

Central regulation of food intake and reproduction

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Abstract

The work described in this thesis investigates the actions of hypothalamic neuropeptides involved in the control of energy homeostasis and reproduction.

Cerebellin 1 (Cbln1) is a 16-amino acid peptide abundantly expressed in the cerebellum and the hypothalamus. I have shown that Cbln1 increases food intake when administered centrally to rats, and that this effect may be partly mediated by neuropeptide Y. I have also demonstrated that Cbln1 mRNA expression within the ventromedial nucleus of the hypothalamus is up-regulated following a 48-hour fast, suggesting that Cbln1 may have a physiological role in the control of food intake.

In addition to hypothalamic centres, the reward system has an important role in the control of feeding. The melanocortin system has a well characterised role in the homeostatic control of food intake. My work suggests that the melanocortin system may also have a previously unknown role within the ventral tegmental area (VTA) in the hedonic control of feeding. Intra-VTA administration of a melanocortin agonist reduced, whereas an antagonist increased, food intake in rats. My work suggests that the VTA melanocortin system may act upstream of dopamine signalling to influence feeding.

Kisspeptin is a hypothalamic neuropeptide with a crucial role in reproduction. The hypothalamus contains two populations of kisspeptin neurones, located in the arcuate nucleus (Arc) and in the anteroventral periventricular nucleus. The physiological role of these two populations is unknown. I used recombinant adeno-associated virus (rAAV) to suppress kisspeptin expression within the Arc in female rats. Animals injected bilaterally into the Arc with rAAV-kisspeptin antisense had significantly fewer oestrous cycles, and an increase in oestrous cycle length, compared to controls.

In summary, these studies have identified novel roles for Cbln1 and the melanocortin system in the control of food intake, and have demonstrated the importance of Arc kisspeptin signalling in the regulation of reproduction.

Declaration of Contributors

The majority of the work in this thesis was performed by the author. All collaboration and assistance is described below:

All radioimmunoassays were carried out under the supervision of Professor M.A. Ghatel (Division of Diabetes, Endocrinology and Metabolism, Imperial College London).

Chapter 2

Acute feeding studies and hypothalamic explant studies were carried out in collaboration with Dr Debabrata Roy (Division of Diabetes, Endocrinology and Metabolism). Assistance with intracerebroventricular cannulation and injection was provided by members of the 'hypothalamic group'.

Competent bacteria were prepared by Dr James Gardiner (Department of Diabetes, Endocrinology and Metabolism).

Chapter 3

Assistance with intra-VTA cannulation and injection was provided by Dr Charlotte Boughton (Division of Diabetes, Endocrinology and Metabolism).

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Recombinant AAV were prepared and tested *in vitro* by Dr James Gardiner (Department of Diabetes, Endocrinology and Metabolism).

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Abbreviations

AAAD	Aromatic amino acid decarboxylase
AAV	Adeno-associated virus
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotrophic hormone
ADAR	Adenosine deaminase
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AP	Area postrema
Arc	Arcuate nucleus
AS	Antisense
AVP	Arginine vasopressin
AVPV	Anteroventral periventricular nucleus
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BNST	Bed nucleus of the stria terminalis
BP	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine-related transcript
Cbln1	Cerebellin 1
CCK	Cholecystokinin
cDNA	Copy deoxyribonucleic acid
CMV	Cytomegalovirus
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CPM	Counts per minute
CPP	Conditioned place preference
CRH	Corticotrophin-releasing hormone
DA	Dopamine
DAT	Dopamine transporter
DMN	Dorsomedial nucleus
DNA	Deoxyribonucleic acid
dNTPSs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GDW	Glass distilled water

GLP-1	Glucagon-like peptide 1
GluD2	Glutamate receptor delta 2 subunit
GnRH	Gonadotrophin releasing hormone
GPCR	G-protein coupled receptor
GTE	Glucose-Tris-EDTA
HPA	Hypothalamo-pituitary-adrenal
HPG	Hypothalamo-pituitary-gonadal
HPLC	High performance liquid chromatography
HPT	Hypothalamo-pituitary-thyroid
ICV	Intracerebroventricular
IP	Intraperitoneal
IR	Immunoreactivity
ISH	<i>In situ</i> hybridisation
ITR	Inverted terminal repeat
LB	Lysogeny broth
LDT	Laterodorsal tegmental nucleus
LH	Luteinising hormone
LHA	Lateral hypothalamic area
LHSS	Lateral hypothalamic self-stimulation
LV	Lateral ventricle
MAOA	Monoamine oxidase A
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MCH	Melanin-concentrating hormone
MPOA	Medial preoptic area
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NDP-MSH	[Nle ⁴ ,D-Phe ⁷]- α -melanocyte stimulating hormone
NKB	Neurokinin B
NPS	Neuropeptide S
NPY	Neuropeptide Y
NSB	Non-specific binding
NTS	Nucleus tractus solitarius
ObRb	Long form of the leptin receptor
PBP	Parabrachial pigmented area of the VTA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFC	Prefrontal cortex
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
Pre-Cbln1	Pre-Cerebellin 1
PVN	Paraventricular nucleus
PYY	Peptide YY

