA, USA). Specific band pass filter sets for DAPI (excitation λ, 360-370 nm, emission λ, 420-460), and eGFP (excitation λ, 480/40 nm, emission λ, 535/50) were used to prevent bleed through from one channel to the next. 50 cells per treatment group were analyzed to quantify the number of internalized endosomes per cell. To determine an IC50 value for hemopressin, we set up a similar experiment, but cells were treated with hemopressin over a nine point log dilution series (100 μM, 10μM, 1μM, 100 nM, 10nM, 1nM, 100pM, 10pM, 1pM) in the presence of 100 nM WIN 55212-2. 40 cells per treatment group were analyzed to quantify the number of internalized endosomes per cell. The percentage inhibition of internalization was calculated relative to the control situation of 100 nM WIN 55212-2 alone.

### 4.3.2 Animals

All experiments (except those using ob/ob or CB1 receptor knockouts) were carried out on adult, male outbred CD1 mice and male, outbred Sprague-Dawley rats (Charles River Laboratories Inc, Sandwich, UK). The male ob/ob mice, homozygous for the obese spontaneous mutation, Lepob, are backcrossed with a C57BL/6N background (B6.V-Lepob/J, Jackson Laboratories, ME, USA). CB1+/− and CB1−/− littermate mice were obtained by breeding of heterozygotes that had been backcrossed six times to a C57BL/6N background, as described previously (Marsicano et al. 2002). All animals were housed under a 12:12 h light/dark cycle (lights on 08.00-20.00), at 22 °C ± 1 °C and 45 ± 10 % humidity. Pelleted food (Beekay International, UK) and water were available ad libitum unless stated otherwise.
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. Experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and local ethical review. The experiment involving the CB1 knockout mice was conducted in accordance to the Guide for the Care and Use of Laboratory Animals of the local Government of Rheinland-Pfalz, Germany.

4.3.3 **Intracerebroventricular surgery**

Under 2% isoflurane (Concord Pharmaceuticals Ltd, Essex, UK) in 1 l/min oxygen, mice and rats were implanted stereotaxically with guide cannulae into the right lateral ventricle (0.2 mm posterior, 1 mm lateral from bregma for mice, and 0.8 mm posterior, 1.5 mm lateral from bregma for rats) according to the atlas of Paxinos and Watson (Paxinos & Watson 1998). The tip of the guide cannula was positioned 1 mm above the injection site (1 mm (mice), and 3 mm (rats) ventral to the surface of the skull). All animals were allowed to recover from surgery for 5 - 7 days before the start of experiments.

4.3.4 **Hemopressin effects on nocturnal feeding behaviour in mice and rats**

All mice and rats were housed singly at least three days prior to the experiment and food was restricted 3 hours before the experiment was due to start. At lights out (20:00), animals were fed pre weighed chow ad libitum. In one experiment, 18 mice (31 ± 1.8g, n = 6) were assigned randomly to receive intraperitoneal (i.p.) injection of vehicle (0.9% w/v NaCl, 10% DMSO, 20% 2-hydroxypropyl-β-cyclodextrin), 500 nmol/kg hemopressin, or 5.4 μmol/kg AM251 (3mg/kg, based on dose described by Tallett et al. 2007a). Injections were made in a volume of 2 ml/kg body weight.

Food intake was determined 1, 2, 4 and 24 hours post injection. Results are presented as mean ± standard error of mean (S.E.M.) for food intake at each time point. Treatments were compared using a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test using the GraphPad Prism statistical package (GraphPad Software, Inc. San Diego, CA, USA). In a second experiment, 24 intracerebroventricular (i.c.v.) cannulated CD1 mice (30 ± 1.4g, n = 6) were assigned randomly to receive i.c.v. injection of vehicle (0.9% w/v NaCl), 1 nmol, 5 nmol or 10 nmol hemopressin. Injections were made in a volume of 2 μl per animal. Treatments were compared using a one-way ANOVA followed by Dunnett’s multiple comparison post hoc test. In a third experiment, 12 i.c.v. cannulated rats (320 ± 12g, n = 6) were assigned randomly to receive i.c.v vehicle (0.9% w/v NaCl) or 10 nmol.
hemopressin. Injections were made in a volume of 2 μl per animal. Treatments were compared using a two tailed t-test.

4.3.5 Effects of hemopressin on feeding behaviour in CB\textsubscript{1} knock out mice

Twelve male CB\textsubscript{1}/- mice and 12 wild-type littermates (26 ± 2.1g) were housed singly one week prior to the experiment. Since the two genotypes normally display significantly different body weights (Cota et al. 2003b) and, therefore, food intake, all the mice were fasted overnight before the start of the experiment. One hour after lights on (08:00) CB\textsubscript{1}/- and CB\textsubscript{1}/+/- animals were assigned randomly to receive i.p. injection of either vehicle or 500 nmol/kg hemopressin in a volume of 2 ml/kg (n = 5/6). Food intake was determined 1, 2, 4 and 12 hours post injection. Treatments were compared using a two-way ANOVA followed by Bonferroni’s multiple comparison post hoc test.

4.3.6 Effects of hemopressin on feeding behaviour in leptin deficient (ob/ob) mice

Fourteen obese ob/ob mice (38 ± 3.5g, n = 7) were assigned randomly to receive i.p. vehicle (0.9% w/v NaCl) or 500 nmol/kg hemopressin. Injections were made in a volume of 2 ml/kg body weight. Food intake was determined 1, 2, 4 and 24 hours post injection. Treatments were compared using a two tailed t-test.

4.3.7 Hemopressin effects CB\textsubscript{1} agonist (CP 55940)-induced hyperphagia in rats

Twenty-four Sprague-Dawley rats (320 ± 18g, n = 5/6) were cannulated into the lateral ventricle under recovery anaesthesia one week before experimentation. Rats were housed singly at least three days prior to the experiment and food was restricted 3 hours before the experiment was due to start. At lights off (20:00) animals were assigned randomly to receive i.c.v vehicle (0.9 % w/v NaCl) or 10 nmol hemopressin. Injections were made in a volume of 2 μl per animal. 20 minutes later, rats received i.p. vehicle (0.9 % w/v NaCl, 2.5 % ethanol) or 0.06 mg/kg CP55940 (Tocris Bioscience Ltd., Brighton, UK) in a volume of 1 ml/kg. The dose of CP55940 was determined in previous published experiments (Dodd et al. 2009). Upon second injection, animals were fed pre-weighed chow ad libitum. Treatments were compared using a one-way ANOVA followed by Bonferroni’s multiple comparison post hoc test.
4.3.8 Behavioural satiety sequence

CD1 mice were transferred to transparent cages three days prior and fasted overnight before the start of the experiment. In one experiment, 16 mice (32 ± 1g, n = 8) were assigned randomly to receive i.p. vehicle or 500 nmol/kg hemopressin in a volume of 2 ml/kg. In a second experiment, 14 mice (30 ± 1.4g, n = 7) were assigned randomly to receive i.c.v. injection of either vehicle or 10 nmol hemopressin in a volume of 2 μl per animal. In a third experiment, 18 mice (32 ± 1.6g, n = 6) were assigned randomly to receive i.p. injection of vehicle (0.9% w/v NaCl, 10% DMSO, 20% 2-hydroxypropyl-β-cyclodextrin), 500 nmol/kg hemopressin, or 5.4 μmol/kg AM251. Following injections, pre-weighed food was presented and the animals were left undisturbed for 90 minutes. Behaviour was scored using momentary time sampling, every 30 seconds for the 90-minute period, after which point food intake was measured (Lawrence et al. 2002, Scott et al. 2005). Food intake was measured again at 3 hours and 24 hours post injection. The behaviours were scored, zero or one, according to the following classifications: feeding (animal at hopper trying to obtain food, chewing, or gnawing), drinking (animal licking at the water spout), grooming (animal scratching, biting or licking any part of its anatomy), resting (animal curled up, resting head with eyes closed), active (animal showing activity, including locomotion, sniffing, rearing), or inactive (animal immobile when aware, or signs of sickness behaviour). Data was collected into 5-minute period bins for display of the group behaviour. Several variables were analysed: food intake, latency to rest (i.e. the time at which animals first displayed resting), the transition from eating to resting (the time bin when the frequency of eating within the group matches the frequency of resting) and the average percentage of time the animals spent in each of the recorded behaviours.

4.4 Results

4.4.1 Hemopressin blocks agonist (WIN 55212-2)-induced eGFP-CB₁ receptor internalization

Previous receptor internalization studies on cultured cells have demonstrated that tagged CB₁ receptors, the vast majority of which are expressed on the plasma membrane under unstimulated conditions, show rapid and persistent endocytosis in response to stimulation with a CB₁ receptor agonist (Hsieh et al. 1999, Coutts et al. 2001, Daigle et al. 2008, Blair et al. 2009). This receptor internalization can be blocked by co-treatment with CB₁ receptor inverse agonists (Hsieh et al. 1999, Coutts et al. 2001). In the present study, we confirmed a direct action of hemopressin on CB₁ receptors by in vitro eGFP-CB₁ internalization assay, in
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. We compared the action of hemopressin with the well-characterized CB1 inverse agonist, AM251, in antagonizing the actions of the agonist WIN 55212-2 (Hsieh et al. 1999). (Fig. 4.1). 2 h treatment of transfected cells with WIN 55212-2 caused a significant increase in eGFP-CB1 receptor internalization. ( Bars represent mean and S.E.M.; n = 50 cells per treatment). **P < 0.01, one-way ANOVA/Dunnett’s post hoc test. Abbreviations: AM251 (AM), hemopressin (HEM), vehicle (VEH), WIN 55212-2 (WIN).
internalization of eGFP-CB₁ receptor into endosomes ($P < 0.01$, Fig. 4.1 b, e). This effect was blocked by co-administration of either AM251 or increasing doses of hemopressin ($IC_{50} = 1.55\mu M$, Fig. 4.1 d, e, supplementary Fig. 4.1). This result supplements other in vitro models demonstrating the action of hemopressin on CB₁ receptors (Heimann et al. 2007). Treatment of transfected cells with either AM251 or hemopressin alone did not cause any internalization of eGFP-CB₁ receptor into endosomes (Fig. 4.1 b, e).

4.4.2 Centrally administered hemopressin results in marked hypophagia in rats and mice

Rimonabant is a well-characterized inverse agonist at the CB₁ receptor and can act in the brain to reduce appetite (Colombo et al. 1998, Di Marzo et al. 2001, Pagotto et al. 2006, Nogueiras et al. 2008). Thus, we proposed that hemopressin might have the same effect. We found that i.c.v. administration of hemopressin caused a dose-dependent decrease of nighttime food intake in freely-behaving, outbred mice and rats. For mice, a dose of 10 nmol per animal, significantly decreased food intake one ($P < 0.05$), two ($P < 0.01$), and four hours post injection ($P < 0.05$, Fig. 4.2a and supplementary Fig. 4.2), whereas for rats, the same dose significantly decreased food intake one hour post injection ($P < 0.05$, Fig. 4.2b). For both species, these doses of hemopressin caused no medium-term adverse effects on feeding behaviour, as cumulative food intake normalized over the following 12-hour period (supplementary Fig. 4.2 for mice; supplementary Fig. 4.3 for rats).

4.4.3 Hypophagia produced by systemic administration of hemopressin is absent in CB₁⁻/⁻ mice

Since hemopressin is a relatively small peptide and appears to be able to cross the blood-brain barrier (Heimann et al. 2007), we next tried systemic (i.p.) administration in outbred mice. Again, hemopressin caused a decrease in normal, nocturnal feeding with a significant effect at 2 hours post injection, comparable to that of the synthetic CB₁ inverse agonist AM251 (hemopressin $P < 0.05$, AM251 $P < 0.01$ Fig. 4.3a). This slight delay in action of hemopressin was observed in repeated experiments and might reflect the peptide accessing sites of action within the brain. Cumulative food intake normalized over the following 12-hour period (supplementary Fig. 4.4). The dose of AM251 was based on behavioural effects (Tallett et al. 2007a), rather than on comparative CB₁ efficacy. This systemic dosing was repeated in over-night fasted, wildtype (CB₁⁺/⁺) and CB₁ receptor knockout (CB₁⁻/⁻) mouse littermates (Marsicano et al. 2002).
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.

Figure 4.2. a) Hemopressin caused a dose-dependent decrease in normal, night-time feeding when administered i.c.v. to outbred mice (n = 6). The response to 10 nmol/animal was significant within 1 hour (*p < 0.05, **p < 0.01; ANOVA/Dunnett’s post hoc test). b) A similar, rapid decrease in food intake was measured in outbred rats when the peptide was injected i.c.v. (n = 6, *p < 0.05; t-test). Data from additional time points are available in Supplementary Figs. 1 and 2. Abbreviations; hemopressin (HEM), vehicle (VEH).

Since the two genotypes have significantly different average body weights, results are expressed as food intake per gram body weight. The fact that i.p. hemopressin decreased food intake in fasted wildtype mice 2 hours post injection (P < 0.05, Fig. 4.3b), shows that it is capable of overcoming a powerful, natural orexigenic drive. This response is lost in the CB1⁻/⁻ mice (Fig. 4.3b), demonstrating that the effect is mediated in vivo by CB1 cannabinoid receptors. Cumulative food intake normalized over the following 12-hour period (supplementary Fig. 4.5).

4.4.4 Systemic administration of hemopressin causes hypophagia in ob/ob mice

Homzygous ob/ob mice are deficient in leptin and express an obese, hyperglycaemic and hypophagic phenotype, with elevated endocannabinoid tone in the hypothalamus (Di Marzo et al. 2001). Like Rimonabant in previous studies (Di Marzo et al. 2001), systemic administration of hemopressin causes marked hypophagia at both 1h and 2h post injection (P < 0.05, Fig. 4.3c). Cumulative food intake normalized over the following 12-hour period (supplementary Fig. 4.6).
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.

**Figure 4.3.**

a) When mice were injected with hemopressin (500 nmol/kg, i.p.) there was a slight delay to full effect, which was not significant until 2 h. \( n = 6, \ *p < 0.05 \); one-way ANOVA/Dunnett’s post hoc test. Hemopressin hypophagic effects are comparable to that of the synthetic CB1 inverse agonist, AM251 (5.4 µmol/kg, i.p.) at 2 h post injection \( **p < 0.01 \); one-way ANOVA/Bonferroni’s post hoc test). b) To demonstrate that the effect of hemopressin is mediated by cannabinoid receptors, 500 nmol/kg was injected i.p. into wildtype (wt, CB1\( ^{++} \)) and null mutant (CB1\( ^{-/-} \)) littermates \( n = 6 \). As the two mouse genotypes have significantly different body weights, the data are expressed as grams of food eaten per gram body weight. Hemopressin reduced food intake in the wildtype, but not CB1\( ^{-/-} \) knockout mice. \( *p < 0.05 \); two-way ANOVA/Bonferroni). c) The hypophagic effects of hemopressin (500 nmol/kg, i.p.) are also present when administered systemically to leptin-deficient, obese ob/ob mice at both 1 h and 2 h post injection \( n = 7, \ *p < 0.05 \); two tailed t-test). Abbreviations; AM251 (AM), hemopressin (HEM), vehicle (VEH).
4.4.5 Hemopressin can functionally antagonise CB₁ agonist (CP 55940)-induced hyperphagia

We and others have shown previously that the CB₁ receptor inverse agonist, rimonabant, can functionally antagonise the orexigenic effect of CB₁ receptor agonists, such as CP55940 (Dodd et al. 2009). To avoid complications with repeated injections in mice, this experiment was carried out in rats. A significant increase in food intake was seen 1 h following CP 55940 administered alone ($P < 0.05$, Fig. 4.4) and a marked attenuation of this orexigenic drive was observed in the presence of hemopressin. At 2 h post injection, a significant decrease in food intake was noted following hemopressin administration when compared with controls at the same time point ($P < 0.05$, Fig. 4.4), and this was significantly attenuated in the presence of CP55940. Cumulative food intake normalized over the following 12-hour period (supplementary Fig. 4.7).

![Figure 4.4](image)

**Figure 4.4.** To demonstrate that the feeding effect of a CB₁ receptor agonist can be blocked pharmacologically, 10 nmol hemopressin (i.c.v) was co-administered with 0.06 mg/kg CP55940 (i.p.). (n = 5/6, *p < 0.05 compared with vehicle/vehicle group. #p < 0.05 compared with hemopressin/CP55940 group; one-way ANOVA/Bonferroni). Abbreviations: hemopressin (HEM), vehicle (VEH).

4.4.6 Hemopressin does not disrupt the behavioural satiety sequence

To demonstrate that hemopressin is reducing food intake without causing any adverse effects, such as nausea, aversion or sedation, we demonstrated that treated mice display a normal behavioural satiety sequence (BSS). Singly-housed mice, which have their food temporarily removed, display a stereotypic sequence of behaviours when food is returned: eating and drinking, through exploration and grooming, before curling up to sleep (Halford et al. 1998). Any factor reducing appetite because of an abnormal, adverse effect will disrupt this sequence, whereas a natural satiety factor will maintain the sequence but shift it “leftwards.” Indeed, there is evidence that rimonabant and its derivative, AM251, reduce
food intake, but also increase scratching in rodent models, probably by an off-target action on opioid receptors (Tallett et al. 2007a, Tallett et al. 2007b, Tallett et al. 2008), also see Fig. 4.6).

During the 90-min test period, mice treated i.p. with hemopressin spent significantly less time feeding and ate significantly less food than controls (both $P < 0.05$; supplementary Table 4.1). No differences were seen between the groups for the average percentage of time spent in the other recorded behaviours. Furthermore, no other unusual behaviours (e.g. excessive scratching, immobility or sickness behaviour) were noted. Similar results were recorded for i.c.v. injection of hemopressin, though here the reduction in time spent feeding did not reach statistical significance (supplementary Table 4.1). When plotted against time, the group receiving vehicle i.p. displayed a normal BSS (Fig. 4.5a). Importantly, hemopressin did not disrupt the sequence, suggesting that it is not reducing feeding by causing any adverse reactions. However, as previously noted for natural satiety factors (Lawrence et al. 2002, Scott et al. 2005), there was an apparent shift of the sequence to the left following hemopressin (Fig. 4.5c, d). The point of transition from eating to resting took place in time bin 8 for mice given hemopressin compared with time bin 10 for controls. The average latency to rest for mice given hemopressin i.p. was found to be significantly shorter than controls (vehicle, $73 \pm 2$ min versus hemopressin, $53 \pm 5$ min; $P < 0.01$; supplementary Table 4.1). The maintenance of the BSS and its shift leftwards are important, therefore we wished to compare this result to that of AM251 which, as with rimonabant, is reported to have an off-target adverse effect in rodents (Tallett et al. 2007a, Tallett et al. 2007b, Tallett et al. 2008). Both hemopressin and AM251 caused a decrease in feeding (Fig. 4.6). However, as reported previously, AM251 caused a significant increase in scratching. No such unusual behaviours were recorded following hemopressin administration.
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. Figure 4.5. Effects of i.p. hemopressin on the BSS. Overnight fasted mice were presented with food following systemic administration of either a) vehicle or b) hemopressin (500 nmol/kg, n = 8). Behaviour was then monitored every 30 seconds for 90 minutes and registered as feeding, drinking, active, grooming, inactive and resting. Data were collected into 5-minute time bins and are presented as percentage of total behaviour. Crossover graphs indicating the point of transition from eating to resting for mice treated with vehicle c), and d) hemopressin. The dashed line represents the time bin in which groups spent an equivalent amount of time eating and resting.
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.
4.5 Discussion

Our results demonstrate that hemopressin, a peptide which acts selectively as an inverse agonist at the CB₁ receptor (Heimann et al. 2007) can: 1) antagonize CB₁ agonist-induced internalization of the CB₁ receptor \textit{in vitro}; 2) induce hypophagia \textit{in vivo} when administered centrally; 3) induce hypophagia \textit{in vivo} when administered systemically, but only in mice with functional CB₁ receptors; 4) can overcome powerful orexigenic drives in fasted or obese mice, and 5) reduce feeding in a behaviourally specific manner.

The endocannabinoid system has diverse roles in cognition, memory, anxiety, motor behaviour, nociception and appetite (Svizenska et al. 2008). Numerous studies have described the orexigenic action of the lipid-based endogenous CB₁ agonists, such as anandamide and 2-arachidonoylglycerol, on feeding behaviour and appetite regulation (Williams & Kirkham 1999, Hao \textit{et al.} 2000, Jamshidi & Taylor 2001, Kirkham \textit{et al.} 2002). An abundance of synthetic compounds also have been synthesised to interfere with cannabinoid CB₁ transmission in attempts to exploit the therapeutic potential offered by targeting this diverse neurotransmitter system. For example, rimonabant has acute central effects on appetite and continuing actions on body weight probably via peripheral interaction with lipid mobilization pathways in white adipose tissue and with cellular glucose uptake systems (Colombo \textit{et al.} 1998, Di Marzo \textit{et al.} 2001, Nogueiras \textit{et al.} 2008). However, the US Food and Drug Administration rejected rimonabant because clinical trials suggested a higher incidence of depression, anxiety and suicidality following prolonged administration (Christensen \textit{et al.} 2007, Nissen \textit{et al.} 2008). Furthermore, in this and in previous studies assessing the behavioural satiety sequence after either rimonabant or its derivative, AM251, reductions in feeding have been associated with off-target actions (probably opioid mediated) leading to excessive scratching (Tallett \textit{et al.} 2007a, Tallett \textit{et al.} 2007b, Tallett \textit{et al.} 2008). By comparison, our behavioural studies have not found any similar adverse reactions in response to hemopressin, either in the short or medium term. Further studies will need to be carried out to determine if hemopressin has any long-term deleterious effects on motivation, or advantageous effects on peripheral metabolism.

Our findings are consistent with other reports showing that synthetic receptor inverse agonists can exhibit hypophagic effects mediated via CB₁ receptors, when administered either centrally or systemically (Arnone \textit{et al.} 1997, Simiand \textit{et al.} 1998, Di Marzo \textit{et al.} 2001,
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice (Rowland et al. 2001, Verty et al. 2004b, Ward & Dykstra 2005). Hemopressin, like rimonabant, can functionally antagonize CB1 agonist-induced hyperphagia (Williams & Kirkham 2002, Dodd et al. 2009) and it is capable of overcoming powerful orexigenic drives in fasted animals. Like rimonabant, hemopressin can also overcome the orexigenic drive produced in leptin deficient, ob/ob mice (Di Marzo et al. 2001). As either fasted mice or leptin-deficient mice are known to have elevated hypothalamic endocannabinoid levels (Di Marzo et al. 2001, Kirkham et al. 2002), the possibility remains that hemopressin may be acting as an neutral antagonist against heightened endocannabinoid tone rather than as an inverse agonist.

The central mechanisms underlying CB1-mediated effects on appetite are unclear. However, a large body of evidence suggests that CB1 receptors may interact not only directly with the known feeding-related circuitry of the hypothalamus but, also, may impinge on dopaminergic and opioid signaling in the striatum which are known to mediate the motivational and rewarding aspects of feeding behaviour (Cota et al. 2003a, Cota et al. 2006, Kirkham 2009). This is further suggested by the ability of CB1 ligands and fatty acid amide hydrolase inhibitors, to elicit robust feeding responses when administered directly into the nucleus accumbens or into nuclei of the hypothalamus (Williams CM 1999, Kirkham et al. 2002, Verty et al. 2005, Soria-Gomez et al. 2007). Interestingly, a number of these studies found no effects of intra-accumbens injection of rimonabant or AM251 on food intake, suggesting that that feeding-related effect of CB1 inverse agonism may depend substantially on an integrated response throughout the forebrain (Werner & Koch 2003, Verty et al. 2004b, Verty et al. 2004a). A recent functional magnetic resonance imaging study in rats showed that regions of the orbitofrontal cortex, striatum (particularly the nucleus accumbens) and the hypothalamus, are functionally responsive to orexigenic or anorectic doses of opposing CB1 ligands (Dodd et al. 2009). An interesting result from the current study is that hemopressin may also act on satiety pathways, perhaps in the brainstem, or via peripheral CB1 receptors in the gut, since it caused a slight advance (leftwards shift) of the behavioural satiety sequence (Gomez et al. 2002).

The expression and functional profile of hemopressin in the brain is yet to be fully elucidated. Recent studies have described the location of hemoglobin α chain mRNA and protein in rat and human neurones, including those in the dopaminergic system (Richter et al. 2009, Schelshorn et al. 2009). Therefore, it is possible that hemopressin, which is derived
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice (Rioli et al. 2003, Lippton et al. 2006, Heimann et al. 2007), may be produced within pathways involved in motivated behaviour. Furthermore, a very recent paper has described N-terminally extended hemopressin sequences which can act as CB1 agonists in vitro (Gomes et al. 2009), while a precedent has already been set for functional opioidergic peptides derived from the hemoglobin β chain (Nyberg et al. 1997). Such biologically active peptides are not processed by the vesicular secretory pathway, so it is yet to be determined if their release can be regulated. As the known, lipid-based endocannabinoids are produced “on demand,” similar processes may regulate the production of small, bioactive peptides, as has been seen with some interleukins (Simi et al. 2007).

Hemopressin is a novel bioactive peptide found in the brain that is capable of functionally antagonizing the actions of endogenous cannabinoid receptor agonists and may be placed to act as a natural suppressant of hedonically-motivated eating. Indeed, the precedent for mutually antagonistic pathways containing receptor agonists and inverse agonists that can subtly modulate food intake (viz α-MSH and agouti-related peptide which antagonize each other at melanocortin receptors) already exists (Lu et al. 1994, Ollmann et al. 1997, Pritchard et al. 2004), and may indicate the existence of such mutual antagonism as a common feature in central appetite regulatory systems.
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.

### 4.6 Supplemental Data

#### 4.6.1 Supplementary Figures

**Supplementary Figure 4.1.** Hemopressin action to inhibit CB1 agonist (WIN 55212-2)-induced eGFP-CB1 receptor internalization. The percentage inhibition of internalization was calculated relative to the control situation of 100 nM WIN 55212-2 alone. The IC\textsubscript{50} value of inhibition for hemopressin is approximately 1.55 \( \mu \text{M} \). (Bars represent mean and S.E.M.; \( n = 40 \) cells per treatment).

**Supplementary Figure 4.2.** Total food intake at 1 h, 2 h, 4 h, and 12 h post injection of CD1 mice treated with vehicle, 1 nmol, 5 nmol, and 10 nmol / animal hemopressin. All treatments were administered intracerebroventricularly in a volume of 2 \( \mu \text{l} \). (Bars represent mean and S.E.M.; \( n = 5/6 \)). *\( P < 0.05 \), **\( P < 0.05 \) ANOVA, post hoc Dunnett’s test.
Supplementary Figure 4.3. Total food intake at 1 h, 2 h, 4 h, and 12 h post injection of rats treated with vehicle, 10 nmol / animal hemopressin. All treatments were administered intracerebroventricularly. (Bars represent mean and S.E.M.; n = 6). *P < 0.05 two-tail unpaired t-test.

Supplementary Figure 4.4. Total food intake at 1 h, 2 h, 4 h, and 12 h post injection of CD1 mice treated with vehicle, hemopressin (500 nmol/kg), and AM251 (5.4 μmol/kg). All treatments were administered intraperitoneally. (Bars represent mean and S.E.M.; n = 6). *P < 0.05, ANOVA, post hoc Dunnett’s test. Abbreviations; AM251 (AM), hemopressin (HEM), vehicle (VEH).
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.

**Supplementary Figure 4.5.** Total food intake per gram of body weight at 1 h, 2 h, 4 h and 12 h post injection of fasted wild-type (wt) or CB1<sup>-/-</sup> mice treated with either vehicle or hemopressin (500 nmol/Kg, i.p.). (Bars represent mean and S.E.M; n = 5/6). *P < 0.05, two-way ANOVA, post hoc Bonferroni’s test.

**Supplementary Figure 4.6.** Total food intake at 1 h, 2 h, 4 h and 12 h post injection of obese ob/ob mice treated with either vehicle or hemopressin (500 nmol/kg, i.p.). (Bars represent mean and S.E.M; n = 7). *P < 0.05, two-way ANOVA, post hoc Dunnett’s test. Abbreviations; hemopressin (HEM), vehicle (VEH).
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. **Supplementary Figure 4.7.** Graph illustrating total food intake at 1 h, 2 h, 4 h and 12 h post injection of rats treated with vehicle / vehicle, hemopressin / vehicle (10 nmol/animal, i.c.v.), vehicle / CP 55940 (0.06mg/kg, i.p.), or hemopressin / CP55940. Bars represent mean and S.E.M. One-way ANOVA followed by Bonferroni’s multiple comparison post hoc test. *P < 0.05 compared with vehicle / vehicle group. **P < 0.05 compared with HEM / CP55940 group, (n = 5-6/group). Abbreviation; hemopressin (HEM)

### 4.6.2 Supplementary Table

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**Supplementary Table.** Group data from the behavioural satiety experiment. Mice were fasted overnight and re-fed the following morning. Food intake was measured at the end of the 90 min observation period and was significantly reduced in both experiments. Percentage time spent in each of the defined behaviours is compared for the two groups. For the intraperitoneal injection, there was a significant decrease in the percentage time spent feeding. This did not quite reach statistical significance for the intracerebroventricular injections. Latency to rest was reduced in both experiments. No other unusual behaviours, such as excessive scratching, were observed following treatment with hemopressin. (n = 8, *p < 0.05, **p < 0.01, two-tailed t test).
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.
The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice. Hemopressin is an inverse agonist of CB1 cannabinoid receptors. *Proc Natl Acad Sci U S A*, **104**, 20588-20593.


The peptide hemopressin acts through CB1 cannabinoid receptors to reduce food intake in rats and mice.


Chapter 5

General Discussion
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5.1 General Discussion

Despite Rimonabant being an effective anti-obesity agent, its recent rejection by the US Food and Drug Administration (FDA) and European Agency for the Evaluation of Medical Products (EMEA), has eroded the perception of the therapeutic importance of targeting the cannabinoid system in the fight against obesity. The FDA and EMEA rejected Rimonabant as clinical trials suggest a higher incidence of depression, anxiety and suicidality following prolonged administration of the drug (Christensen et al. 2007, Nissen et al. 2008). Since Rimonabant crosses the blood brain barrier, it seems likely that its prolonged central actions are resulting in the deleterious effects shown with long-term treatment (Christensen et al. 2007, Nissen et al. 2008).

Several studies in rodents have shown that acute treatment of Rimonabant or AM251 results in increased stress-induced serum corticosterone levels, and impaired habituation to restraint stress; both of which are thought to be precursors for depressive and anxiety-related disorders (Marsicano et al. 2002, Steiner et al. 2008). In addition, CB₁ knock-out mice show several symptoms of melancholic depression, such as anhedonia, reduced eating, weight loss, heightened anxiety, increased HPA axis activity and hippocampal atrophy (Patel et al. 2005).

Since CB₁ receptors are expressed in brain regions implicated in the modulation of emotional responses related to mood and anxiety (such as the prefrontal cortex, amygdala, periaqueductal grey and hippocampus), it seems unsurprising that drug-induced impairment of all endocannabinoid signalling produces behavioural changes that resemble symptoms of psychiatric disorders, such as anxiety and depression (Mackie 2005). Antagonism of the endogenous cannabinoid system within the brain may, therefore, interfere with the set point at which the endocannabinoid system equilibrates rewarding and aversive emotions. The adverse effect of long-term treatment with synthetic CB₁ inverse agonists such as Rimonabant may also be due to the “on-demand” characteristic of the cannabinoid system (Di Marzo et al. 1998). This means that in a physiological situation, endocannabinoid synthesis, and thus CB₁ activation, will only occur in activated neurones. Conversely, pharmacological
treatment with synthetic CB₁ ligands will activate all CB₁ receptors in the brain regardless of their specific physiological role.

If the cannabinoid system is to remain a viable target, future drugs will need to differentiate between either physiologically relevant CB₁ receptors, different neuronal circuitry, or between brain and periphery. Despite Rimonabant’s acute central effects on appetite, its continuing actions on body weight are probably via peripheral interaction with lipid mobilisation pathways in white adipose tissue and with cellular glucose uptake systems (Nogueiras et al. 2008). Therefore, the development of future CB₁ inverse agonists which do not cross the blood brain barrier may hold significant therapeutic importance in regulating adiposity.

The extent of hemopressin’s involvement with the additional actions exposed by synthetic inverse agonists has not yet been investigated. Future studies will need to explore the effects of hemopressin on energy expenditure, lipid metabolism and long-term feeding behaviour. Additionally, the use of biotinylated hemopressin may offer important insights into how and where the peptide penetrates the brain. If hemopressin does have a useful action on peripheral tissues, for example to affect lipolysis, the peptide could possibly be modified so that its uptake through the blood brain barrier is blocked.

Even if no significant therapeutic potential is offered by an endogenous CB₁ inverse agonist, the discovery that hemopressin is bioactive both in vitro and in vivo is very exciting. Furthermore, the recent discovery that N-terminally extended forms of hemopressin exist within the brain and act as CB₁ agonists in vitro opens up the exciting potential for the existence of a novel and unexplored neurotransmitter system within the brain.

5.2 N-terminally extended forms of hemopressin

The hemopressin peptide was discovered using a substrate capture technique on rat brain homogenate, which evaluated novel ligands that bind to endopeptidase 24.15
(Rioli et al. 2003). Although this affinity purification approach is highly accurate, it does not offer any insight as to the relative abundance of the peptide within the brain. This question was addressed in a recent study by Gomes et al (2009), which used mass spectrometry (MS) data from peptidomic analyses of mouse brain extracts to investigate the relative abundance of hemopressin or hemopressin-like peptides within the mouse brain. Using this approach Gomes et al did not find the presence of hemopressin. Instead they identified two slightly longer, N-terminally extended hemopressin peptides containing 11 and 12 amino acid residues, VDPVNFKLSSH (VD-Hpα) and RVDPVNFKLSSH (RVD-Hpα) (Gomes et al. 2009). Furthermore, their meta-analysis of peptidomics data showed that RVD-Hpα is represented within a number of screens of the mouse brain, and in several brain regions such as the hypothalamus, nucleus accumbens, olfactory bulb, cerebellum, prefrontal cortex and striatum.

In contrast to hemopressin, which acts as a CB₁ inverse agonist (Heimann et al. 2007), both VD-Hpα and RVD-Hpα appear to act in vitro as CB₁ agonists (Gomes et al. 2009). This study, which investigated response levels of extracellular signal-regulated kinases 1/2 phosphorylation and intracellular Ca²⁺ release, indicates that the “extended hemopressin” CB₁ ligands appear to activate a distinct signal transduction pathway from that of lipid-based (2-AG) and synthetic (Hu-210) CB₁ ligands (Gomes et al. 2009).