rAAV	Recombinant adeno-associated virus
rAAV-EGFP	Recombinant adeno-associated virus expressing EGFP
rAAV-Kisspeptin-AS	Recombinant adeno-associated virus expressing kisspeptin antisense
RIA	Radioimmunoassay
RT	Reverse transcription
SAP	Shrimp alkali phosphatase
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SF1	Steroidogenic factor 1
SON	Supraoptic nucleus
SSC	Saline sodium citrate
STAT3	Signal transducer and activator of transcription 3
TAE	Tris-acetate-EDTA buffer
TEA	Triethanolamine
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TH	Tyrosine hydroxylase
TNF- α	Tumour necrosis factor- α
TPP	Tegmental pedunculopontine nucleus
TRH	Thyrotrophin-releasing hormone
tRNA	Transfer ribonucleic acid
TSH	Thyroid stimulating hormone
UCP-1	Uncoupling protein-1
UV	Ultraviolet
VMN	Ventromedial nucleus
VTA	Ventral tegmental area
V/V	Volume by volume
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
W/V	Weight by volume
α -MSH	Alpha- melanocyte stimulating hormone
β -MSH	Beta- melanocyte stimulating hormone
γ -MSH	Gamma-melanocyte stimulating hormone

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Chapter 1: General introduction

1.1. The endocrine system

Maintenance of a stable internal environment is essential for the survival of most organisms. In mammals, this stability is primarily achieved by two systems: the autonomic nervous system (ANS) and the endocrine system. Whilst the ANS signals rapidly via neural impulses and neurotransmitter release, the endocrine system communicates via chemical messengers, or hormones. Hormones are secreted by endocrine glands directly into the bloodstream and are transported to their often distant specific target cells. The endocrine system is therefore slower and less localised than the ANS.

Although distinct, the ANS and endocrine system overlap in function and each influences the other. The hypothalamus is a key interface between both systems and can be considered a neuroendocrine organ, as it contains neurones which can release neurosecretions either across synapses, or directly into the bloodstream.

The hypothalamus controls the release of hormones from the pituitary gland into the systemic circulation, which act upon target cells in peripheral tissues. Together, the hypothalamus and pituitary regulate numerous functions including reproduction, metabolism, growth and the stress response. An important characteristic of the endocrine system is its ability to regulate itself via negative feedback. Hormones released from peripheral endocrine organs act at the hypothalamus and pituitary to inhibit the stimuli for their own release, hence ensuring that the activity of the endocrine system remains within certain limits.

1.2. Energy homeostasis and reproduction

Body weight and fertility are closely linked. Pubertal onset and normal functioning of the reproductive axis are dependent on adequate body energy stores, and energy deficiency resulting from under-nutrition or excessive exercise results in a reduction in reproductive capacity in both humans and animals (Klentrou and Plyley, 2003; De Souza et al., 1998; Glass et al., 1976; Terry et al., 2005). At the opposite end of the spectrum, obesity is also linked to disorders of fertility (Rachon and Teede, 2010).

The hypothalamus plays a crucial role in the regulation of both energy homeostasis and reproduction. By responding to and integrating numerous metabolic signals, the hypothalamus couples energy reserves with fertility. One such metabolic signal, which has a vital role in the control of both energy homeostasis and reproduction, is the adipokine leptin.

1.3. Leptin

Leptin is a peptide hormone encoded by the *ob* gene and secreted by white adipose tissue (Zhang et al., 1994). Leptin circulates at levels proportional to body fat stores (Maffei et al., 1995; Considine et al., 1996) and is able to cross the blood-brain barrier by a saturable process (Banks et al., 1996). Although leptin is generally thought to be a signal of long-term energy stores, circulating levels may become temporarily dissociated from body adipose tissue levels. For example following a 48 hour fast, serum leptin levels rapidly decrease and rise following re-feeding in rodents (Ahima et al., 1996; Frederich et al., 1995).

Mice genetically deficient in leptin (*ob/ob*) or the leptin receptor (*db/db*) are severely obese and hyperphagic. Additionally, male and female *ob/ob* mice do not enter puberty and are infertile (Swerdloff et al., 1976). Leptin administration to *ob/ob* mice reduces food intake and body weight (Halaas et al., 1995), and restores fertility (Chehab et al., 1996). This suggests that leptin has a key role in the regulation of both energy homeostasis and reproduction.

Peripheral and central administration of leptin to rodents reduces food intake and body weight, and increases energy expenditure (Halaas et al., 1995; Campfield et al., 1995; Van Heek et al., 1997). Although cases of leptin deficiency resulting in severe early-onset obesity have been identified in humans (Montague et al., 1997), most obese people have elevated serum leptin compared to lean controls (Considine et al., 1996). This suggests that common obesity is associated with leptin resistance. Accordingly, diet-induced obese mice and obese humans are insensitive to the anorectic effect of peripherally administered leptin (Van Heek et al., 1997; Heymsfield et al., 1999).

Leptin signals via the long form of the leptin receptor, ObRb, a member of the type 1 cytokine receptor family (Tartaglia et al., 1995). ObRb is expressed in hypothalamic and non-hypothalamic sites including the reward system and the brainstem (Hakansson et al., 1998; Mercer et al., 1996b; Munzberg, 2008).

Starvation induces neuroendocrine changes designed to conserve energy stores and aid survival. These include a reduction in reproductive capacity and thyroid hormone-induced thermogenesis. These changes are also observed in *ob/ob* mice, suggesting that a lack of leptin signalling induces these adaptations. Leptin administration corrects the neuroendocrine abnormalities observed in food-deprived mice (Ahima et al., 1996). The severe phenotype induced by leptin deficiency, and the apparent lack of effect of excess leptin in obesity, suggest that the main role

of leptin is to signal starvation, rather than nutrient excess, to the CNS. This signalling is primarily mediated via the hypothalamus.