We have confirmed the agonistic action of RVD-Hpα on CB₁ receptors in preliminary results from our laboratory, as similarly to synthetic CB₁ agonists, RVD-Hpα significantly increases eGFP-CB₁ receptor internalization in vitro (Fig. 5.1). In a nocturnal feeding experiment in pre-satiated mice, RVD-Hpα also caused a dose-dependent increase in feeding (Fig. 5.2a) (Dodd and Luckman, unpublished). However, during a subsequent daytime experiment, i.c.v. injections did not cause a significant increase in feeding (Fig 5.2b), although there is a possibility that larger group sizes may produce significant results. It also worth noting, that the animals in the latter experiment were not pre-satiated, which is a normal requisite for cannabinoid agonists to be effective in increasing food intake.
VD-Hpα and RVD-Hpα are found in the striatum and hypothalamus, and their expression is higher in Cpe\textit{fat/fat} mice, which lack the enzyme carboxypeptidase E (Gelman \textit{et al.}). Gelman \textit{et al} suggest that hemopressin may be a “break-down” product of endogenous extended peptides, that themselves can be regulated by a carboxypeptidase E-independent mechanism. However, the fact that Cpe\textit{fat/fat} mice express an obese and hyperphagic phenotype (Wardman \textit{et al.}, Naggert \textit{et al.} 1995, Cawley \textit{et al.} 2004, Gomes \textit{et al.} 2009), is interesting as the increased RVD-Hpα may underlie the observed phenotypes (our unpublished results). Taken with our published work describing hemopressin effects on feeding behaviour (Dodd \textit{et al.} 2010), we would like to propose a model in which the hemopressin peptides form novel, mutually antagonist CB\textsubscript{1} pathways capable of subtly modulating food intake, similar to that seen with α-MSH and AgRP which antagonise each other at melanocortin receptors (Lu \textit{et al.} 1994, Ollmann \textit{et al.} 1997, Pritchard \textit{et al.} 2004).

Many neuropeptides are involved in cell-cell communication, the majority of which are produced in the endoplasmic reticulum via classical secretory pathways (e.g. Enkephalin, NPY) (Gelman & Fricker 2010). This usually involves cleavage of a peptide precursor at specific, well-defined sites by selective proteases (Fricker 1988, Zhou \textit{et al.} 1999). Following synthesis, these peptide precursors are transported to the trans-Golgi where they are further cleaved into mature peptides and packaged into synaptic vesicles (Zhou \textit{et al.} 1999).

These “classical” neuropeptides are then secreted upon depolarization into the synaptic cleft in an activity-dependent manner (Gelman & Fricker 2010). As hemopressin and hemopressin-like peptides are derived from the α-haemoglobin chain (HBA\textsubscript{1}), a cytosolic protein, in order to act as neuropeptide transmitters, they would need to be synthesised and released from the cytosol of the cell in a “non-classical” fashion (Gelman & Fricker 2010).
Effect of RVD-Hpα on eGFP-CB1 receptor internalization. The percentage internalization was calculated relative to the control situation of 100 nM WIN 55212-2 alone. The EC50 value of internalization for RVD-Hpα is approximately 5.08 μM. (Bars represent mean and S.E.M.; n = 40 cells per treatment). Methods are identical to that described in Chapter 4.3.1, with the exception that RVD-Hpα is used instead of hemopressin (Dodd, Luckman, unpublished).

Figure 5.1. Effect of RVD-Hpα on eGFP-CB1 receptor internalization. The percentage internalization was calculated relative to the control situation of 100 nM WIN 55212-2 alone. The EC50 value of internalization for RVD-Hpα is approximately 5.08 μM. (Bars represent mean and S.E.M.; n = 40 cells per treatment). Methods are identical to that described in Chapter 4.3.1, with the exception that RVD-Hpα is used instead of hemopressin (Dodd, Luckman, unpublished).

Figure 5.2. Effect of RVD-Hpα 1 hour post injection on feeding behaviour when administered both systemically (i.p., n=5/6, vehicle = saline, treatments in nmol), and b) centrally (i.c.v., n=4/5, vehicle=saline). In a) CD1 mice were given a 30 minute pre-feed at lights before injection, and nocturnal feeding behaviour was assessed. In b) CD1 mice were cannulated as described in Chapter 4.3.3, and feeding behaviour was assessed during the daytime. Bars represent mean and S.E.M. One-way ANOVA followed by Dunnett’s multiple comparison post hoc test. **p < 0.01 compared with vehicle (Dodd, Luckman, unpublished).
In the case of the hemopressins, it is likely that proteasomes (cytosolic protease complexes which cleave proteins into peptides of 4-25 amino acids) are involved in its synthesis. Hemopressin peptides are within the expected size of proteasome products, and the specificity of the various proteasome catalytic units are consistent with the cleavage sites needed to produce these peptides. The idea that bioactive peptide fragments are produced from cytosolic proteins is by no means novel, as both the α and β chains of haemoglobin are precursors for the peptides hemorphins and neokyotorphins (Fukui et al. 1983, Brantl et al. 1986, Liebmann et al. 1989, Moeller et al. 1997, Lee et al. 2003). These haemoglobin-derived peptides are bioactive and interact with opiate receptors and angiotensin receptors, respectively (Fukui et al. 1983, Brantl et al. 1986).

In addition, a number of non-haemoglobin-derived bioactive peptides that represent fragments of cytosolic proteins have also been described, including:

- Diazepam-binding inhibitor, a peptide product of acyl-CoA-binding protein, that binds to GABA$_A$ receptors;
- Microcryptide-1, a peptide product of cytochrome $c$ oxidase subunit capable of activating neutrophils; and

There is a developing theory that bioactive peptides such as hemopressin and RDV-Hpα can be generated “on demand”, in a similar fashion to non-classical neurotransmitters (e.g. 2-AG, nitric oxide), rather than being stored and then released by stimulus-secretion coupling (Di Marzo et al. 1998, Vincent 2010).

An important avenue of future research is to identify the cell types that express haemoglobin, and to identify the pathways involved in the synthesis and secretion of hemopressin and hemopressin-like peptides. Although α and β haemoglobin are traditionally viewed as erythrocytic proteins, haemoglobin has been found in several cell types, including activated macrophages, lung epithelial type II and Clara cells,

Overall, it seems apparent that the HBA1 precursor of the hemopressin peptides is expressed within a number of cells, notably neurones, and that it does not represent blood contamination. The absence of hemopressin described by Gomes et al could possibly reflect differential processing in rats versus the mice used in their study (Gomes et al. 2009). It is clear, however, that further investigation is required to investigate the existence and regulation of hemopressin and hemopressin-like proteins within the brain, and to ultimately unravel the enzymatic pathways required for the processing of these bioactive peptides.

5.3 Functional circuitry underlying appetite

Since CB₁ receptors are located presynaptically, and strength of downstream coupling varies with brain region, expression studies alone do not provide a firm basis for interpreting sites of cannabinoid action. The results of this thesis shed light on the functional actions of cannabinoids, and how activation of CB₁ receptors translates into feeding behaviour. By investigating functional responses to behaviourally relevant doses of CB₁ ligands using the complementation of c-Fos immunohistochemistry and phMRI, we have identified a central role for a cortico-striatal-hypothalamic link which is activated by CB₁ receptors to induce appetite.

Taken together with research discussed in Chapter 1 regarding central appetitive pathways and the role of cannabinoids in energy balance, it seems evident that cannabinoids may facilitate cross talk between “hedonistic” and “homeostatic” aspects of brain circuitry. As cannabinoids play a predominantly neuromodulatory role in the CNS, it is possible that information regarding preference, motivation and reward, represented by activation of the orbitofrontal cortex and ventral striatum,
influences the fundamental hypothalamic control on feeding. Cannabinoids may therefore modulate this “higher cognitive” input, allowing subtle control over the BCC, and ultimately bias the energy balance equilibrium to the influence of these “hedonistic” centres.

An interesting parallel can be made by comparing the responses seen following an orexigenic dose of the “hedonistic” CB₁ agonist, to that of the primarily “homeostatic” stimulus, 2-DG. We find a considerable overlap in areas of the hypothalamus, such as the Arc, DMN, VMN and LH (Fig. 5.3). Care must be taken not to over interpret independent data sets. However, although by no means setting a novel hypothesis, it does add additional confirmation that these areas of the hypothalamus are fundamental driving forces behind feeding. This also illustrates the level of accuracy and potential offered by fMRI in unravelling functional circuitry at a whole-brain level.

Due to the surrogate nature of phMRI, which fundamentally measures acute haemodynamic changes, it is important to note the techniques, potential limitations to the technique. One of the key limitations is the origins of the BOLD signal. Positive BOLD signals are triggered by oxygen depletion in response to increased metabolic demand of neuronal firing and synaptic activity (Logothetis & Wandell 2004, Nair 2005). In fact, a linear relationship between positive BOLD and neuronal activity has been described (Heeger et al. 2000, Rees et al. 2000). The functional origins underlying the negative BOLD signal are, however, far more speculative. These signals are thought to arise from a complex interplay between decreased neuronal metabolic demand (Shmuel et al. 2002, Shmuel et al. 2006, Devor et al. 2007), the “vascular-steal” effect (Harel et al. 2002, Shmuel et al. 2002), and increases in oxygen metabolism that exceed the arterial blood compensation. Notwithstanding, numerous studies show strong evidence to suggest that negative BOLD is indicative of a true suppression of neuronal activation (Shmuel et al. 2002, Stefanovic et al. 2004, Devor et al. 2007).
A further limitation to small animal phMRI is the use of anaesthetics, which can suppress the coupling between functional activation and metabolic and blood flow responses (Van der Linden et al. 2007). Although necessary to reduce motion artefacts during the acquisition of the scan, anaesthetics generally decrease both basal brain activity and brain activation during stimulation (Lindauer et al. 1993). It does, however, appear that some anaesthetics are more suitable for use in phMRI protocols than others. The anaesthetic used in these studies was α-chloralose (Ueki et al. 1992, Hyder et al. 1994, Peeters et al. 1999), which has been shown to best preserve cerebral blood flow, cerebral glucose metabolism, and stabilise cardiovascular parameters in response to somatosensory stimulation (Ueki et al. 1992, Lindauer et al. 1993, Bonvento et al. 1994). Furthermore, α-chloralose produces no effects on hypothalamic monoamine levels (Heimburger et al. 1992) or dopamine in the striatum (Massott & Longo 1978). However, it is important to note that differences remain in stimulus-evoked activation in α-chloralose anaesthetised animals compared with conscious animals (Lahti et al. 1999, Peeters et al. 2001). Furthermore, as with all general anaesthetics, α-chloralose has several limitations, including toxicity complications, poor control of depth of anaesthesia and poor analgesic properties (resulting in possible muscle reflex and movement) (Steward et al. 2005). In an attempt to measure potential detrimental interaction between anaesthetic and neuro-vasculature coupling, the studies described in this thesis have all measured transcutaneous blood gases during the fMRI scan.

Elevation of pCO₂ as a result of anaesthesia can lead to masking of the stimulus-induced brain activity through increased cerebral blood flow (CBV), cerebral blood volume (CBF) and the Boer Effect (Strebel et al. 1993, Bonvento et al. 1994, Sicard et al. 2003). Although very little research has been conducted in this area, a key study by Ramos et al (2005) has demonstrated that BOLD contrast in the somatosensory cortex of anaesthetised rats following front paw stimulation is masked by tcpCO₂ levels that exceed above 20% of the baseline value (Fig. 5.4) (Ramos-Cabrér et al. 2005). Importantly, the BOLD signal is recovered providing that normalized tcpCO₂ values remain no more than 20% above the baseline value.
By measuring tcpCO₂, this thesis (see Chapters 2 and 3) has measured the potential for detrimental effects of anaesthesia on the BOLD signal. It is clear, however, that in order to further increase the reliability of future fMRI studies in anaesthetised animals, it will be essential to develop a clearer understanding of the mechanisms by which anaesthetics interact with pCO₂ and ultimately effect BOLD contrast.

**Figure. 5.3** Comparison of fMRI brain maps depicting functional responses to orexigenic doses of a primarily “hedonistic” (CB₁ agonist), and “homeostatic” (2-DG) stimulus. Statistical parametric maps indicate overlap between the Arc, DMN, LH, and VMN, all of which are key hypothalamic nuclei implicated in feeding behaviour.
Figure 5.4. fMRI experiments, showing the utility of continuous on-line tcpCO₂ monitoring during fMRI studies. The tcpCO₂ values of three different rats, anesthetized with α-chloralose (expressed as percentage changes from baseline values), are plotted against time. A robust and reproducible activation-induced BOLD signal change in the somatosensory cortex was observed following front paw stimulation, when the relative tcpCO₂ levels did not exceed a threshold level of 120% (red horizontal line) over baseline values (100%). Figure taken from (Ramos-Cabar et al. 2005).
5.4 Future Work

5.4.1 Determine the expression profile of hemopressin in the rodent brain

For hemopressin to be placed as a significant endogenous regulator of appetite, it is important to show that the peptide is not only present within the brain, but is also localised to specific brain regions. As previously discussed, both \( HBA1 \) mRNA and protein are present within the rat brain and have been demonstrated in neurones of the cortex, hippocampus, striatum and substantia nigra by \textit{in situ} hybridisation histology and immunohistochemistry (Richter et al. 2009, Schelhorn et al. 2009). However, neither technique would be suitable for specifically localising hemopressin, as the peptide is itself part of the haemoglobin \( \alpha_1 \)-chain sequence. In addition, attempting to capture such small peptides (< 1 kDa) on a gel for Western blot analysis is practically impossible, and additionally would not provide high spatial localisation within the brain.

For these reason, we are employing \textit{in situ} imaging mass spectroscopy (IMS) on \textit{ex vivo} rat brain sections. For this technique, rat brains are rapidly dissected, snap frozen and sectioned using a cryostat onto non-ionising metallic slides. The sections are then coated using a matrix of crystallised molecules which act as proton sources, allowing the analyte to be ionised during matrix-assisted laser desorption/ionisation (MALDI) and tandem (MS/MS) mass spectrometry. When the sections are analysed using MALDI and MS/MS, ionised molecules travel down a linear flight path and their mass to charge (m/z) ratio is determined by the time it takes to reach the detector (Chaurand \textit{et al.} 2005). This allows molecules, such as peptides, contained within the tissue to be visualised as a spectrum of m/z peaks.

As the laser used to ionise the tissue has a resolution of <100\( \mu \)M, the technique is able to map the proteomic distribution of entire brain sections with high spatial resolution (Chaurand \textit{et al.} 2005). Additionally, the molecular identification of peaks can be achieved by tandem MS/MS, whereby analyte ions undergo further fragmentation and a second round of m/z separation. This allows the sequence
identification of specific peptides that differ by just a single amino acid (Chaurand et al. 2005).

In collaboration with Dr Emrys Jones at The Wolfson Molecular Imaging Centre, we have achieved a limit of detection of approximately 6 fmol per pixel at a m/z peak of 1090 (the molecular weight of hemopressin is 1089 + 1[H]^+ = 1090), when brain sections have been spiked with exogenous hemopressin. Subsequently, following on-section trypsinisation and MS/MS, we have confirmed that the peptide we detect at peak 1090 is hemopressin (Fig. 5.5).

![Figure 5.5. MS/MS analysis of hemopressin. m/z peak confirmation of hemopressin amino acid sequence through fragmentation sequencing using MS/MS (Jones, Dodd, Luckman, unpublished).](image)

From our initial experiments looking for endogenously expressed hemopressin in rat forebrain sections, we found that not only is hemopressin present within the brain, but that it is specifically localised to the Arc (Fig. 5.6). The integral role of the Arc has been discussed in Chapter 1 and, the presence of hemopressin within this nucleus is extremely exciting, as it now places hemopressin directly in a key position to modulate appetite. It is important to note that the m/z 1090 does not appear to be present in the other forebrain structures, we have viewed, including those involved in reward. An important next step will be to make an exhaustive examination of the whole brain.
Since our preliminary data suggests that the extended N-terminally extended hemopressin peptide, RVD-Hpα, may act as a CB₁ agonist and have opposing actions on feeding behaviour to that of hemopressin (Fig 5.1, and 5.2), it will also be important to use in situ IMS to evaluate the expression patterns all hemopressin-related peptides within the brain.

A further important question is whether hemopressin or RVD-Hpα are regulated within the brain in response to energy status. For this we could compare the concentrations of hemopressin or RVD-Hp in different states of energy balance (e.g. fasted and diet-induced obese). Although the in situ IMS technique itself is not strictly quantifiable, new methods have recently been established (Kultima et al. 2009). For instance, one could label mediobasal hypothalamic homogenates from control and experimental (e.g. fasted or diet-induced obese) groups of animals with heavy or light isotopic esters of trimethylammoniumbutyrate (TMAB), respectively (Fricker 2007). TMAB reacts with N-terminal amines and/or lysine side chains within the peptidome. The two group samples are then pooled and the relative abundance of TMAB isotopes can be detected by MS/MS, giving the relative abundance of each peptide in the two samples.

5.4.2 Genetic dissection of HBA1-derived peptides

As previously mentioned, the hemopressin family is derived from α-haemoglobin chain or HBA1 gene. Mass spectroscopic analysis has identified the presence of RVD-Hp and VD-Hp in the brain, but not in the blood or heart tissue, suggesting that α-haemoglobin chain derivatives, endogenously expressed in brain tissue, do not represent contamination from the blood (Gomes et al. 2009, Gelman & Fricker 2010). As HBA1 mRNA and protein are present within the neurones within the cortex, hippocampus, striatum, substantia nigra and hypothalamus, this suggests that HBA1 derivatives may be positioned to play important physiological roles (Biagioli et al. 2009, Richter et al. 2009, Schelshorn et al. 2009). Therefore, genetic dissection of HBA1 could offer an important insight into physiological roles of haemoglobin-derived peptides.
To investigate this theory, the HBA1 gene could have loxP sites genetically engineered either side of one of its exons. The resulting HBA1\textsuperscript{floxflox} mice should not have a phenotype. However, they could be crossed initially with nestin-Cre mice. The resulting double transgenic mice would lack expression selectively in all neurones after Cre-mediated excision of the loxP-flanked HBA1 gene (nestin-Cre-HBA1\textsuperscript{floxflox}). Mutant animals can be compared with nestin-Cre-HBA1\textsuperscript{wtwt} littermates to investigate the phenotypic response of knocking out of HBA1 specifically within neurones. If successful, HBA1\textsuperscript{floxflox} mice could be crossed with a number of other mice expressing Cre recombinase under the control of cell-specific promoters. This
would allow the specific dissection of \textit{HBA1} from specific neuronal populations such as dopaminergic, GABA, POMC or NPY neurones. As \textit{HBA1} mRNA has been found in dopaminergic neurones \cite{Schelshorn2009}, the crossing of a \textit{HBA1}\textsuperscript{flox/flox} mouse with tyrosine hydroxylase-eGFP-Cre mice will result in a double transgenic mouse (tyrosine hydroxylase-eGFP-Cre-\textit{HBA1}\textsuperscript{flox/flox}) that lacks \textit{HBA1} specifically within dopaminergic neurones. As these cells will fluoresce, this will allow electrophysiological recordings to show how dopaminergic cell functioning is dependent on co-localised \textit{HBA1}-derived peptides. This approach could also be used to target specific neuronal cell types in the Arc, such as POMC, NPY, to help unravel the function of \textit{HBA1}-derived peptides in these cells.

5.5 Summary of Key Findings

5.5.1 Homeostatic Stimulus (2-DG)

- To characterise brain regions responsive to an orexigenic dose of 2-DG, we combined BOLD phMRI with whole brain c-Fos functional activity mapping. Using these techniques we showed activation of previously characterised 2-DG-responsive regions, such as the hypothalamus and brainstem; and also identified the orbitofrontal cortex and striatum (nucleus accumbens and ventral pallidum) as novel 2-DG-responsive regions.

5.5.2 Hedonistic Stimulus (Cannabinoids)

- The \textit{CB}_1 receptor agonist (CP55940) and the \textit{CB}_1 receptor inverse agonist (Rimonabant) significantly increase and decrease food intake, respectively, in pre-satiated rats.

- Co-administration of the agonist and inverse agonist together results in food intake comparable to that of controls, suggesting that the observed feeding behaviour is \textit{CB}_1 receptor mediated.
• Using the combination of BOLD phMRI with whole brain c-Fos functional activity mapping, we showed that CB₁ receptor agonist, CP55940, significantly activated several key feeding and reward/motivation areas, including the frontal cortices, limbic striatum, amygdala and hypothalamus.

• BOLD activity in the above areas was reversed by the CB₁ receptor inverse agonist, suggesting that activation is specific to CB₁ activation, and that the two CB₁ ligands are functionally antagonising each other at the same receptors.

• This thesis highlights the cortico-striatal-hypothalamic pathway as a target site of cannabinoid signaling involved in appetite.

5.5.3 Hemopressin (novel endogenous CB₁ receptor inverse agonist)

• Hemopressin antagonises CB₁ receptor agonist-induced internalisation of the CB₁ receptor in vitro.

• Central and systemic injection of hemopressin induces hypophagia in vivo. The effect of hemopressin on feeding behaviour is lost in mice lacking functional CB₁ receptors.

• Systemic administration of hemopressin can overcome powerful orexigenic drives in fasted or leptin-deficient obese mice, and does so in a behaviourally specific manner.

• Preliminary, in situ imaging mass spectrometry data confirms that hemopressin is expressed endogenously within the brain, and that the expression is localised specifically to the Arc.

• The N-terminally extended hemopressin peptide (RVD-Hpα) acts as a CB₁ agonist both in vitro and in vivo. RVD-Hpα increases eGFP-CB₁ receptor
internalisation in a heterologous cell model, and increases nocturnal feeding behaviour when administered systemically to pre-fed mice.

- This thesis suggests the possible existence of novel, mutually antagonistic pathways within the brain regulating appetite.
5.6 References


Chapter 6

Manuscript Offprints
Glucose is a vital component of cellular function, and the maintenance of an adequate supply of glucose is of major importance to both central and peripheral tissues to support life (Levin et al. 1999; Williams et al. 2001). The constant level of glucose in the blood is regulated homeostatically by the neural and endocrine interplay between the brain and the regulatory mechanisms it controls in peripheral organs, notably the liver and the pancreas (Anand et al. 1964; Oomura et al. 1964; Donovan et al. 1991; Hevener et al. 1997; Cryer et al. 2003). It is likely that disruption or defects in the defense of a brain’s glucostatic ‘set-point’ may contribute to a number of pathological conditions, including diabetes and obesity (Levin and Sullivan 1987; Levin et al. 1999). Further understanding of how the brain regulates this glucose set-point could, therefore, have important therapeutic potential.

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Abbreviations used: 2-DG, 2-deoxy-D-glucose; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Arc, arcuate nucleus of the hypothalamus; BNST, bed nucleus of the stria terminalis; BOLD, blood-oxygen-level-dependent; CeA, central amygdala; DMN, dorso-medial nucleus; IC, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; NTS, the nucleus of the tractus solitarius; PAG, periaqueductal grey; PB, phosphate buffer; PBN, parabrachial nucleus; phMRI, pharmacological-challenge magnetic resonance imaging; PRN, pontine reticular nucleus; PVA, thalamic paraventricular nucleus; PVN, paraventricular nucleus; SO, superior olive; SPM5, statistical parametric mapping; VMH, ventromedial hypothalamus; VMN, ventromedial nucleus; VP, ventral pallidum.

Glucose is a vital component of cellular function, and the maintenance of an adequate supply of glucose is of major importance to both central and peripheral tissues to support life (Levin et al. 1999; Williams et al. 2001). The constant level of glucose in the blood is regulated homeostatically by the neural and endocrine interplay between the brain and the regulatory mechanisms it controls in peripheral organs, notably the liver and the pancreas (Anand et al. 1964; Oomura et al. 1964; Donovan et al. 1991; Hevener et al. 1997; Cryer et al. 2003). It is likely that disruption or defects in the defense of a brain’s glucostatic ‘set-point’ may contribute to a number of pathological conditions, including diabetes and obesity (Levin and Sullivan 1987; Levin et al. 1999). Further understanding of how the brain regulates this glucose set-point could, therefore, have important therapeutic potential.

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Abbreviations used: 2-DG, 2-deoxy-D-glucose; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Arc, arcuate nucleus of the hypothalamus; BNST, bed nucleus of the stria terminalis; BOLD, blood-oxygen-level-dependent; CeA, central amygdala; DMN, dorso-medial nucleus; IC, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; NTS, the nucleus of the tractus solitarius; PAG, periaqueductal grey; PB, phosphate buffer; PBN, parabrachial nucleus; phMRI, pharmacological-challenge magnetic resonance imaging; PRN, pontine reticular nucleus; PVA, thalamic paraventricular nucleus; PVN, paraventricular nucleus; SO, superior olive; SPM5, statistical parametric mapping; VMH, ventromedial hypothalamus; VMN, ventromedial nucleus; VP, ventral pallidum.

Glucose is a vital component of cellular function, and the maintenance of an adequate supply of glucose is of major importance to both central and peripheral tissues to support life (Levin et al. 1999; Williams et al. 2001). The constant level of glucose in the blood is regulated homeostatically by the neural and endocrine interplay between the brain and the regulatory mechanisms it controls in peripheral organs, notably the liver and the pancreas (Anand et al. 1964; Oomura et al. 1964; Donovan et al. 1991; Hevener et al. 1997; Cryer et al. 2003). It is likely that disruption or defects in the defense of a brain’s glucostatic ‘set-point’ may contribute to a number of pathological conditions, including diabetes and obesity (Levin and Sullivan 1987; Levin et al. 1999). Further understanding of how the brain regulates this glucose set-point could, therefore, have important therapeutic potential.

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Research over the past four decades has identified a subset of neurones within the brain that are able to modulate their firing activity in response to changes in extracellular glucose levels (Anand et al. 1964; Oomura et al. 1969; Oomura and Yoshimatsu 1984; Dunn-Meynell et al. 2002; Kang et al. 2004; Levin et al. 2004). These neurones can be split broadly into two populations: those that increase their firing rate in response to elevations in extracellular glucose concentration (termed glucose excited); and those which are activated by a decrease in extracellular glucose concentration or cellular glucoprivation (termed glucose inhibited) (Song et al. 2001; Routh 2002; Thorens 2003; Yang et al. 2004; Burdakov et al. 2005). Both types of neurone are widely distributed in the brain, but are particularly represented in the hypothalamus and the brainstem, regions involved in the control of energy homeostasis and food intake (Oomura et al. 1974; Mizuno and Oomura 1984; Kow and Pfaff 1985; Silver and Erecinska 1998).

Glucose-sensing neurones are thought to act as sentinels of the body’s glucostatic ‘set-point,’ so that in times of hypo- or hyperglycaemia, they initiate a constellation of integrated counter-regulatory responses, the most notable of which are modulation of the hypothalamo-pituitary-adrenal axis, glucagon/insulin secretion, and energy intake (DiRocco and Grill 1979; Borg et al. 1995; Hevener et al. 1997; Ritter et al. 2000). The physiological importance of central glucose sensing has been further highlighted in a number of studies whereby peripheral injection of gold thioglucose, which sensing has been further highlighted in a number of studies et al. Routh 2002; Thorens 2003; Yang et al. 2004; Burdakov et al. 2005). Both types of neurone are widely distributed in the brain, but are particularly represented in the hypothalamus and the brainstem, regions involved in the control of energy homeostasis and food intake (Oomura et al. 1974; Mizuno and Oomura 1984; Kow and Pfaff 1985; Silver and Erecinska 1998).

Materials and methods

Animals

All experiments were carried out using adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Sandwich, UK). Animals were group housed in The University of Manchester animal unit in a constant environment of 21 ± 2°C and 45 ± 10% humidity, on a 12:12 hours light-dark cycle with the dark phase commencing at 20:00 hours. Rat chow (Beekay International, Hull, UK) and tap water were available ad libitum unless stated otherwise. All procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act, 1986 and local ethical review.

Feeding and c-Fos protein immunohistochemistry

Rats (275 ± 20 g; n = 5/6 per group) were housed singly 5 days prior to the experiment and received food and water ad libitum. During this acclimatisation period, rats were handled daily and food intake monitored. Rats were assigned randomly to receive subcutaneous (s.c.) injections of either vehicle (0.9% NaCl) or 200 mg/kg body weight (b.w.) 2-DG (Sigma-Alrich Corp. Ltd., Poole, UK) between the hours of 09:00 and 14:00. All injections were given in a volume of 1 mL/kg body weight. The dose of 2-DG was based on literature (Nonavinakere and Ritter 1983; Tepper and Kanarek 1984; Giraudo et al. 1998) and previous in-house experiments. Food was weighed just before injection and again 90 min later before the animals were culled. The animals were deeply anaesthetized with 5% isoflurane (Concord Pharmaceuticals Ltd., Dunmow, Essex, UK) in oxygen (1 L/min) and perfused transcardially with heparinised saline (10 000 units/L heparin in 0.9% NaCl), followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1M, pH 7.4). The brains were post-fixed overnight and then kept for 2 days in 30% paraformaldehyde solution.

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avidin-biotin-peroxidase complex (GE Healthcare, Buckinghamshire, UK) diluted 1 : 500 in PB and, finally, visualized with nickel-intensified diaminobenzidine (Vector Laboratories, UK).

c-Fos immunoreactivity was first examined qualitatively to determine areas expressing c-Fos-positive neurones. Areas that showed qualitative changes in immunoreactivity were then analysed blind by counting c-Fos expressing nuclei in the areas of interest. The areas that showed qualitative changes were the nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), thalamic paraventricular nucleus (PVA), hypothalamic PVN, Arc, VMN, dorsomedial nucleus (DMN), LH, central amygdala (Cea), periaqueductal grey (PAG), and NTS. Brain areas were photographed using an Axioview upright microscope (Zeiss, Hertfordshire, UK) and an Axiocam colour CCD camera (Zeiss). The number of c-Fos-expressing cell nuclei was quantified in areas defined according to a standard atlas (Paxinos and Watson, 1986). Results are presented as mean and SEM for food intake at 90 min and the number of c-Fos-immunoreactive cells per section in each brain area. Treatments were compared using the two-way unpaired t-test using the Prism statistical package (GraphPad Software Inc, San Diego, CA, USA).

Blood-oxygen-level-dependent fMRI
Fourteen rats (260 ± 25 g, n = 7) were assigned randomly to receive subcutaneous injections of vehicle (0.9% w/v NaCl) or 2-DG (200 mg/kg) (Sigma-Aldrich). Animals were anaesthetized with 2.5% isoflurane (Concord Pharmaceuticals) in oxygen (2 L/min) to allow cannulation of a tail vein and subsequent anaesthetic maintenance by intravenous (i.v.) α-chloralose (Sigma-Aldrich). A bolus of α-chloralose (60 mg/kg body weight; i.v.) was injected manually over a period of 5 min whilst the isoflurane and oxygen were turned off. Then α-chloralose was infused continuously at a rate of 30 mg/kg/h i.v. by infusion pump for the remainder of the experiment. For imaging, rats were secured into an in-house built cradle with a nose cone to minimize movement. Temperature (RS 51 K-type thermometer; RS Components Ltd, Northants, UK), respiration rate (MR10 respiration monitor; Graseby Medical Ltd, Hertfordshire, UK) and transcutaneous pCO2 and transcutaneous pO2 were monitored (see Appendix S1), whereas the rats were allowed to breathe spontaneously. Imaging was carried out using a 7-Tesla, horizontal-bore magnet (Magnex Scientific Ltd, Abingdon, UK) with a transmit/receive birdcage volume coil connected to a SMIS computer console (Surrey Medical Imaging Systems Limited, Guildford, UK). For anatomical reference images, a T2-weighted fast spin echo was used (repetition time = 60 ms, number of samples = 256, number of views = 128, number of averages = 16). For functional images, a T2*-weighted gradient echo was used to measure BOLD signal (repetition time = 172 ms, echo time = 15 ms, number of samples = 128, number of views = 64, number of averages = 4, voxel size = 0.313 mm × 1 mm × 0.313 mm, each volume took 70 s to acquire). Eleven contiguous slices, each of 1 mm thickness were aligned horizontally through the brain (Paxinos and Watson, 1986). A total of 60 brain volumes over a period of 70 min were acquired in all. 2-DG or vehicle was administered during volume 12.

Data were analysed with Statistical Parametric Mapping (SPM5) programme using a random effects model (The Wellcome Trust Centre for Neuroimaging, London, UK; (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/)). Individual brains were re-aligned and co-registered to the first volume, spatially normalized and smoothed to a full width half maximum of 0.939 mm isotropic Gaussian kernel. In a first-level analysis, a series of contrasts were constructed between five successive time blocks each consisting of 12 consecutive volumes (14 min each). The contrasts compared time blocks following injection (4 time blocks, each 12 volumes) to that of the pre-infusion period (1 time block, 12 volumes). These images were combined in a second-level random effects analysis using a two sample t-test. T contrasts were then constructed to discern the positive and negative effects of 2-DG compared with vehicle. The resulting T2 contrast statistical parametric maps were overlaid onto a T2*-weighted anatomical template image (Schwarz et al. 2006), with a threshold level of p < 0.05 uncorrected. For the unbiased (operator-independent) identification of the BOLD MRI data, regions of interest were delineated using a 3D digital reconstruction of the Paxinos and Watson rat brain atlas (Paxinos and Watson 1998), co-registered with the rat brain template (Pic atlas) (Schwarz et al. 2006). Only clusters within regions containing ≥ 3 voxels were considered for further analysis. To provide a measure of response in these areas, Z scores and mean percentage BOLD contrast changes for the maximally responding voxel in each cluster were obtained using SPM5.

**Results**

**Effects 2-DG on food intake and c-Fos immunoreactivity in freely-behaving rats**

An unpaired, two-tailed t-test revealed a significant increase in food intake in 2-DG-treated animals compared with controls, 90 min post-injection (p = 0.0024, Fig. 1). As this dose gave a robust orexigenic response, it was also used in the subsequent phMRI experiment.

Quantitative analysis of the number of c-Fos-positive neurones in each of the brain areas of interest showing qualitative activity (Fig. 2) revealed a significant increase in counts following 2-DG administration compared with vehicle. Increased numbers of c-Fos-positive cells were seen in 

Fig. 1 Bar graph illustrating total food intake at 90 min post-injection of animals treated with vehicle or 2-deoxy- D-glucose (2-DG) (200 mg/kg b.w.). Each treatment was administered subcutaneously. Error bars show SEM. Unpaired two-tailed t-test. **p < 0.01 compared with vehicle (n = 5–6/group).
the AcbC (p = 0.0098), AcbSh (p < 0.0098, Fig. 3a and d), VP (p < 0.0001), PVA (p < 0.0001, Fig. 3b and e), PVN (p < 0.0001, Fig. 3c and f), Arc (p = 0.0134, Fig. 4a and d), DMN (p < 0.0001, Fig. 4b and e), LH (p < 0.0001), CeA (p = 0.0019, Fig. 4c and f), PAG (p < 0.0001) and NTS (p < 0.0001). None of the brain areas analysed showed a

Fig. 2 Graph illustrating the number of c-Fos-positive neurones per section in brain regions after subcutaneous administration of vehicle or 2-deoxy-D-glucose (2-DG) (200 mg/kg b.w.). Bars represent mean and SEM (n = 5/group). Data analysed using an unpaired two-tailed t-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared with vehicle. Abbreviations; nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), thalamic paraventricular nucleus (PVA), hypothalamic paraventricular nucleus (PVN), arcuate nucleus (Arc), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamus (LH), central amygdala (CeA), periaqueductal grey (PAG), and the nucleus of the solitary tract (NTS).