1.4. The hypothalamus

The hypothalamus is present in the brain of all vertebrates. It is located in the ventral part of the diencephalon and lies inferior to the thalamus, surrounding the third ventricle. The hypothalamus regulates several functions including body temperature, reproduction, electrolyte balance and circadian rhythm. By detecting and responding to internal and external stimuli, the hypothalamus plays an integral role in maintaining bodily homeostasis.

The hypothalamus can be divided into three distinct zones: the periventricular, medial, and lateral zones (Berthoud, 2002). Each zone contains numerous discrete collections of neuronal cell bodies, or nuclei (Figure 1.1).

1.5. Hypothalamic regulation of food intake and energy expenditure

A role for the hypothalamus in energy homeostasis was first suggested in the mid-20th century, when it was discovered that bilateral lesions of the lateral hypothalamus resulted in anorexia in rats, whereas destruction of the ventromedial hypothalamus led to obesity and hyperphagia (Hetherington and Ranson, 1940; Anand and Brobeck, 1951). The lateral hypothalamus was therefore designated the ‘feeding centre’ and the ventromedial nucleus the ‘satiety centre’. The ‘dual centre’ hypothesis is now considered too simplistic and it is instead thought that circuits between and within discrete hypothalamic nuclei communicate via specific neurotransmitters to regulate food intake and energy expenditure. Additionally, many extra-hypothalamic areas including the brainstem, reward centres and higher cortical areas are now known to be involved in the control of feeding (Berthoud, 2002).

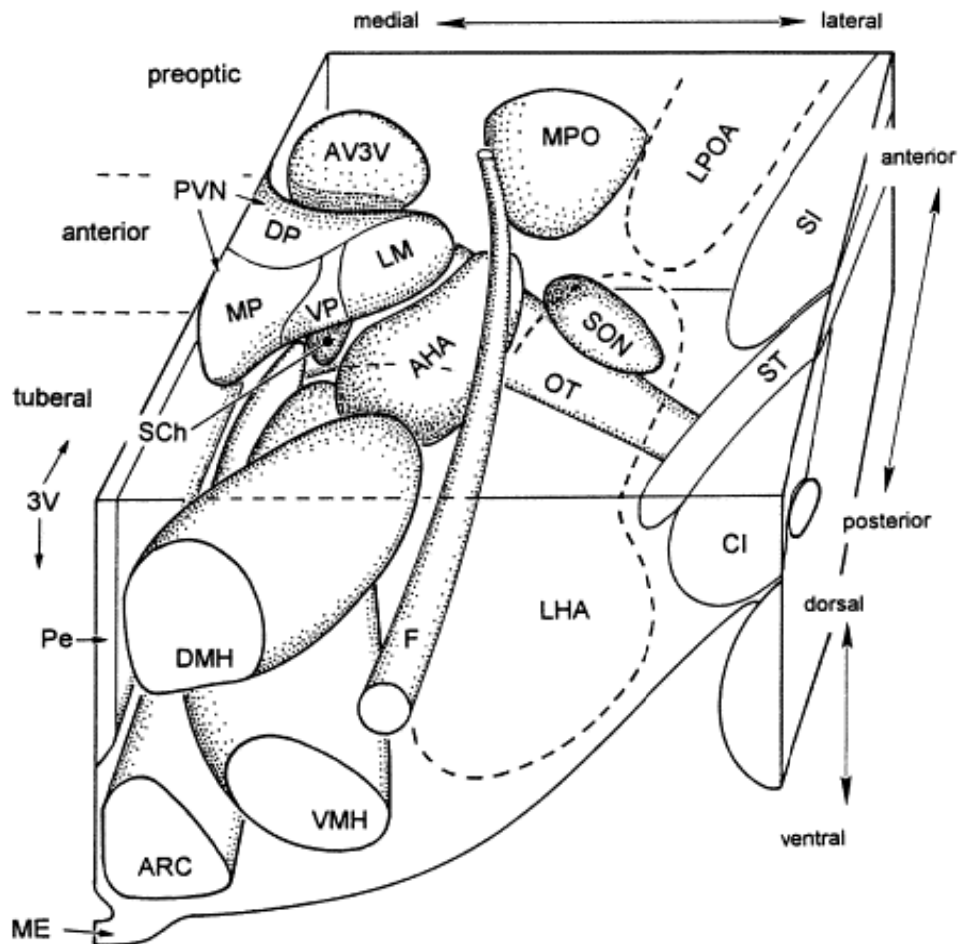


Figure 1.1: Three-dimensional view of the rat hypothalamus (right hemisphere) as viewed from the dorsal and caudal perspective. Abbreviations: AHA, anterior hypothalamic area; ARC, arcuate nucleus; AV3V, anteroventral area of third ventricle; CI, capsula interna; DP, dorsal parvocellular subnucleus of paraventricular nucleus (PVN); DMN, dorsomedial nucleus; F, fornix; LHA, lateral hypothalamic area; LM, lateral magnocellular subnucleus of PVN; LPOA, lateral preoptic area; ME, median eminence; MP, medial parvocellular PVN; MPO, medial preoptic area; OT, optic tract; Sch, suprachiasmatic nucleus; SON, supraoptic nucleus; SI, substantia innomminata; ST, subthalamic nucleus; VMN, ventromedial nucleus; VP, ventral parvocellular subnucleus of PVN. Taken from (Berthoud, 2002).

1.5.1. Arcuate nucleus

The Arcuate nucleus (Arc) of the hypothalamus is an elongated collection of neuronal cell bodies which lies adjacent to the floor of the third ventricle (Schwartz et al., 2000). The Arc is situated directly above the median eminence, a circumventricular organ which lacks a complete blood-brain barrier, and is thus ideally situated to detect and respond to circulating signals of short- and long-term nutritional status (Broadwell and Brightman, 1976). Numerous peripheral signals act at the Arc to influence energy homeostasis, including adipokines such as leptin (Glaum et al., 1996), insulin (Wilcox et al., 1989), gut hormones, such as ghrelin (Hewson and Dickson, 2000), and metabolites, such as glucose (Lynch et al., 2000).

The Arc contains two major groups of neurones which influence energy homeostasis. Located medially are a subpopulation of neurones which co-express the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) (Hahn et al., 1998). A second, lateral sub-population co-express the anorexigenic peptide cocaine-and amphetamine-regulated transcript (CART), and the precursor protein pro-opiomelanocortin (POMC), which is cleaved to produce the anorectic alpha-melanocyte stimulating hormone (α -MSH) (Elias et al., 1998a). In addition to exerting opposing actions, a small population of NPY neurones directly inhibit adjacent POMC/CART neurones via local release of gamma-aminobutyric acid (GABA) (Cowley et al., 2001).

Both NPY/AgRP and POMC/CART neurones express the leptin receptor (Mercer et al., 1996a; Cheung et al., 1997). Leptin inhibits the activity of NPY/AgRP neurones and stimulates the activity of POMC/CART neurones, hence promoting a state of negative energy balance (Elias et al., 1998a; Elias et al., 1999).

NPY/AgRP co-expressing neurones of the Arc project to various hypothalamic nuclei implicated in the control of food intake, including the paraventricular nucleus (PVN), lateral hypothalamus (LH) and dorsomedial nucleus (DMN). Additionally, extra-hypothalamic targets include the ventral tegmental area (VTA), the amygdala, and the bed nucleus of the stria terminalis (BNST) (Broberger et al., 1998). POMC/CART co-expressing neurones also project widely to hypothalamic and extra-hypothalamic sites (Elias et al., 1998a; Elias et al., 1998b).

1.5.2. Paraventricular nucleus

The PVN is situated adjacent to the superior part of the third ventricle and contains two functionally distinct cell divisions. Magnocellular neurones synthesise arginine vasopressin (AVP) and oxytocin and project to the posterior lobe of the pituitary gland. Parvocellular neurones produce corticotrophin releasing hormone (CRH) and thyrotrophin releasing hormone (TRH) and project to other hypothalamic nuclei, the brainstem, and the median eminence (Swanson and Kuypers, 1980). At the median eminence, parvocellular nerve terminals release CRH and TRH into the pituitary portal circulation (Swanson and Sawchenko, 1983). CRH and TRH regulate the hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-thyroid (HPT) axes respectively.

The PVN plays a crucial role in energy homeostasis. Bilateral lesions of the PVN cause hyperphagia (Fukushima et al., 1987) and intra-hypothalamic administration of either CRH or TRH reduces food intake and increases energy expenditure (Suzuki et al., 1982; Arase et al., 1988). CRH and TRH neurones in the PVN are hypothesised to act downstream of Arc NPY/AgRP and POMC/CART neurones (Schwartz et al., 2000). NPY/AgRP and POMC/CART neurones project to the PVN, and intra-PVN administration of NPY stimulates, whereas α -MSH inhibits, food intake in rats (Stanley and Leibowitz, 1984; Giraudo et al., 1998).

The PVN receives inputs from hypothalamic nuclei including the Arc, DMN and VMN, and from extra-hypothalamic sites such as the brainstem and cortico-limbic structures (Berthoud, 2002). In addition to projecting to the posterior pituitary and median eminence, the PVN also has direct inputs to sympathetic and parasympathetic pre-ganglionic neurones (Jansen et al., 1997). The PVN is therefore able to integrate information from a variety of sources to initiate appropriate endocrine and autonomic responses.

1.5.3. Ventromedial nucleus

The VMN lies superior to the Arc and has reciprocal connections with the brainstem and most hypothalamic nuclei involved in the regulation of food intake (Canteras et al., 1994). It was originally designated the 'satiety centre' of the brain, as bilateral lesions of the VMN induce obesity and hyperphagia in rats (Anand and Brobeck, 1951). It has since been suggested that the observed phenotype may have been caused by damage to the neighbouring Arc and PVN (Aravich and Sclafani, 1983). However, mice lacking steroidogenic factor 1 (SF1), a relatively

VMN-specific transcription factor, have abnormal VMN cellular morphology (Dellovade et al., 2000) and are obese (Majdic et al., 2002). VMN SF1 neurones are leptin-responsive and loss of leptin signalling in SF1 neurones causes obesity (Dhillon et al., 2006).