Fig. 3 Photomicrographs showing c-Fos-labelled nuclei in selected brain areas following administration of vehicle (a, b, c), or 2-deoxy-D-glucose (200 mg/kg b.w., d, e, f). Abbreviations; anterior commissure (ac), nucleus accumbens shell (AcbSh), hypothalamic paraventricular nuclei (PVN), and thalamic paraventricular nuclei (PVA). Scale bars = 200 μm.
significant decrease in c-Fos immunoreactivity following 2-DG administration. No statistically significant difference between groups was seen in the VMN ($p = 0.1681$).

phMRI: Effects of 2-DG on BOLD signal in α-chloralose-anaesthetized rats

In the present study we show that administration of 2-DG induced significant positive BOLD signal in the agranular insular cortex, somatosensory cortex, hippocampus, inferior colliculus (IC), olfactory tubercle, caudate putamen, septum, substantia innominata, Arc, VMN, mesencephalic region, locus coeruleus (LC), PBN, pontine reticular nucleus (PRN), and the superior olive (SO) (Table 1, Fig. 5).

In addition, administration of 2-DG induced significant negative BOLD signal in the cingulate cortex, entorhinal cortex, medial pre-frontal cortex, ventral orbitofrontal cortex, ventral hippocampus, olfactory nucleus, AcbC, AcbSh, caudate putamen, bed nucleus of the stria terminalis (BNST), diagonal band, nucleus of posterior limb of the anterior commissure, VP, CeA, thalamus, zona incerta, DMN, LH, PVN, PAG and raphé nucleus (Table 1, Fig. 5).

phMRI: blood gases

As detailed in Appendix S1, blood O$_2$ and CO$_2$ were measured transcutaneously throughout the imaging experiments. CO$_2$ was very stable in all animals throughout the experiment (before and after infusion of 2-DG or vehicle, Figure S1a). Oxygen was more variable, but there was no evidence for systematic effects of time or infusion of either vehicle or 2-DG (Figure S1b). Further details are given in the Appendix S1.

Discussion

The central sites mediating 2-DG-induced responses are poorly understood outside the realm of the classic homeostatic centres of the hypothalamus and caudal brainstem. In the present study, we have shown ‘whole-brain’ responses to a behaviourally relevant glucoprivic dose of 2-DG, using the complementary techniques of BOLD phMRI and c-Fos protein functional activity mapping.

Concurring with previous studies, intraperitoneal administration of 200 mg/kg 2-DG resulted in acute hyperphagia (King et al. 1978; Tepper and Kanarek 1984; Ritter and Taylor 1989, 1990; Giraudo et al. 1998). Functional brain activity following 2-DG administration was compared with vehicle injections, using the neuronal activity marker protein, c-Fos. 2-DG increased c-Fos expression in the PVA, PVN, Arc, VMN, DMN, LH, CeA, and the NTS, supporting previous studies investigating 2-DG-induced c-Fos immuno-

Fig. 4 Photomicrographs showing c-Fos-labelled nuclei in selected brain areas following administration of vehicle (a, b, c), or 2-deoxy-D-glucose (200 mg/kg b.w., d, e, f). Abbreviations; third ventricle (3V), central amygdala (CeA), hypothalamic arcuate (Arc), dorsomedial nucleus (DMN), and ventromedial nucleus (VMN). Scale bars = 200 μm.
reactivity (Ritter and Dinh 1994; Solomon et al. 2006). Previous studies, however, limit reporting to selected brain areas and do not provide whole-brain coverage. The current study provides a complete whole-brain analysis and demonstrates, additionally, increased c-Fos immunoreactivity in the striatum (VP, AcbC, AcbSh) and PAG following treatment with 2-DG. A clear deviation between this and previous studies is the lack of c-Fos induction in some regions of the pons and medulla oblongata. Other, similar studies have reported robust c-Fos immunoreactivity in the PBN, LC, SO and ventrolateral medulla (Ritter and Dinh 1994; Ritter et al. 1998). The latter have tended to use higher doses of 2-DG and to remove food from the animals after injection, which might affect the response. However, interestingly, although these areas were not detected in the present study using c-Fos immunohistochemistry, they were identified using phMRI, highlighting complementarity between the two techniques.

c-Fos immunohistochemistry provides high spatial resolution and whole-brain coverage, although c-Fos expression does not automatically follow neuronal activity (Luckman et al. 1994), which can lead to false negative results. In addition, c-Fos is seldom translated in response to reduced activity so, in these instances, neuronal inhibition can not be discerned (Hughes and Dragunow 1995). c-Fos protein induction occurs over a period of 30–90 min, thus limiting temporal resolution. For these reasons, it is essential to complement functional immunohistochemistry with other functional imaging techniques to provide additional data and to aid interpretation. Systemic administration of 2-DG produced significantly enhanced BOLD activity in several brain areas including the frontal cortices (orbitofrontal, cingulate, insular cortex), mesolimbic system (AcbC, AcbSh, VP, BNST), striatum (caudate putamen, globus pallidus), amygdala (CeA), thalamus (PVA, IC), midbrain (PAG), hypothalamus (Arc, DMN, LH, PVN, VMN) and pons (LC, PBN, PRN, SO).

In terms of functionality, the combination of BOLD and c-Fos immunohistochemistry highlights a number of regions that show changes in both measures following 2-DG. Positive BOLD signals are triggered by oxygen depletion in response to increased metabolic demand of neuronal firing and synaptic activity [for review see (Logothetis and Wandellar).

Table 1 Regions of significant blood-oxygen-level-dependent activation relative to vehicle following administration of 2-DG (200 mg/kg, s.c.) detected by pharmacological-challenge magnetic resonance imaging (n = 7). Columns show Z scores, for the peak-responding voxel in brain areas showing significant changes in blood-oxygen-level-dependent signal following 2-DG treatment (analysed using a two-sample t-test)

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>phMRI Z-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agranular insular</td>
<td>2.12</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>– 2.62</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>2.18 3.74</td>
</tr>
<tr>
<td>Medial pre-frontal cortex</td>
<td>2.41</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>2.39 – 3.11</td>
</tr>
<tr>
<td>Ventral orbitofrontal cortex</td>
<td>–</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
</tr>
<tr>
<td>Hippocampus medial</td>
<td>3.15 –</td>
</tr>
<tr>
<td>Hippocampus posteroendorsal</td>
<td>2.36 –</td>
</tr>
<tr>
<td>Hippocampus subiculum</td>
<td>3.4 –</td>
</tr>
<tr>
<td>Hippocampus ventral</td>
<td>– 3.23</td>
</tr>
<tr>
<td>Hippocampus anteroendorsal</td>
<td>2.57 –</td>
</tr>
<tr>
<td>Olfactory region</td>
<td></td>
</tr>
<tr>
<td>Olfactory nuclei</td>
<td>– 3.74</td>
</tr>
<tr>
<td>Olfactory tubulic</td>
<td>2.77 –</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td></td>
</tr>
<tr>
<td>Accumbens core</td>
<td>– 3.91</td>
</tr>
<tr>
<td>Accumbens shell</td>
<td>– 3.96</td>
</tr>
<tr>
<td>BNST</td>
<td>– 3.92</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>2.12 3.34</td>
</tr>
<tr>
<td>Diagonal band</td>
<td>– 3.71</td>
</tr>
<tr>
<td>IPAC</td>
<td>– 2.91</td>
</tr>
<tr>
<td>Septum</td>
<td>1.79 –</td>
</tr>
<tr>
<td>Substantia innominata</td>
<td>2.15 – 3.34</td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td>–</td>
</tr>
<tr>
<td>Amygdala</td>
<td></td>
</tr>
<tr>
<td>Central amygdala</td>
<td>– 2.55</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
</tr>
<tr>
<td>Dorso lateral thalamus</td>
<td>– 2.67</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>3.89 –</td>
</tr>
<tr>
<td>Midline thalamus</td>
<td>– 2.37</td>
</tr>
<tr>
<td>Zonale nerta</td>
<td>– 3.05</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
</tr>
<tr>
<td>Arcuate nuclei</td>
<td>3.15 –</td>
</tr>
<tr>
<td>Dorso medial nucleus</td>
<td>– 1.9</td>
</tr>
<tr>
<td>Lateral hypothalamus</td>
<td>– 3.05</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>– 3.15</td>
</tr>
<tr>
<td>Ventromedial nucleus</td>
<td>3.1 –</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
</tr>
<tr>
<td>Mesencephalic region</td>
<td>3.06 –</td>
</tr>
<tr>
<td>Periaqueductat grey</td>
<td>– 2.58</td>
</tr>
<tr>
<td>pons</td>
<td></td>
</tr>
<tr>
<td>Locus cerellosus</td>
<td>2.33 –</td>
</tr>
<tr>
<td>Parabrachial nucleus</td>
<td>2.17 –</td>
</tr>
<tr>
<td>Pontine reticulate nucleus</td>
<td>2.56 –</td>
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Table 1 (Continued)

<table>
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<tr>
<th>Brain Region</th>
<th>phMRI Z-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe nucleus</td>
<td>– 2.58</td>
</tr>
<tr>
<td>Superior olive</td>
<td>3.36 –</td>
</tr>
</tbody>
</table>

BNST, bed nucleus of the stria terminalis; IPAC, interstitial nucleus of posterior limb of the anterior commissure; phMRI, pharmacological-challenge magnetic resonance imaging.
In fact a linear relationship between positive BOLD and neuronal activity has been described (Heeger et al. 2000; Rees et al. 2000). Therefore areas showing increased signal using both techniques following 2-DG administration in the present study (Arc and VMN) potentially reflect increased synaptic and cellular electrical activity.

The functional origins underlying the negative BOLD signal are far more speculative, as they are thought to arise from a complex interplay between decreased neuronal metabolic demand (Shmuel et al. 2002, 2006; Devor et al. 2007) and the ‘vascular-steel’ effect (Harel et al. 2002; Shmuel et al. 2002). Despite this, numerous studies show strong evidence to suggest that negative BOLD is indicative of a true suppression of neuronal activation (Shmuel et al. 2002; Stefanovic et al. 2004; Devor et al. 2007). Taken in this context, the brain areas depicted in the present study showing a decrease in BOLD signal and an increase in c-Fos immunoreactivity following 2-DG administration (AcbC, AcbSh, VP, DMN, PVN, LH, CeA, PVA, PAG) potentially reflect decreased synaptic activity and increased cellular activity; an effect which could be occurring because of disinhibition in these regions.

The present study does, however, illustrate clear evidence of dissociation between BOLD fMRI and c-Fos immunoreactivity in a number of brain regions. For example, the IC showed a robust change in BOLD activity but failed to show any changes in c-Fos immunoreactivity. The possible reasons for this dissociation are discussed in detail elsewhere (Stark et al. 2006; Preece et al. 2009).

In light of the widespread effects of 2-DG on metabolism, it is interesting to note the relatively small number of brain sites expressing significant c-Fos immunoreactivity and BOLD activity in response to 2-DG. In addition, previous studies have found that both 2-DG-induced hyperphagia and c-Fos immunoreactivity persist in many of the brain areas

Fig. 5 Group ($n = 7$) statistical parametric maps showing changes in blood-oxygen-level-dependent (BOLD) contrast, with a significance threshold set to $p < 0.01$ uncorrected, following acute administration of 2-deoxy-D-glucose (2-DG) (200 mg/kg, s.c.). BOLD blobs in red indicate regions of increased activity compared with vehicle, whereas blobs in blue are regions of decreased activity. The colour bar represents $t$-values. Values above images represent approximate distances from bregma (mm). The histogram represents the total area under the BOLD signal percentage change curve produced following treatment with vehicle or 2-DG for each of the corresponding brain regions. Bars represent mean and SEM. Arc, arcuate nucleus; CeA, central amygdala; DMN, dorsomedial hypothalamic nucleus; IC, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; AcbShell, nucleus accumbens shell; Orb, orbitofrontal cortex; PAG, periaqueductal grey; PBN, parabrachial nucleus; PRN, pontine reticular nucleus; PVN, paraventricular hypothalamic nucleus; SO, superior olive; VP, ventral pallidum; VMN, ventromedial nucleus.
described above following subdiaphragmatic vagotomy, implying that these effects are a consequence of the direct central metabolic effect of 2-DG (Miselis and Epstein 1975; Ritter and Dinh 1994). The functional responses to 2-DG observed in the current study may represent direct actions of 2-DG on neurones in each of these brain areas, or may represent transynaptic activation of these sites following more selective activation of specific populations of specialized glucoreceptive neurones within the brain.

Interpretation of the current results in the context of feeding centres of the brain highlights the hypothalamus and hindbrain as potential sites of action underlying the hyperphagia. Studies attempting to establish the primary site coordinating to the counter-regulatory hyperphagic response initiated by 2-DG-induced hypoglycaemia, have resulted in the emergence of two separate views. A substantial body of evidence points to an integral role of the ventromedial hypothalamus (VMH, comprising both the Arc and the VMN). Breakthrough studies demonstrated that perfusion of glucose directly into the VMH of systemically-induced VMN). Breakthrough studies demonstrated that perfusion of glucose directly into the VMH of systemically-induced hypoglycaemic animals abolishes the normal counter-regulatory responses, whereas infusion of 2-DG into the VMH caused a prompt increase in plasma glucose, glucagon, and catecholamines (Borg et al. 1994, 1995), suggesting that the neurones sensing glucopenia may be localised in the VMH.

A recent study found that manipulation of VMH glucosensing by blocking VMH glucokinase mRNA expression reduced glucoprivic feeding, but exerted no effect on spontaneous feeding behaviour, implying behavioural specificity in the role of the VMH in food intake (Dunn-Meynell et al. 2009). In light of this evidence, the significant functional activation of the VMH by 2-DG seen in the present study further illustrates that VMH glucosensing plays an important role in the counter-regulatory behavioural and neuroendocrine responses to glucoprivation.

A second body of evidence points to a primary role for the hindbrain (DiRocco and Grill 1979). Direct injection of 2-DG into the LH, VMH, amygdala or striatum has no effect on feeding behaviour, despite a marked response to intracerebroventricular administration (Berthoud and Mogenson 1977). This intracerebroventricular injection of 2-DG failed to activate feeding in the presence of an obstruction to the cerebral aqueduct (Ritter et al. 1981), suggesting that 2-DG-induced glucopenia may be sensed pre-dominantly by neuronal populations in the hindbrain. Food intake can be stimulated by direct injections of the glucose anti-metabolite 5-thioglucose into areas of the hindbrain, such as the ventrolateral and dorsomedial medulla (Ritter et al. 2000). These regions, along with other hindbrain nuclei highlighted by phMRI in the present study (LC, PBN, PRN), contain neurones that respond either directly or indirectly to a glucoprivic dose of 2-DG (Ritter and Dinh 1994; Ritter et al. 1998). Neurones in the NTS sense glucose both directly and indirectly via vagal afferents from peripheral glucosensors in the hepatic portal vein. Confirming previous reports (Ritter and Dinh 1994; Moriyama et al. 2003), the current study found a marked increase in c-Fos immunoreactivity in the NTS, but no significant change in BOLD signal. There are various explanations for this. First, there may be a drug/anaesthetic interaction in this area not seen elsewhere in the brain; second, there may only be a delayed response in these regions which were not detected maximally in the time frame examined; and third, the response in NTS neurones is transcriptional but not electrical. Either way, counter regulation in response to glucoprivation is likely to require an integrated brain response.

As the present study has mapped functional responses of the whole-brain, it has also identified a number of novel 2-DG-responsive brain regions. Areas of particular interest are the Orb (cortex), AcbSh, and VP (mesolimbic system). These regions make up a corticostriatal connection with the hypothalamus, by which aspects of motivation, salience and reward can impinge on the hypothalamic control of feeding behaviour (Swanson 2000; Fulton 2009). Furthermore, the hypothalamus can be viewed as the head of a brainstem control column which implements the feeding behaviour. A recent study investigating functional brain responses to an orexigenic dose of a cannabinoid CB₁ receptor agonist, also highlights this corticostriatal-hypothalamic pathway (Dodd et al. 2009). Thus, we have provided evidence for a common integrated circuit involved in the induction of feeding behaviour to different types of stimulus.

In summary, we have characterised the whole-brain response to 2-DG-induced glucoprivation using c-Fos protein functional activity mapping and BOLD phMRI. By using the complementarity of two functional techniques, we have identified the well-characterised connections of the hypothalamus and brainstem, whilst highlighting areas of the frontal cortices (orbitofrontal, cingulate, insular cortex), mesolimbic system (AcbC, AcbSh, VP, BNST), striatum (caudate putamen, globus pallidus), amygdala (CeA) and thalamus (PVA, IC) as additional brain regions responding to 2-DG-induced glucoprivation. This study, therefore, provides an insight into how the whole-brain responds to 2-DG to co-ordinate complex counter-regulatory responses, whilst further illustrating the accuracy and valuable potential of phMRI in investigating central pharmacological activity.

Acknowledgements

The authors wish to thank the technical assistance of Ms Karen Davies for maintenance of the MRI magnet and console. Dr Shane McKie and Dr Jennifer Stark for advice, and help on using SPM5. Dr Adam Schwarz (Eli-Lilly) and GlaxoSmithKline for providing us with a copy of the rat brain template and PIC-atlas image software. GTD was supported by a Biotechnology and Biosciences Research Council Integrative Mammalian Physiology priority post-graduate.
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Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and Methods.

Figure S1. Line graph illustrating time course changes of (a) transcutaneous pCO2 and (b) transcutaneous pO2 following subcutaneous administration of vehicle, or 2-DG (200 mg/kg) in α-chloralose anesthetized rats (n = 7).

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References


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CENTRAL CANNABINOID SIGNALING MEDIATING FOOD INTAKE: A PHARMACOLOGICAL-CHALLENGE MAGNETIC RESONANCE IMAGING AND FUNCTIONAL HISTOLOGY STUDY IN RAT

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Abstract—Endocannabinoids have a variety of effects by acting through cannabinoid 1 (CB1) receptors located throughout the brain. However, since CB1 receptors are located presynaptically, and because the strength of downstream coupling varies with brain region, expression studies alone do not provide a firm basis for interpreting sites of action. Likewise, to date most functional studies have used high doses of drugs, which can bias results toward non-relevant adverse effects, and which mask more behaviourally-relevant actions. Here we use a low, orexigenic dose of the full CB1 agonist, CP55940, to map responsive brain regions using the complementary techniques of pharmacological-challenge functional magnetic resonance imaging (phMRI) and immediate-early gene activity. Areas of interest demonstrate a drug interaction when the CB1 receptor inverse agonist, rimonabant, is co-administered. This analysis highlights the corticostriatal–hypothalamic pathway, which is central to the motivational drive to eat. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: pharmacological-challenge MRI, appetite, SR141716A, reward, hypothalamus, nucleus accumbens.