Brain-derived neurotrophic factor (BDNF), a neurotrophin essential for brain development, is highly expressed within the VMN (Xu et al., 2003). BDNF is a potent anorexigenic factor; central infusion of BDNF reduces food intake, induces weight loss and improves glucose homeostasis in diabetic db/db mice (Pelleymounter et al., 1995; Ono et al., 1997). BDNF mRNA expression within the VMN is reduced by fasting (Xu et al., 2003) and mice heterozygous for the BDNF gene are heavier and eat more than their wild-type littermates (Kernie et al., 2000). BDNF is hypothesised to act downstream of the melanocortin system to reduce food intake, as VMN BDNF mRNA expression is down-regulated in MC4R knockout mice, and administration of an MC4R agonist rescues fasting-induced suppression of BDNF expression (Xu et al., 2003).

1.5.4. Dorsomedial nucleus

The dorsomedial nucleus of the hypothalamus (DMN) is located immediately dorsal to the VMN. Unlike the VMN, ablation of the DMN causes hypophagia and reduced body weight gain (Dalton et al., 1981). The DMN receives afferent inputs from hypothalamic nuclei including the Arc, and from the brainstem (Thompson and Swanson, 1998). Projections from the DMN are largely intra-hypothalamic; the PVN is particularly densely innervated (Thompson et al., 1996).

The DMN contains a population of NPY-expressing neurones, and DMN NPY expression is up-regulated in states of energy deprivation, for example following intense exercise, food restriction or lactation (Lewis et al., 1993; Smith, 1993). The DMN contains a high density of the leptin receptor ObRb and is highly responsive to peripheral leptin administration (Elmqvist et al., 1998a).

1.5.5. Lateral hypothalamic area

The lateral hypothalamic area (LH) is a large, heterogeneous region located lateral to the VMN. It has reciprocal connections with hypothalamic nuclei including the Arc and PVN and projects widely to the hippocampus, thalamus and amygdala (Saper et al., 1979; Elias et al., 1998b). The LH was originally termed the 'feeding centre' of the brain, as early studies demonstrated that bilateral lesions resulted in hypophagia (Anand and Brobeck, 1951). Subsequently, it was discovered that the LH is the site of production of two groups of orexigenic neuropeptides: melanin-concentrating hormone (MCH) and the orexins. Both MCH and orexin-expressing neurones receive dense innervations from arcuate NPY/AgRP and α -MSH fibres (Elias et al., 1998b) and hence indirectly signal downstream of leptin.

In mammals, MCH-expressing neuronal cell bodies are found exclusively in the zona incerta and lateral hypothalamus (Skofitsch et al., 1985). ICV administration of MCH acutely stimulates feeding (Qu et al., 1996) and reduces energy expenditure by suppressing the HPT axis (Kennedy et al., 2001). Furthermore, hypothalamic MCH expression is up-regulated by fasting and is elevated in leptin deficient ob/ob mice compared to wild-type controls (Qu et al., 1996). Mice lacking MCH are lean and hypophagic (Shimada et al., 1998).

A distinct neuronal population within the LH express prepro-orexin, which is processed to produce orexin A and orexin B. Prepro-orexin mRNA expression is increased by fasting and ICV administration of orexin A or B increases food intake (Sakurai et al., 1998). Orexin knockout mice have a phenotype very similar to human narcolepsy (Chemelli et al., 1999) and the orexins are hence thought to be important mediators of sleep and wakefulness. The effects of the orexins on appetite may therefore be at least partly secondary to effects on wakefulness.

The LH is also hypothesised to coordinate the homeostatic control of food intake and the rewarding, hedonistic aspect of feeding. The LH has reciprocal connections with the limbic system (Saper et al., 1979; Usuda et al., 1998) and the effects of the mesocorticolimbic dopamine system on food intake may be mediated by LH neurones (Stratford and Kelley, 1999).

1.6. Extra-hypothalamic regions involved in the control of energy homeostasis

1.6.1. The brainstem

The brainstem, consisting of the midbrain, pons and medulla, is a site of convergence for meal-related satiety signals. It plays a vital role in the control of energy homeostasis and is able to regulate food intake even when severed from the forebrain in rats (Grill and Smith, 1988).

The nucleus tractus solitarius (NTS) of the brainstem is particularly important in the control of energy homeostasis. The NTS lies along the length of the medulla and receives afferent input from the glossopharyngeal, trigeminal, and facial nerves, which convey taste and sensory information from the tongue and face. The NTS is also the termination site of vagal sensory neurones originating in the gastrointestinal tract. These neurones relay information regarding visceral chemo- and mechanosensation. Many gut hormones, including cholecystokinin (CCK) and ghrelin mediate their effects on food intake partly via vagal signalling to the brainstem (Smith et al., 1981; Date et al., 2002).

The NTS lies in close proximity to the area postrema (AP), a circumventricular organ which, like the Arc, lacks a complete blood-brain barrier. The AP is therefore able to detect meal-related blood-borne signals and, due to the rich projections from the AP to the NTS, relay this information directly to the NTS.

The NTS also has reciprocal connections with the forebrain and projects particularly strongly to the PVN and DMN (Ter Horst et al., 1989). Neuronal populations expressing POMC, NPY and glucagon-like peptide 1 (GLP-1) are located in the NTS and project widely to the forebrain.

1.6.2. The mesocorticolimbic pathway

In addition to homeostatic circuits in the hypothalamus and brainstem, the cortico-limbic structures involved in cognition, emotion and reward also play an important role in the control of feeding. This is particularly relevant in today's obesogenic environment in which there is a surplus of easily available palatable food. There is extensive cross-talk between the hypothalamus and areas involved in the non-homeostatic control of feeding. It has been suggested that these pathways allow cognitive and emotional factors to override homeostatic signals, and hence promote food intake even in the absence of an energy deficit (Berthoud, 2006).

Dopaminergic neurones projecting from the ventral tegmental area (VTA) in the midbrain to the nucleus accumbens (NAc), olfactory tubercle, hippocampus and pre-frontal cortex comprise the mesocorticolimbic dopamine pathway (Ikemoto, 2007) (Figure 1.2). This pathway is an important component of the CNS motivational circuitry and has a major role in mediating the rewarding properties of substances including palatable food and drugs of abuse.

Rewarding stimuli, including cocaine, opiates and palatable food, increase dopamine release within the NAc (Pettit and Justice, Jr., 1989; Wilson et al., 1995; Di Chiara and Imperato, 1988). The reinforcing effect of extracellular dopamine release in the NAc is demonstrated by the finding that rats will repeatedly self-administer the dopamine-releasing agent amphetamine into the NAc (Hoebel et al., 1983).

Over time, repeated stimulation of reward pathways induced by chronic drug use induces neuroadaptations within the mesocorticolimbic dopaminergic pathway (Volkow et al., 2004). Repeated ingestion of palatable food may induce similar changes and contribute to compulsive over-eating and obesity (Wang et al., 2001).

Dopamine has long been known to influence energy homeostasis. Dopamine depletion within the ventrolateral striatum disrupts feeding behaviour, and dopamine-deficient mice are severely hypophagic (Zhou and Palmiter, 1995). The dopamine transporter (DAT) mediates dopamine clearance from the synaptic cleft, and hence terminates dopamine signalling. DAT mRNA expression levels are altered in the VTA following food restriction (Patterson et al., 1998), further supporting a role for mesocorticolimbic dopamine in the control of food intake.

It is well known that food deprivation enhances the rewarding attributes of drugs of abuse (Bell et al., 1997; Carroll and Meisch, 1981). Starvation induces a series of metabolic changes

including hypoleptinaemia and hypoinsulinaemia. Recent work has suggested that low levels of both insulin and leptin may mediate the effect of food deprivation on reward circuitry. Food-conditioned place preference can be abolished by peripheral leptin treatment to food restricted rats (Figlewicz et al., 2001), and ICV administration of leptin or insulin reduces sucrose self-administration in rats (Figlewicz et al., 2006). However, this effect is blunted if animals are fed a high fat diet for several weeks prior to the study (Figlewicz et al., 2006), suggesting that high fat feeding induces central resistance to leptin and insulin.

ICV insulin administration increases DAT mRNA expression and hence reduces dopaminergic signalling within the VTA (Figlewicz et al., 1994). Insulin and leptin receptors are localised on VTA dopaminergic neurones (Figlewicz et al., 2003), suggesting that in addition to acting at the Arc to reduce food intake, leptin and insulin may also act directly on the mesocorticolimbic dopamine system to reduce the rewarding properties of food. Additionally, hypothalamic neuropeptides including the melanocortins (Tracy et al., 2008; Davis et al., 2010) and the orexins (Zheng et al., 2007) may influence food intake by modulating reward pathways.

In addition to dopamine, other neurotransmitters including glutamate and GABA are implicated in reward and reinforcement. GABAergic interneurons within the VTA tonically inhibit dopaminergic neurones (Matthews and German, 1984; David et al., 1997). In contrast, excitatory glutamatergic inputs to the VTA from cortical structures and the brainstem increase VTA dopamine transmission (Carter, 1982).

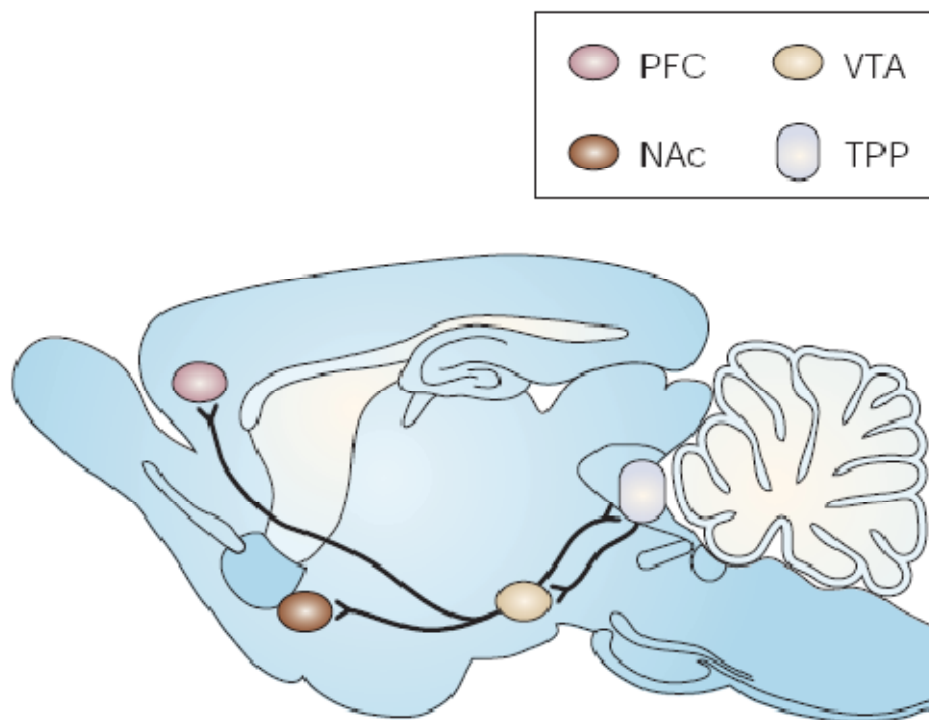


Figure 1.2: Sagittal section of the rat brain showing the mesocorticolimbic dopaminergic pathway, which originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAc) and prefrontal cortex (PFC). The VTA also has reciprocal connections with the tegmental pedunculo pontine nucleus (TPP). Taken from (Laviolette and van der Kooy, 2004).