Endogenous cannabinoids are released widely in the brain and act primarily as retrograde transmitters on presynaptically located receptors (Freund et al., 2003; Di Marzo et al., 2004; Kawamura et al., 2006). Endocannabinoids are synthesised and released by the postsynaptic neurone ‘on demand’, and impart an inhibitory action on further synaptic transmission (Di Marzo et al., 1998; Schlicker and Kathmann, 2001; Wilson and Nicoll, 2002; Di Marzo et al., 2004). Cannabinoid 1 (CB1) receptor is expressed throughout the brain (Herkenham et al., 1991; Matsuda et al., 1993; Egertova and Elphick, 2000), while receptor CB2 is much less represented, instead being found mostly in the periphery (Munro et al., 1993; Van Sickle et al., 2005). Cannabinoids have been used to target pain relief and to treat the symptoms of multiple sclerosis and glaucoma, through their actions on peripheral nervous tissues (Robson, 2005). However, the diverse central actions of endocannabinoids (implicated in the modulation of nociception, locomotion, body temperature, memory and appetite) and the relative paucity of information regarding their sites of action have hindered their development as pharmaceutical agents. Recently, rimonabant (Acomplia; SR141716) did not receive approval for the treatment of obesity in the United States, and was withdrawn in 2008 from the European market due to some concerns that prolonged use may occasionally lead to depression, anxiety or suicidality (Christensen et al., 2007). Rimonabant is an inverse agonist of the CB1 receptor (Bouaboula et al., 1997) and is capable of producing weight-reducing effects over extended periods, notably when used in conjunction with lifestyle changes (Van Gaal et al., 2005). However, its action to specifically reduce appetite is relatively short lived, and any continued weight loss is via peripheral interaction with lipid mobilisation in adipose tissue and cellular glucose uptake (Nogueiras et al., 2008). Further validation of the CB1 receptor as a target for pharmaceutical intervention is concentrating on the development of inverse agonists or neutral antagonists, which may not induce undesirable psychotic side effects, including those which do not pass the blood–brain barrier. An integral step in order to aid future development of therapeutically viable cannabinoid-based drugs is to gain further understanding of how cannabinoids act in the brain.

A number of histological techniques demonstrate high levels of CB1 expression in the cortex, cerebellum, basal ganglia and striatum, and particularly low levels of expression in the hypothalamus (Herkenham et al., 1991; Matsuda et al., 1993; Egertova and Elphick, 2000). However, these studies do not discern the functionality of cannabinoids or how they mediate particular behavioural outputs. Functional studies using activity markers, such as the protein product of the c-fos gene, have highlighted a number of cannabinoid-responsive areas central to motivation and reward processes, with particular emphasis on the nucleus accumbens (Miyamoto et al., 1997; McGregor et al., 1998; Arnold et al., 2001; Allen et al., 2003). High doses of...
cannabinoids also induce c-Fos protein in the hypothalamic paraventricular nucleus (PVN) and the central amygdala (CeA), two areas often associated with stress responses. Furthermore, pharmacological challenge magnetic resonance imaging (phMRI) studies also have tended to use exceptionally high doses, often orders of magnitude higher than that required to produce behavioural responses (Shah et al., 2004; Chin et al., 2008). Here, using behaviourally relevant doses and complementary functional techniques, we are able to provide strong evidence for target sites of cannabinoid signalling with whole-brain coverage.

**EXPERIMENTAL PROCEDURES**

**Animals and drugs**

All experiments were carried out using male Sprague–Dawley rats (250±22 g; Charles River Laboratories, Inc., Sandwich, UK). Animals were group housed in the University of Manchester animal unit in a constant environment of 21±2 °C and 45±10% humidity, on a 12-h light/dark cycle. Rat chow (Beekay International, Hull, UK) and tap water were available *ad libitum* unless stated otherwise. All procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act, 1986, and local ethical review.

Rimonabant was a kind gift from Sanofi-Aventis (Longjumeau, France), and CP55940 was purchased from Tocris Bioscience Ltd. (Brighton, UK). The vehicle for rimonabant was a saline solution containing 1 drop of Tween 80 (Sigma-Aldrich Corp. Ltd.). A bolus of 2.5% chloralose-HBC (Sigma-Aldrich Corp. Ltd.) was injected manually over a period of 5 min, while the isoflurane and oxygen were turned off. Then α-chloralose was infused continuously at a rate of 30 mg/kg/h i.v. by infusion pump for the remainder of the experiment. To confirm that neither treatment interacted with the anaesthesia to produce confounding actions on circulating blood gas levels, the effects of CP55940 and rimonabant on the transcutaneous partial pressure of blood CO\(_2\) and O\(_2\) were tested in separate anaesthetised rats (see Supplemental data). For imaging, rats were secured into an in-house built cradle with a nose cone to minimise movement.

**Effects of CB1 agonist and inverse agonist on food intake in pre-satiated rats**

Rats were housed singly for one day and food was restricted 6 h before the experiment was due to start. At lights out (20:00 h), animals were fed preweighed chow *ad libitum* for 30 min to partially satiate them. In the first experiment, rats were assigned randomly to receive a single i.p. dose of CP55940 (0, 0.01, 0.03, 0.06, and 0.1 mg/kg body weight) in a volume of 1 ml/kg of vehicle at 20:30 h. In the second experiment, rats were assigned randomly to receive two i.p. injections at 20:30 and 21:00 h. The first injection was either 1 mg/kg rimonabant, or its vehicle. The second injection consisted of 0.06 mg/kg CP55940 or its vehicle. Injections were made in a volume of 1 ml/kg body weight. The dose of rimonabant was based on the literature (Arnone et al., 1997; Solinas and Goldberg, 2005; Thornton-Jones et al., 2005), while that of CP55940 was determined from the first experiment.

Food intake was determined 1, 2, and 12 h following CP55940. Results are presented as mean and standard error of the mean for food intake at each time point (n=6 per group). Treatments were compared using a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison post hoc test using the GraphPad Prism statistical package (GraphPad Software, Inc. San Diego, CA, USA).

**c-Fos protein immunohistochemistry**

Eighteen rats were assigned randomly to receive i.p. injections of either vehicle, 0.06 mg/kg CP55940 or 1 mg/kg rimonabant, between 09:00 and 11:00 h. Ninety minutes post injection the animals were deeply anaesthetised with sodium pentobarbital (100 mg/kg body weight i.p.; Rhône-Poulenc, Lyon, France) and perfused transcardially with heparinised saline (10,000 U/L heparin in 0.9% NaCl) followed by 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.3). The brains were post fixed overnight and then kept for two days in 30% sucrose in 0.1 M of PB to cryoprotect the tissue before freezing on dry ice. Thirty-micrometre sections (120 μm apart) were cut in the coronal plane throughout the entire rostrocaudal extent of the brain and incubated in 20% methanol, 0.2% Triton X-100, and 1.5% hydrogen peroxide for 30 min to deactivate endogenous peroxidases. The sections were then incubated at room temperature for 1 h in the blocking buffer: 0.1 M PB, 0.3% Triton X-100, and 1% normal sheep serum and then overnight at 4 °C in rabbit anti-c-Fos antibody (Oncogene Science Inc., Bayer Health Care, Cambridge, MA, USA) diluted to 1:10,000 in blocking buffer. After washing, the sections were incubated sequentially at room temperature for 1 h in goat anti-rabbit IgG–biotin complex (Vector Laboratories, Peterborough, UK) diluted 1:500 in blocking buffer followed by avidin–biotin–peroxidase complex (GE Healthcare, Bucks, UK) diluted 1:500 in PB and, finally visualised with nickel-intensified diaminobenzidine (Vector Laboratories). c-Fos immunoreactive neurones were examined with the experimenter blinded to the treatments. Images were collected on an AxioVision upright microscope (Zeiss, Hertfordshire, UK) using an AxioCam colour CCD camera. Images were then processed using AxioVision software to quantify the number of c-Fos-expressing nuclei in areas defined according to a standard atlas (Paxinos and Watson, 1986). The results are presented as mean and standard error of the mean for the number of c-Fos-immunoreactive cells in each brain area per section. Treatments were compared using a one-way ANOVA followed by Bonferroni’s multiple comparison post hoc test.

**Blood oxygen level dependent (BOLD) phMRI**

Twenty-four rats were assigned randomly to receive pretreatment of vehicle or rimonabant (1 mg/kg body weight, i.p.) followed by subsequent administration of either vehicle or CP55940 (0.06 mg/kg body weight, i.p., n=6 per group). Animals were anaesthetised with 2.0–2.5% isoflurane (Concord Pharmaceuticals Ltd., Dunmow, Essex, UK) in oxygen (2 l/min) to allow cannulation of a tail vein and subsequent anaesthetic maintenance by i.v. α-chloralose-HBC (Sigma-Aldrich Corp. Ltd.). A bolus of α-chloralose (60 mg/kg body weight; i.v.) was injected manually over a period of 5 min, while the isoflurane and oxygen were turned off. Then α-chloralose was infused continuously at a rate of 30 mg/kg/h i.v. by infusion pump for the remainder of the experiment. To confirm that neither treatment interacted with the anaesthesia to produce confounding actions on circulating blood gas levels, the effects of CP55940 and rimonabant on the transcutaneous partial pressure of blood CO\(_2\) and O\(_2\) were tested in separate anaesthetised rats (see Supplemental data). For imaging, rats were secured into an in-house built cradle with a nose cone to minimise movement.

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**Fig. 1.** Graph illustrating total food intake at 1 h post injection of animals treated with vehicle, 0.01, 0.03, 0.06, and 0.1 mg/kg body weight CP55940. All treatments were administered i.p. Bars represent mean and SEM. One-way ANOVA followed by Bonferroni’s multiple comparison post hoc test. *****P<0.01 compared with vehicle (n=6/group). Abbreviation: SEM, standard error of the mean.
Effects of CB1 agonist CP55940 on food intake in pre-satiated rats

An effect of treatment was detected at the 1-h post-injection time point, revealed by one-way ANOVA ($F_{4,25} = 11.77, P < 0.001$; Fig. 1). CP55940 showed a dose-dependent increase in food intake, though the highest dose caused hypolocomotion. Bonferroni’s multiple comparison post hoc analysis identified a significant increase in food intake following 0.06 mg/kg CP55940 ($P < 0.001$). As this dose showed a robust oredi-
genic response, it was used in subsequent experiments. At the 2-h time point (data not shown) the hyperphagic response to 0.06 mg/kg was no longer statistically significant (F_{4,25}=4.649, P>0.05). At the 12-h time point (data not shown) there were no longer any differences between groups (F_{4,25}=1.905, P=0.1409).

Effect of CB1 inverse agonist rimonabant on CP55940-induced hyperphagia in pre-satiated rats

An effect of treatment was detected at the 1-h time point, revealed by one-way ANOVA (F_{3,20}=10.92, P<0.0002; Fig. 2), and not at the 2- or 12-h (F_{3,20}=0.732, P<0.1928, and F_{3,20}=0.9144, P<0.4517, respectively; data not shown) post-injection time points. At the 1-h time point, Bonferroni’s multiple comparison post hoc analysis identified a significant increase in food intake following CP55940 alone, and a significant decrease in food intake following rimonabant alone (1 mg/kg body weight) compared with both the vehicle/vehicle control group and the rimonabant/CP55940 co-administration group (both P<0.01). The effect of either drug was effectively cancelled out when they were co-administered, resulting in food intake being comparable to that of the control group.

Fig. 4. Photomicrographs showing c-Fos-labelled nuclei in selected brain areas following administration of vehicle (A, D, G, J), rimonabant (1 mg/kg body weight, B, E, H, K), or CP55940 (0.06 mg/kg body weight, C, F, I, L). Abbreviations: third ventricle (3V), anterior commissure (ac). Scale bars = 200 μm.
Effect of CB1 agonist CP55940 and CB1 inverse agonist rimonabant on c-Fos immunoreactivity in freely behaving rats

All brain sections for each treatment were initially analysed qualitatively to ascertain brain regions showing c-Fos immunoreactivity. Qualitative analysis of the whole brain highlighted the following brain areas of interest: medial orbitofrontal cortex (MO), ventral orbitofrontal cortex (VO), lateral orbitofrontal cortex (LO), agranular insular cortex (AI), cingulate cortex (Cg), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), ventral pallidum (VP), medial amydala (MeA), CeA, lateral hypothalamus (LH), and the hypothalamic arcuate nucleus (Arc), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and PVN.

Quantitative analysis of the number of c-Fos-positive neurones in each of these brain areas of interest (Fig. 3) revealed a significant increase in counts following CP55940 administration compared with vehicle in the following regions: LO, VO, MO, Cg, AI (Fig. 4A–C), VP, AcbC, AcbSh (Fig. 4D–F), CeA (Fig. 4G–I), LH and PVN. None of the brain areas analysed showed a significant decrease in c-Fos immunoreactivity following CP55940 administration. The only significant increase in immunoreactivity following rimonabant administration was in the PVN; however, significant decreases in immunoreactivity were seen in the CeA (Fig. 4G–I) and VMN (Fig. 4J–L). No significant differences for either treatment were seen in the Arc or DMN. N.B. All rats for immunostaining were killed 90 min post-drug treatment.

pHrMI: interaction of the CB1 inverse agonist rimonabant with the CB1 agonist CP55940

Using statistical analysis and the PickAtlas (Schwarz et al., 2006) it was possible to identify regions of the brain in which an interaction had occurred between the two CB1 acting drugs. Only regions responding to CP55940 that also were modulated by rimonabant (an interaction, see Fig. 5 for explanation) were considered for analysis, illustrating more accurately where cannabinoids act within the brain. Brain areas responding to CP55940 that show an interaction with rimonabant are described in Table 1, with Z-scores and BOLD percentage change of the peak-responding voxel in a cluster for each area of interest.

Administration of CP55940-induced positive BOLD signal in the piriform cortex, somatosensory cortex, rostral olfactory nucleus, olfactory tubercle, AcbC, AcbSh, ventrolateral caudate putamen, diagonal band, interstitial nucleus of posterior limb of the anterior commissure, VP, MeA, Arc, VMN and caudal substantia innominata (Fig. 6, Table 1). In addition, administration of CP55940 induced negative BOLD signal in the AI, LO, entorhinal cortex, AI, caudal olfactory nucleus, bed nucleus of the stria terminals, dorsolateral caudate putamen, globus pallidus, septum, CeA, dorsolateral thalamus, ventrolateral thalamus, LH, PVN, mesencephalic region, rostral substantia innominata, zona incerta and superior olive (Fig. 6A, Table 1). Rimonabant showed a significant interaction in all of the above areas; that is, rimonabant administration resulted in a significant attenuation of the CP55940-induced positive and negative BOLD signals (Fig. 6B).

DISCUSSION

High doses of cannabinoids cause hypokinesia, catalepsy and hypothermia, and have strong effects on cognition and memory (Fitton and Pertwee, 1982; Pertwee et al., 1993; Rubino et al., 1994; McGregor et al., 1996, 1998; Howlett et al., 2002). We have used CP55940 at a dose sufficient to cause acute hyperphagia, but at which further effects are minimised. Experiments were carried out in satiated animals to diminish homeostatic drive, allowing assessment of the rewarding motivation to eat. CP55940 produced dose-dependent hyperphagia, with significance at 0.06 mg/kg body weight. This dose was used for the subsequent mapping studies. Higher doses resulted in marked hypophagia due to hypolocomotion, which has been noted previously (McGregor et al., 1996; Gallate et al., 1999). One milligram per kilogram body weight rimonabant attenuated food intake, confirming earlier studies that also have implicated it in reward/motivation (Amon et al., 1997; Ward and Dykstra, 2005). When given together, the actions of the drugs were cancelled out, confirming rimonabant’s ability to attenuate anandamide- or tetrahydrocannabinol-induced hyperphagia (Williams and Kirkham, 2002), as well as attenuating Fig. 5. Examples of negative and positive BOLD interactions. (A) A negative interaction in the highest responding voxel of the accumbens shell (AcbSh). Here, CP55940 significantly increases BOLD signal compared with controls. However, when the two drugs are co-administered, rimonabant significantly attenuates the CP55940-induced BOLD increase. (B) A positive interaction in the highest responding voxel of the bed nucleus of the stria terminals (BNST). Here, CP55940 significantly decreases BOLD signal compared with controls. However, when the two drugs are co-administered, rimonabant significantly attenuates the CP55940-induced BOLD decrease.
Table 1. CP55940-induced changes in BOLD contrast and interactions with rimonabant

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Z-score</th>
<th>Change (%)</th>
<th>Interaction with rimonabant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>-2.86</td>
<td>-4.86</td>
<td>↑</td>
</tr>
<tr>
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<td>-4.50</td>
<td>↑</td>
</tr>
<tr>
<td>LO</td>
<td>3.29</td>
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<td>↓</td>
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<tr>
<td>Piriform cortex</td>
<td>2.61</td>
<td>2.93</td>
<td></td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>2.01</td>
<td>3.27</td>
<td>↓</td>
</tr>
<tr>
<td>Olfactory region</td>
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<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>Olfactory nucleus, rostral</td>
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<td>2.93</td>
<td>↓</td>
</tr>
<tr>
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<td>2.93</td>
<td>↓</td>
</tr>
<tr>
<td>Basal ganglia</td>
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<td></td>
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<tr>
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<td>1.96</td>
<td>4.14</td>
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<tr>
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<tr>
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<td>IPAC</td>
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<td>Septum</td>
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<td>-5.66</td>
<td>↓</td>
</tr>
<tr>
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</tr>
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<tr>
<td>Amygdala</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CeA</td>
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<td>↑</td>
</tr>
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</tr>
<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arc</td>
<td>1.86</td>
<td>5.41</td>
<td></td>
</tr>
<tr>
<td>Lateral hypothalamic area</td>
<td>-2.27</td>
<td>-5.71</td>
<td>↑</td>
</tr>
<tr>
<td>PVN</td>
<td>-2.64</td>
<td>-5.21</td>
<td>↓</td>
</tr>
<tr>
<td>VMN</td>
<td>1.88</td>
<td>5.43</td>
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<tr>
<td>Midbrain</td>
<td></td>
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<tr>
<td>Mesencephalic region</td>
<td>-2.98</td>
<td>-7.59</td>
<td>↑</td>
</tr>
<tr>
<td>Superior olive</td>
<td>-2.93</td>
<td>-7.86</td>
<td>↓</td>
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</table>

Regions of significant activation relative to vehicle associated with the main effect of CP55940 (0.06 mg/kg body weight, i.p.), and their interaction with rimonabant (1 mg/kg body weight, i.p.) detected by phMRI (n=6/group). Columns show Z-scores and mean BOLD percentage changes, for the peak-responding voxel in brain areas showing significant changes in BOLD signal following CP55940 treatment (analysed using a full factorial ANOVA). The direction of rimonabant’s interaction with these brain areas is depicted by ↑ = positive interaction (rimonabant significantly attenuates the CP55940-induced negative BOLD signal), and ↓ = negative interaction (rimonabant significantly attenuates the CP55940-induced positive BOLD signal). Abbreviations: BNST, bed nucleus of the stria terminalis; IPAC, interstitial nucleus of posterior limb of the anterior commissure.

increased motivational ‘break point’ for a sweet reward following CP55940 (Gallate et al., 1999).

As rimonabant and CP55940 have high specificity and affinity for CB1 receptors (Miller et al., 2004; Pertwee, 2005; Xie et al., 2007), the simplest hypothesis is that rimonabant is functionally antagonising CP55940 at the same receptors in the brain. CP55940 significantly increased or decreased BOLD signal in several key feeding reward/motivational areas, including the frontal cortices, limbic striatum, amygdala and hypothalamus. Furthermore, BOLD activity in all areas was reversed by rimonabant, suggesting it is specific to CB1 activation, and that rimonabant functionally antagonises CP55940 at the same receptors. The regional activation by CP55940 contrasts with two previous studies that used phMRI and the non-selective cannabinoid agonists, HU210 and A-834735 (Shah et al., 2004; Chin et al., 2008), where a more global activation of the brain was noted, presumably due to non-selective actions.

CP55940 increased c-Fos in the AcbSh, AcbC, PVN, LH and CeA, supporting previous CB1 studies (McGregor et al., 1998; Arnold et al., 2001; Allen et al., 2003; Singh et al., 2005; Soria-Gomez et al., 2007). Most previous publications, however, were limited to selected areas, and did not attempt whole-brain coverage. The present study provides a complete analysis and, in addition, showed increases in cortical and pallidal regions (LO, VO, MO, Cg, Al and VP). However, in contrast, the current experiment produced a weak response to rimonabant, increasing c-Fos in the PVN, and decreasing it in the CeA and VMN. Several studies demonstrate a robust induction of c-Fos following rimonabant in the striatum, hypothalamus and frontal cortices (Alonso et al., 1999; Singh et al., 2004). However, these used doses up to 10 times greater and could, therefore, be distinguishing non-specific effects of rimonabant. In support of our observations, intra-AcbSh administration of AM251, which is similar in structure to rimonabant, did not induce c-Fos in the striatum or hypothalamus at a low, hypophagic dose (Soria-Gomez et al., 2007).

BOLD phMRI and c-Fos immunohistochemistry produce complementary results, as discussed in detail elsewhere (Stark et al., 2006; Preece et al., 2009). Here we highlight a number of regions showing positive changes in both measures following CP55940, including the AcbC, AcbSh, VP, and VMN, which reflects increased neuronal activation. The Arc and MeA displayed increased BOLD signal, but no induction of c-Fos, which could reflect an increase in inhibitory inputs or a lack of a transcriptional response to the stimulus. BOLD signal is directly related to overall metabolic demand, which is strongly influenced by synaptic activity (Arthurs and Boniface, 2002; Lauritzen and Gold, 2003). Areas displaying a decrease in BOLD and an increase in c-Fos (Al, LO, LH, CeA, PVN), might reflect disinhibition by CP55940. Thus, reduced activity primarily in inhibitory synapses could lead to the observed reduction in BOLD, but with increased activity in postsynaptic neurones. Dissociations between c-Fos induction and BOLD activity are likely due to CB1 receptor’s negative coupling to adenylate cyclase in presynaptic terminals (Howlett, 1985). Using radiolabelled [3H]CP55940, Herkenham and colleagues (1991) found CB1 heterogeneously expressed throughout the brain. Many areas activated following CP55940 (AcbC, AcbSh, VP, PVN, LH, VMN), contain only moderate to sparse densities of CB1 binding. Although expression is relatively low in these areas, functional studies using GTPγS binding suggest that some
CB1 receptors, particularly those in the hypothalamus, are more strongly coupled to G-proteins than in any other brain region (Breivogel et al., 1997). This offers an explanation why the relatively low doses of cannabinoid ligands used in this study can elicit significant functional responses in regions of low CB1 receptor expression.

Interpreting drug interactions in specific brain areas is difficult without further experimentation, but results can be put into the context of known circuitry. In ‘minimal circuit analysis’, behavioural control columns (BCCs) for motivated behaviours, such as feeding, are regulated by cascading projections from the telencephalon (Swanson, 2000). The BCC contains elements of the brainstem controlling feeding that are headed by the hypothalamus. Excitatory projections from the cortex to the BCC send collaterals to the striatum. The major output of the striatum, GABAergic medium spiny neurones (MSNs), send collaterals to the BCC and the pallidum. Finally, the pallidum sends an inhibitory input to the BCC, which is functionally a disinhibitory projection as it is inhibited by the striatum, which is in turn activated by the cortex. The basal ganglia and BCC also send feedback through thalamocortical projections. In this study, each of these areas showed significant changes in BOLD signal in response to CB1 stimulation.

A corticostriatal–hypothalamic pathway is relevant to feeding, since local injection of glutamate antagonist to block cortical input to the AcbSh produces a behaviourally selective hyperphagia (Maldonado-Irizarry et al., 1995; Kelley and Swanson, 1997; Haberny et al., 2004). Injection of cannabinoids into the AcbSh also results in marked hyperphagia, which is antagonised by rimonabant or AM251 (Kirkham et al., 2002; Soria-Gomez et al., 2007). In addition, intra-AcbSh cannabinoids increase c-Fos in the LH, suggesting the existence of a CB1-mediated functional relationship between the AcbSh and feeding centres in the hypothalamus (Soria-Gomez et al., 2007). AcbSh GABAergic neurones receive glutamatergic afferents from the prefrontal cortex (Christie et al., 1985; Bennett and Bolam, 1994), representing a critical link between cortex and striatum. However, it is not clear whether glutamatergic input is predominantly direct to MSNs or local GABAergic interneurones. Local interneurones do express mRNA for glutamic acid decarboxylase and CB1 (Hohmann and Herkenham, 2000). Furthermore, the interneurones have a significant inhibitory effect on MSNs (Jaeger et al., 1994; Kawaguchi et al., 1995; Koos and Tepper, 1999). Since both glutamatergic and GABAergic synapses within the AcbSh possess CB1 receptors that can inhibit synaptic transmission (Manzoni and Bockaert, 2001; Robbe et al., 2001; Lopez-Moreno et al., 2008), it is possible that the local action of cannabinoids reduces the activity of interneurones, leading to a disinhibition of MSNs. Since MSNs constitute approximately 90% of the AcbSh population (Tepper et al., 2004) this could result in the increases in BOLD activity and c-Fos noted here. Since a feature of CB1 receptors is that they are located on both glutamatergic and GABAergic terminals, one difficulty in interpreting function is that an agonist may have low-dose effects on one distinct population and high-dose effects on another.

The AcbSh is the only striatal region to send a direct projection to the LH, suggesting it has unique access to hypothalamic feeding centres (Groenewegen and Rüschen, 1984; Heimer et al., 1991; Kelley et al., 2005) and provides a link whereby reward/motivational circuits impinge on hypothalamic control of feeding. This study shows a decrease in BOLD signal and increase in c-Fos in the LH following CP55940. Furthermore, a recent electrophysiological study demonstrated that orexigenic effects of cannabinoids include a presynaptic CB1-mediated suppression of GABAergic transmission to postsynaptic LH neurones (Jo et al., 2005). Taken together, this suggests that cannabinoids could exert their orexigenic effects by disinhibiting LH neurones, which impart drive to other regions of the feeding BCC. If the LH neurones constitute the final pathway to the BCC, the effects of exogenous cannabinoids in upstream brain systems would have little bearing on the behavioural output.
The current study demonstrates CB1 agonist-induced hyperphagia is reversed by pre-treatment with a receptor inverse agonist. Using phMRI to identify specific areas where rimonabant functionally antagonises the CP55940-induced BOLD signal, we have deciphered regions which are potentially responsible for cannabinoidegic effects on food intake. By complementing phMRI with c-Fos mapping, we highlight areas involved in the direct regulation of food intake (hypothalamus) and those concerned with reward and motivation (striatum, prefrontal cortex). We have demonstrated that phMRI can produce meaningful insights into behavioural function, and that it is a powerful tool for investigating both drug mechanisms and functional neural circuits at the whole-brain level.

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APPENDIX

Supplementary data

Hemopressin is a short, nine amino acid peptide (H-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-OH) isolated from rat brain that behaves as an inverse agonist at the cannabinoid receptor CB1, and is shown here to inhibit agonist-induced receptor internalization in a heterologous cell model. Since this peptide occurs naturally in the rodent brain, we determined its effect on appetite, an established central target of cannabinoid signaling. Hemopressin dose-dependently decreases night-time food intake in normal male rats and mice, as well as in obese ob/ob male mice, when administered centrally or systemically, without causing any obvious adverse side effects. The normal, behavioral satiety sequence is maintained in male mice fasted overnight, though refeeding is attenuated. The anorectic effect is absent in CB1 receptor null mutant male mice, and hemopressin can block CB1 agonist-induced hyperphagia in male rats, providing strong evidence for antagonism of the CB1 receptor in vivo. We speculate that hemopressin may act as an endogenous functional antagonist at CB1 receptors and modulate the activity of appetite pathways in the brain.

Introduction

Hemopressin is a product of the hemoglobin α chain, discovered in rat brain using an enzyme-substrate capture technique and so named as it can cause small decreases in blood pressure (Rioli et al., 2003; Lippton et al., 2006). Subsequently, hemopressin was found also to have nonopioid antinociceptive effects (Dale et al., 2005). In vitro studies show that the peptide acts as a CB1 receptor inverse agonist, and can interact with both peripheral and central pain pathways in vivo (Heimann et al., 2007). To date, all known endogenous cannabinoids, such as 2-arachidonoylglycerol and anandamide, are fatty acid derivatives (Bisogno, 2008; Petroisino et al., 2009). These endocannabinoids are released by postsynaptic neurons "on demand," following the Ca2+ influx produced in response to postsynaptic depolarization or activation of metabotropic receptors (Kano et al., 2009). When released into the synaptic cleft, endocannabinoids activate presynaptic CB1 receptors, and impart an inhibitory action on further presynaptic transmission. The administration of exogenous CB1 agonists, such as Δ9-tetrahydrocannabinol (THC), the active ingredient of Cannabis sativa, or the synthetic compounds CP55940 [(−)-cis-3-[2-hydroxy-4-(1-1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol] and WIN 55212-2 [(R)-(−)−[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-d,e]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate], increase food intake by increasing motivational reward (Cota et al., 2003a;Pagotto et al., 2006). By comparison, the synthetic compound, rimonabant (SR141716A), is an inverse agonist at the CB1 receptor and is capable of producing weight-reducing effects over extended periods in rodents and humans (Van Gaal et al., 2005; Di Marzo, 2008). The action of rimonabant to reduce specifically motivational appetite is relatively short lived, and any continued weight loss is thought to be mediated mainly via peripheral CB1 interaction with lipid mobilization pathways in adipose tissue and liver, energy expenditure and cellular glucose uptake (Di Marzo, 2008; Nogueiras et al., 2008; Kunos et al., 2009).

We hypothesize that hemopressin may be a naturally occurring inverse agonist of brain CB1 receptors, capable of antagonizing central orexigenic pathways.

Materials and Methods

Cell culture and transfection. COS-7 Monkey Kidney Fibroblasts cells (Invitrogen) were grown on coverslips in a 24-well plate, in a medium of DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. Throughout the experiment cells were kept under 5% CO2 in air at 37°C and passage numbers P1–P20 of undifferentiated cells were used for experiments. At ~90% confluence, cells were transfected with pEGFP-N1-CB1, plasmid (mouse CB1 cDNA was cloned into a pEGFP-N1 vector which encodes the GFPmut1 variant (Clontech Labs), leading to an eGFP fusion at the C terminus of CB1) using Lipofectamine according to the manufacturer’s protocol (Invitrogen). Following an overnight transfection, the growth medium was changed, and cells were treated with vehicle (0.25% DMSO), 100 μM AM251, 100 μM, 10 μM, and 100 μM hemopressin, in the absence, or presence of 100 μM WIN 55212-2 (all Tocris Bioscience). Cells were stimulated with drugs for 2 h, and then fixed in an ice-cold solution of 4% paraformaldehyde, 4% sucrose in 0.1 M phosphate buffer for 45 min. Slides were coverslipped using VectorShield hard set (Vector Labs) containing 4’,6-diamidino-2-phenylindole (DAPI) to stain cell nuclei. Images of trans-
Modified cells were viewed by an experimenter blinded to treatment group using an Olympus BX51 upright microscope with a 60×/1.4 UP-PlanApo objective. Images were captured at random using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Specific bandpass filter sets for DAPI (excitation λ, 360–370 nm, emission λ, 420–460), and eGFP (excitation λ, 480/40 nm, emission λ, 535/50) were used to prevent bleed through from one channel to the next. Fifty cells per treatment group were analyzed to quantify the number of internalized endosomes per cell. To determine an IC50 value for hemopressin, we set up a similar experiment, but cells were treated with hemopressin over a nine point log dilution series (100 μM, 10 μM, 1 μM, 100 nm, 10 nm, 1 nm, 100 pm, 10 pm, 1 pm) in the presence of 100 nm WIN 55212-2. Forty cells per treatment group were analyzed to quantify the number of internalized endosomes per cell. The percentage inhibition of internalization was calculated relative to the control situation of 100 nm WIN 55212-2 alone.

**Animals.** All experiments (except those using ob/ob or CB1 receptor knock-outs) were performed on adult, male outbred CD1 mice and male, outbred Sprague Dawley rats (Charles River Laboratories Inc.). The male ob/ob mice, homozygous for the obese spontaneous mutation, Leprdb/db, are backcrossed with a C57BL/6N background (B6.V-Leprdb/db). Jackson Laboratories). CB1+/− and CB1−/− littermate mice were obtained by breeding of heterozygotes that had been backcrossed six times to a C57BL/6N background, as described previously (Marsicano et al., 2002). All animals were housed under a 12:12 h light/dark cycle (lights on 8:00 A.M. to 8:00 P.M.), at 22°C and 45 ± 10% humidity. Pelleted food (Beekay International) and water were available ad libitum unless stated otherwise. Experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and local ethical review. The experiment involving the CB1 knock-out mice was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the local Government of Rheinland-Pfalz, Germany.

**Intracerebroventricular surgery.** Under 2% isoflurane (Concord Pharmaceuticals Ltd) in 1/min oxygen, mice and rats were implanted stereotaxically with guide cannulae into the right lateral ventricle (0.2 mm posterior, 1 mm lateral from bregma for mice, and 0.8 mm posterior, 1.5 mm lateral from bregma for rats) according to the atlas of Paxinos and Watson (1998). The tip of the guide cannula was positioned 1 mm above the injection site (1 mm (mice), and 3 mm (rats) ventral to the surface of the skull). All animals were allowed to recover from surgery for 5–7 d before the start of experiments.

**Hemopressin effects on nocturnal feeding behavior in mice and rats.** All mice and rats were housed singly at least 3 d before the experiment and food was restricted 3 h before the experiment was due to start. At lights out (8:00 P.M.), animals were fed preweighed chow ad libitum.

In one experiment, 18 mice (31 ± 1.8 g, n = 6) were assigned randomly to receive intraperitoneal injection of vehicle (0.9% w/v NaCl, 10% DMSO, 20% 2-hydroxyprolyl-β-cycloextrin), 500 nmol/kg hemopressin, or 5.4 μmol/kg AM251 (3 mg/kg, based on dose described by Tallett et al., 2007b). Injections were made in a volume of 2 ml/kg body weight. Food intake was determined 1, 2, 4, and 24 h after injection. Results are presented as mean ± SEM for food intake at each time point. Treatments were compared using a one-way ANOVA followed by Dunnnett’s multiple-comparison post hoc test using the GraphPad Prism statistical package (GraphPad Software). In a second experiment, 24 intracerebroventricularly cannulated CD1 mice (30 ± 1.4 g, n = 6) were assigned randomly to receive intracerebroventricular injection of vehicle (0.9% w/v NaCl) or AM251, hemopressin. Injections were made in a volume of 2 μl per animal. Treatments were compared using a one-way ANOVA followed by Dunnnett’s multiple-comparison post hoc test. In a third experiment, 12 intracerebroventricularly cannulated rats (320 ± 12 g, n = 6) were assigned randomly to receive vehicle (0.9% w/v NaCl) or 10 nmol hemopressin intracerebroventricularly. Injections were made in a volume of 2 μl per animal. Treatments were compared using a two-tailed t test.