1.7. Central regulators of energy homeostasis

1.7.1. NPY

NPY is one of the most widely distributed neurotransmitters in the central nervous system (Allen et al., 1983) and is an extremely potent stimulator of food intake (Levine and Morley, 1984). Within the hypothalamus, NPY-expressing neurones are most abundant in the Arc and mainly project to the ipsilateral PVN (Bai et al., 1985). Arcuate NPY neurones are stimulated by ghrelin (Kohno et al., 2003) and inhibited by leptin and insulin (Wang et al., 1997b; Schwartz et al., 1991).

Intra-PVN administration of NPY to rats acutely induces carbohydrate-specific hyperphagia (Stanley et al., 1985). Additionally, chronic administration of NPY into the third ventricle or directly into the PVN increases food intake, reduces energy expenditure and ultimately causes obesity (Stanley et al., 1986; Billington et al., 1991; Zarjevski et al., 1993). The reduction in energy expenditure is partly mediated by a reduction in brown adipose tissue (BAT)-mediated thermogenesis (Egawa et al., 1991). Chronic ICV administration of NPY also induces hyperinsulinaemia and hepatic lipogenesis, independently of changes in food intake (Zarjevski et al., 1993).

Levels of NPY expression and release in the rodent hypothalamus reflect nutritional status, by increasing during fasting and decreasing after re-feeding (Kalra et al., 1991; Brady et al., 1990). Additionally, long-term down-regulation of Arc NPY expression using anti-sense RNA reduces body weight gain and food intake in adult rats (Gardiner et al., 2005). This suggests that NPY has a physiological role in the control of food intake.

NPY is a member of the pancreatic polypeptide family, which also includes peptide tyrosine tyrosine (PYY) and pancreatic polypeptide (PP). These peptides bind to members of the Y receptor family, of which there are at least 6 members (Y1-Y6). NPY binds preferentially to Y1 and Y5 receptors, and ICV administration of a Y1 or Y5 antagonist inhibits NPY-induced and nocturnal food intake (O'Shea et al., 1997; Daniels et al., 2002).

1.7.2. Melanocortin system

Within the CNS, POMC is synthesised exclusively in neurones of the Arc and NTS of the brainstem (Young et al., 1998). However, POMC-expressing fibres project widely throughout the forebrain and brainstem (Joseph et al., 1985). POMC can be post-translationally cleaved to produce several bioactive peptides including adrenocorticotrophic hormone (ACTH) and α , β and γ -melanocyte stimulating hormone (MSH).

ICV α -MSH administration reduces food intake in fasted rats (Tsujii and Bray, 1989) and arcuate POMC mRNA expression levels are reduced by fasting and increased following leptin administration or re-feeding (Schwartz et al., 1997). In addition to suppressing food intake, α -MSH stimulates the hypothalamo-pituitary-thyroid (HPT) axis and hence increases energy expenditure. Alpha-MSH nerve fibres terminate in close proximity to thyrotrophin-releasing hormone (TRH) neurones in the PVN (Fekete et al., 2000), and ICV administration of an α -MSH analogue increases plasma levels of thyroid-stimulating hormone (TSH) (Kim et al., 2000b). Additionally, central α -MSH administration increases sympathetic nerve activity in BAT and increases BAT temperature (Yasuda et al., 2004).

To date, five melanocortin receptors (MC1R-MC5R) have been cloned. Alpha-MSH exerts its effects on energy homeostasis via the melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R).

The MC4R is widely expressed in the brain, particularly in hypothalamic nuclei involved in the control of feeding such as the PVN, DMN and LH (Mountjoy et al., 1994). ICV administration of MC4R agonists potently reduces food intake, whereas MC4R antagonists stimulate food intake (Benoit et al., 2000). Inactivation of the MC4R results in hyperphagia, obesity, and hyperinsulinaemia in mice and humans (Huszar et al., 1997; Yeo et al., 1998).

The role of the MC3R is less well defined. MC3R mRNA is abundant throughout the hypothalamus, particularly in the Arc (Roselli-Reh fuss et al., 1993). Although mice lacking the MC3R have increased fat mass, they are not hyperphagic (Chen et al., 2000). However, mice lacking both the MC3R and MC4R are heavier than those lacking only the MC4R, suggesting that the two receptors have distinct roles in the control of energy homeostasis (Chen et al., 2000).

Two endogenous antagonists of the melanocortin system have been identified: agouti and AgRP. Expression of agouti is usually restricted to hair follicles, whereas AgRP is located almost exclusively within NPY neurones of the Arc (Shutter et al., 1997). AgRP is a selective

MC3R/MC4R antagonist (Ollmann et al., 1997). ICV administration of AgRP blocks the anorexigenic effect of α -MSH and can elicit an increase in food intake which lasts for 24 hours or longer (Rossi et al., 1998; Small et al., 2003). Additionally, recent work using optogenetic techniques has demonstrated that stimulation of Arc AgRP neurones is sufficient to induce feeding independently of melanocortin receptor antagonism (Aponte et al., 2011). Chronic ICV AgRP administration for 7 days reduces oxygen consumption and energy expenditure, leading to an increase in body weight (Small et al., 2003). Mice engineered to over-express AgRP are obese and hyperphagic (Ollmann et al., 1997).