**Effects of hemopressin on feeding behavior in CB1−/− mice.** Twelve male CB1−/− mice and 12 wild-type littermates (26 ± 2.1 g) were housed singly 1 week before the experiment. Since the two genotypes normally display significantly different body weights (Cota et al., 2003b) and, therefore, food intake, all the mice were fasted overnight before the start of the experiment. One hour after lights on (8:00 A.M.) CB1−/− and
CB1+/+ animals were assigned randomly to receive intraperitoneal injection of either vehicle or 500 nmol/kg hemopressin in a volume of 2 ml/kg (n = 5/6). Food intake was determined 1, 2, 4, and 12 h after injection. Treatments were compared using a two-way ANOVA followed by Bonferroni’s multiple-comparison post hoc test.

Effects of hemopressin on feeding behavior in leptin-deficient (ob/ob) mice. Fourteen obese ob/ob mice (38 ± 3.5 g, n = 7) were assigned randomly to receive intraperitoneally vehicle (0.9% w/v NaCl) or 500 nmol/kg hemopressin. Injections were made in a volume of 2 ml/kg body weight. Food intake was determined 1, 2, 4, and 24 h after injection. Treatments were compared using a two-tailed t-test.

Hemopressin effects CB1 agonist (CP55940)-induced hyperphagia in rats. Twenty-four Sprague Dawley rats (320 ± 18 g, n = 5/6) were cannulated into the lateral ventricle under recovery anesthesia 1 week before experimentation. Rats were housed singly at least 3 d before the experiment and food was restricted 3 h before the experiment was due to start. At lights off (8:00 P.M.) animals were assigned randomly to receive vehicle (0.9% w/v NaCl) or 10 nmol hemopressin intracerebroventricularly. Injections were made in a volume of 2 μl per animal. Twenty minutes later, rats received, intraperitoneally, vehicle (0.9% w/v NaCl, 2.5% ethanol) or 0.06 mg/kg CP55940 (Tocris Bioscience Ltd., Brighton, UK) in a volume of 1 ml/kg. The dose of CP55940 was determined in previous published experiments (Dodd et al., 2009). Upon second injection, animals were fed preweighed chow ad libitum. Treatments were compared using a one-way ANOVA followed by Bonferroni’s multiple-comparison post hoc test.

Behavioral satiety sequence. CD1 mice were transferred to transparent cages 3 d prior and fasted overnight before the start of the experiment. In one experiment, 16 mice (32 ± 1 g, n = 8) were assigned randomly to receive intraperitoneally vehicle or 500 nmol/kg hemopressin in a volume of 2 ml/kg. In a second experiment, 14 mice (30 ± 1.4 g, n = 7) were assigned randomly to receive intracerebroventricular injection of either vehicle or 10 nmol hemopressin in a volume of 2 μl per animal. In a third experiment, 18 mice (32 ± 1.6 g, n = 6) were assigned randomly to receive intraperitoneal injection of vehicle (0.9% w/v NaCl, 10% DMSO, 20% 2-hydroxypropyl-β-cyclodextrin), 500 nmol/kg hemopressin, or 5.4 μmol/kg AM251. Following injections, preweighed food was presented and the animals were left undisturbed for 90 min. Behavior was scored using momentary time sampling, every 30 s for the 90 min period, after which point food intake was measured (Lawrence et al., 2002; Scott et al., 2005). The behaviors were scored, 0 or 1, according to the following classifications: feeding (animal at hopper trying to obtain food, chewing, or gnawing), drinking (animal licking at the water spout), grooming (animal scratching, biting or licking any part of its anatomy), resting (animal curled up, resting head with eyes closed), active (animal showing activity, including locomotion, sniffing, rearing), or inactive (animal immobile when aware, or signs of sickness behavior). Data were collected into 5 min period bins for display of the group behavior. Several variables were analyzed: food intake, latency to rest (i.e., the time at which animals...
first displayed resting), the transition from eating to resting (the time bin when the frequency of eating within the group matches the frequency of resting) and the average percentage of time the animals spent in each of the recorded behaviors.

**Results**

**Hemopressin blocks agonist (WIN 55212-2)-induced eGFP-CB₁ receptor internalization**

Previous receptor internalization studies on cultured cells have demonstrated that tagged CB₁ receptors, the vast majority of which are expressed on the plasma membrane under unstimulated conditions, show rapid and persistent endocytosis in response to stimulation with a CB₁ receptor agonist (Hsieh et al., 1999; Coutts et al., 2001; Daigle et al., 2008; Blair et al., 2009). This receptor internalization can be blocked by cotreatment with CB₁ receptor inverse agonists (Hsieh et al., 1999; Coutts et al., 2001). In the present study, we confirmed a direct action of hemopressin on CB₁ receptors by *in vitro* eGFP-CB₁ internalization assay, in which we compared the action of hemopressin with the well characterized CB₁ inverse agonist, AM251, in antagonizing the actions of the agonist WIN 55212-2 (Hsieh et al., 1999) (Fig. 1). Two hours’ treatment of transfected cells with WIN 55212-2 caused a significant internalization of eGFP-CB₁ receptor into endosomes (*p* < 0.01; Fig. 1b,e). This effect was blocked by coadministration of either AM251 or increasing doses of hemopressin (IC₅₀ = 1.55 μM, Fig. 1d,e; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This result supplements other *in vitro* models demonstrating the action of hemopressin on CB₁ receptors (Heimann et al., 2007). Treatment of transfected cells with either AM251 or hemopressin alone did not cause any internalization of eGFP-CB₁ receptor into endosomes (Fig. 1b,e).

**Centrally administered hemopressin results in marked hypophagia in rats and mice**

Rimonabant is a well characterized inverse agonist at the CB₁ receptor and can act in the brain to reduce appetite (Colombo et al., 1998; Di Marzo et al., 2001; Pagotto et al., 2006; Nogueiras et al., 2008). Thus, we proposed that hemopressin might have the same effect. We found that intracerebroventricular administration of hemopressin caused a dose-dependent decrease of night-time food intake in freely behaving, outbred mice and rats. For mice, a dose of 10 nmol per animal, significantly decreased food intake 1 (*p* < 0.05), 2 (*p* < 0.01), and 4 h after injection (*p* < 0.05, Fig. 2a; supplemental Fig. 2, available at www.jneurosci.org as supplemental material), whereas for rats, the same dose significantly decreased food intake 1 h after injection (*p* < 0.05; Fig. 2b). For both species, these doses of hemopressin caused no medium-term adverse effects on feeding behavior, as cumulative food intake normalized over the following 12 h period (for mice and rats, respectively, see supplemental Figs. 2, 3, available at www.jneurosci.org as supplemental material).

**Hypophagia produced by systemic administration of hemopressin is absent in CB₁⁻/⁻ mice**

Since hemopressin is a relatively small peptide and appears to be able to cross the blood–brain barrier (Heimann et al., 2007), we next tried systemic (intraperitoneal) administration in outbred mice. Again, hemopressin caused a decrease in normal, nocturnal feeding with a significant effect at 2 h postinjection, comparable to that of the synthetic CB₁ inverse agonist AM251 (hemopressin *p* < 0.05, AM251 *p* < 0.01; Fig. 3a). This slight delay in action of hemopressin was observed in repeated experiments and might reflect the peptide accessing sites of action within the brain. Cumulative food intake normalized over the following 12 h period (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The dose of AM251 was based on behavioral effects (Tallett et al., 2007b), rather than on comparative CB₁ efficacy.

This systemic dosing was repeated in over-night fasted, wild-type (CB₁⁺/⁺) and CB₁ receptor knock-out (CB₁⁻/⁻) mouse litter-
Hemopressin can functionally antagonize CB₁ agonist

Cumulative food intake normalized over the following 12 h period (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Hemopressin does not disrupt the behavioral satiety sequence

To demonstrate that hemopressin is reducing food intake without causing any adverse effects, such as nausea, aversion or sedation, we demonstrated that treated mice display a normal behavioral satiety sequence (BSS). Singly housed mice, which have their food temporarily removed, display a stereotypic sequence of behaviors when food is returned: eating and drinking, through exploration and grooming, before curling up to sleep (Halford et al., 1998). Any factor reducing appetite because of an abnormal, adverse effect will disrupt this sequence, whereas a natural satiety factor will maintain the sequence but shift it “leftwards.” Indeed, there is evidence that rimonabant and its derivative, AM251, reduce food intake, but also increase scratching in rodent models, probably by an off-target action on opioid receptors (Tallett et al., 2007a,b, 2008) (also see Fig. 6).

During the 90 min test period, mice treated intraperitoneally with hemopressin spent significantly less time feeding and ate significantly less food than controls (both \( p < 0.05 \); supplemental Table 1, available at www.jneurosci.org as supplemental material). No differences were seen between the groups for the average percentage of time spent in the other recorded behaviors. Furthermore, no other unusual behaviors (e.g., excessive scratching, immobility or sickness behavior) were noted. Similar results were recorded for intracerebroventricular injection of hemopressin, though here the reduction in time spent feeding did not reach statistical significance (supplemental Table 1, available at www.jneurosci.org as supplemental material). When plotted against time, the group receiving vehicle intraperitoneally displayed a normal BSS (Fig. 5a). Importantly, hemopressin did not disrupt the sequence, suggesting that it is not reducing feeding by causing any adverse reactions. However, as previously noted for natural satiety factors (Lawrence et al., 2002; Scott et al., 2005), there was an apparent shift of the sequence to the left following hemopressin administration (Fig. 5c,d). The point of transition from eating to resting took place in time bin 8 for mice given hemopressin compared with time bin 10 for controls. The average latency to rest for mice given hemopressin intraperitoneally was found to be significantly shorter than controls (vehicle, 73 ± 2 min vs hemopressin, 53 ± 5 min; \( p < 0.01 \); supplemental Table 1, available at www.jneurosci.org as supplemental material). The maintenance of the BSS and its shift leftwards are important, therefore we wished to compare this result to that of AM251 which, as with rimonan-

mates (Marsicano et al., 2002). Since the two genotypes have significantly different average body weights, results are expressed as food intake per gram body weight. The fact that intraperitoneal hemopressin decreased food intake in fasted wild-type mice 2 h after injection \( (p < 0.05; \) Fig. 3b), shows that it is capable of overcoming a powerful, natural orexigenic drive. This response is lost in the CB₁/⁻/⁻ mice (Fig. 3b), demonstrating that the effect is mediated in vivo by CB₁ cannabinoid receptors. Cumulative food intake normalized over the following 12 h period (supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

Systemic administration of hemopressin causes hypophagia in ob/ob mice

Homozygous ob/ob mice are deficient in leptin and express an obese, hypoglycemic and hypophagic phenotype, with elevated endocannabinoid tone in the hypothalamus (Di Marzo et al., 2001). Like Rimonabant in previous studies (Di Marzo et al., 2001), systemic administration of hemopressin causes marked hypophagia at both 1 and 2 h postinjection \( (p < 0.05; \) Fig. 3c). Cumulative food intake normalized over the following 12 h period (supplemental Fig. 6, available at www.jneurosci.org as supplemental material).

Hemopressin can functionally antagonize CB₁ agonist (CP55940)-induced hypophagia

We and others have shown previously that the CB₁ receptor inverse agonist, rimonabant, can functionally antagonize the orexigenic effect of CB₁ receptor agonists, such as CP55940 (Dodd et al., 2009). To avoid complications with repeated injections in mice, this experiment was performed in rats. A significant increase in food intake was seen 1 h following CP55940 adminis-
bant, is reported to have an off-target adverse effect in rodents (Tallett et al., 2007a,b, 2008). Both hemopressin and AM251 caused a decrease in feeding (Fig. 6). However, as reported previously, AM251 caused a significant increase in scratching. No such unusual behaviors were recorded following hemopressin administration.

Discussion

Our results demonstrate that hemopressin, a peptide which acts selectively as an inverse agonist at the CB₁ receptor (Heimann et al., 2007) can: (1) antagonize CB₁, agonist-induced internalization of the CB₁ receptor in vitro; (2) induce hypophagia in vivo when administered centrally; (3) induce hypophagia in vivo when administered systemically, but only in mice with functional CB₁ receptors; (4) overcome powerful orexigenic drives in fasted or obese mice; and (5) reduce feeding in a behaviorally specific manner.

The endocannabinoid system has diverse roles in cognition, memory, anxiety, motor behavior, nociception and appetite (Svízenská et al., 2008). Numerous studies have described the orexigenic action of the lipid-based endogenous CB₁ agonists, such as anandamide and 2-arachidonoylglycerol, on feeding behavior and appetite regulation (Williams and Kirkham, 1999; Hao et al., 2000; Jamshidi and Taylor, 2001; Kirkham et al., 2002). An abundance of synthetic compounds also have been synthesized to interfere with cannabinoid CB₁ transmission in attempts to exploit the therapeutic potential offered by targeting this diverse neurotransmitter system. For example, rimonabant has acute central effects on appetite and continuing actions on body weight probably via peripheral interaction with lipid mobilization pathways in white adipose tissue and with cellular glucose uptake systems (Colombo et al., 1998; Di Marzo et al., 2001; Nogueiras et al., 2008). However, the US Food and Drug Administration rejected rimonabant because clinical trials suggested a higher incidence of depression, anxiety and suicidality following prolonged administration (Christensen et al., 2007; Nissen et al., 2008). Furthermore, in this and in previous studies assessing the behavioral satiety sequence after either rimonabant or its derivative, AM251, reductions in feeding have been associated with off-target actions (probably opioid mediated) leading to excessive scratching (Tallett et al., 2007a,b, 2008). By comparison, our behavioral studies have not found any similar adverse reactions in response to hemopressin, either in the short or medium term. Further studies will need to be performed to determine whether hemopressin has any long-term deleterious effects on motivation, or advantageous effects on peripheral metabolism.

Our findings are consistent with other reports showing that synthetic receptor inverse agonists can exhibit hypophagic effects mediated via CB₁ receptors, when administered either centrally or systemically (Arnone et al., 1997; Simiand et al., 1998; Di Marzo et al., 2001; Rowland et al., 2001; Verty et al., 2004a; Ward and Dykstra, 2005). Hemopressin, like rimonabant, can functionally antagonize CB₁ agonist-induced hyperphagia (Williams and Kirkham, 2002; Dodd et al., 2009) and it is capable of overcoming powerful orexigenic drives in fasted animals. Like rimonabant, hemopressin can also overcome the orexigenic drive produced in leptin-deficient, ob/ob mice (Di Marzo et al., 2001). As either fasted mice or leptin-deficient mice are known to have elevated hypothalamic endocannabinoid levels (Di Marzo et al., 2001; Kirkham et al., 2002), the possibility remains that hemopressin may be acting as an neutral antagonist against heightened endocannabinoid tone rather than as an inverse agonist.

The central mechanisms underlying CB₁-mediated effects on appetite are unclear. However, a large body of evidence suggests that CB₁ receptors may interact not only directly with the known feeding-related circuitry of the hypothalamus but, also, may impinge on dopaminergic and opioid signaling in the striatum which are known to mediate the motivational and rewarding aspects of feeding behavior (Cota et al., 2003a, 2006; Kirkham, 2009). This is further suggested by the ability of CB₁ ligands and fatty acid amide hydrolase inhibitors, to elicit robust feeding responses when administered directly into the nucleus accumbens or into nuclei of the hypothalamus (Williams and Kirkham, 1999; Kirkham et al., 2002; Verty et al., 2005; Soria-Gómez et al., 2007). Interestingly, a number of these studies found no effects of intra-accumbens injection of rimonabant or AM251 on food intake, suggesting that that feeding-related effect of CB₁ inverse agonism may depend substantially on an integrated response throughout the forebrain (Werner and Koch, 2003; Verty et al., 2004a,b). A recent functional magnetic resonance imaging study in rats showed that regions of the orbitofrontal cortex, striatum (particularly the nucleus accumbens) and the hypothalamus, are functionally responsive to orexigenic or anorectic doses of opposing CB₁ ligands (Dodd et al., 2009). An interesting result from the current study is that hemopressin may also act on satiety pathways, perhaps in the brainstem, or via peripheral CB₁ receptors in the gut, since it caused a slight advance (leftwards shift) of the behavioral satiety sequence (Gómez et al., 2002).

The expression and functional profile of hemopressin in the brain is yet to be fully elucidated. Recent studies have described the location of hemoglobin α chain mRNA and protein in rat and human neurons, including those in the dopaminergic system (Richter et al., 2009; Schelhorn et al., 2009). Therefore, it is possible that hemopressin, which is derived from the hemoglobin α chain gene (Rioli et al., 2003; Lippton et al., 2006; Heimann et al., 2007), may be produced within pathways involved in motivated behavior. Furthermore, a very recent paper has described N-terminally extended hemopressin sequences which can act as CB₁ agonists in vitro (Gomes et al., 2009), while a precedent has already been set for functional opioidergic peptides derived from the hemoglobin β chain (Nyberg et al., 1997). Such biologically active peptides are not processed by the vesicular secretory pathway, so it is yet to be determined whether their release can be regulated. As the known, lipid-based endocannabinoids are produced “on demand,” similar processes may regulate the production of small, bioactive peptides, as has been seen with some interleukins (Simi et al., 2007).

Hemopressin is a novel bioactive peptide found in the brain that is capable of functionally antagonizing the actions of endogenous cannabinoid receptor agonists and may be placed to act as a natural suppressant of hedonically motivated eating. Indeed, the precedent for mutually antagonistic pathways containing receptor agonists and inverse agonists that can subtly modulate food intake (viz α-MSH and agonist-related peptide which antagonize each other at melanocortin receptors) already exists (Lu et al., 1994; Ollmann et al., 1997; Pritchard et al., 2004), and may indicate the existence of such mutual antagonism as a common feature in central appetite regulatory systems.

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Chapter 7 - References


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