1.7.3. Cocaine and Amphetamine Related Transcript

Cocaine and amphetamine related transcript (CART) is widely distributed throughout the central and peripheral nervous system, and is abundantly expressed in hypothalamic nuclei involved in the control of food intake including the Arc, PVN, DMN, SON, and VMN (Douglass et al., 1995). In the Arc, over 90% of CART neurones co-express POMC mRNA (Elias et al., 1998a).

ICV administration of active CART peptide fragments inhibits basal and starvation-induced food intake in rats (Lambert et al., 1998; Kristensen et al., 1998). ICV injection of antiserum raised against CART stimulates feeding, and CART mRNA within the Arc is down-regulated in fasted animals, suggesting that endogenous CART is a physiological anorectic agent (Lambert et al., 1998; Kristensen et al., 1998). CART mRNA expression is increased following leptin administration, therefore, similarly to POMC, CART expression is positively regulated by leptin (Kristensen et al., 1998).

Despite these findings, ICV CART administration has been noted to induce a movement-associated tremor in rats, implying that its effects on food intake may be secondary to behavioural changes (Kristensen et al., 1998). Additionally, injection of CART into specific hypothalamic nuclei including the Arc and PVN acutely increases food intake in non-diabetic (Abbott et al., 2001) and diabetic (Hou et al., 2010) rats, suggesting that the role of CART in the control of energy homeostasis is more complex than originally thought.

1.8. Energy expenditure

Under most conditions, homeostatic mechanisms ensure that energy intake closely matches energy expenditure to maintain body weight at a relatively constant level. However, if energy intake exceeds energy expenditure, weight gain and obesity develop.

Energy expenditure can be subdivided into obligatory thermogenesis, which refers to the loss of heat resulting from all metabolic processes that support life; physical activity; and adaptive thermogenesis. Adaptive thermogenesis is heat production in response to environmental temperature or diet. The HPT axis is an important mediator of adaptive thermogenesis.

1.8.1. The hypothalamo-pituitary-thyroid axis

The HPT axis plays a vital role in the control of metabolism and energy expenditure.

TRH is a tri-peptide primarily produced by neurones in the parvocellular PVN, although it is also present in other areas of the CNS (Winokur and Utiger, 1974). TRH-expressing neurones terminate in the median eminence in close proximity to capillaries of the hypothalamo-hypophyseal portal system. TRH is transported by this capillary plexus to the adenohypophysis, where it stimulates the biosynthesis and release of TSH (Harris et al., 1978).

TSH binds to receptors on the thyroid gland and increases the synthesis and release of the thyroid hormones, thyroxine (T4) and the more biologically active tri-iodothyronine (T3). T4 is converted to T3 in peripheral tissues by the iodothyronine deiodinase enzyme type 2 deiodinase. T3 and T4 exert negative feedback at the hypothalamus and pituitary gland to inhibit the release of TRH and TSH respectively.

T3 and T4 increase the basal metabolic rate of many tissues, thus increasing oxygen consumption. Thyroid hormones also stimulate adaptive thermogenesis, the heat production observed following cold exposure or consumption of an energy-dense meal. One mechanism by which T3 drives thermogenesis is by increasing the expression and activity of uncoupling protein 1 (UCP-1) in brown adipose tissue (Branco et al., 1999). UCP-1 uncouples the mitochondrial proton gradient from ATP production, leading to the release of energy as heat.

Starvation suppresses the HPT axis to decrease energy expenditure and hence conserve resources. This down-regulation is characterised by reduced prepro-TRH mRNA expression within the PVN, low TSH and reduced circulating T3 and T4 (Blake et al., 1991; Connors et al.,

1985). The fall in circulating leptin during fasting is thought to play a key role in mediating this down-regulation, as leptin administration to fasted animals restores plasma thyroid hormones to fed levels (Ahima et al., 1996). Leptin acts directly at TRH neurones to increase pro-TRH biosynthesis, and indirectly via arcuate NPY/AgRP and POMC neurones. Both NPY/AgRP and POMC/CART- expressing neurones project to TRH neurones within the PVN (Fekete et al., 2000; Legradi and Lechan, 1999; Toni et al., 1990). Alpha- MSH increases, whereas NPY and AgRP reduce plasma TSH and thyroid hormones (Kim et al., 2000b; Fekete et al., 2001).

1.9. Hypothalamic regulation of reproduction

In mammals, reproductive function is controlled by the hypothalamo-pituitary-gonadal (HPG) axis. The hypothalamus is the location of neuronal cell bodies expressing gonadotrophin-releasing hormone (GnRH), a neuropeptide which drives the release of the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. LH and FSH act at the gonads to regulate steroidogenesis and gametogenesis (Figure 1.3).

The hypothalamus also contains neurones expressing kisspeptin, a 54-amino acid peptide which has a crucial role in the control of the HPG axis. Loss of function of either kisspeptin or its receptor, GPR54, results in impaired pubertal development and infertility (Seminara et al., 2003; de Roux N. et al., 2003; d'Anglemont de Tassigny et al., 2007). Kisspeptin is hypothesised to act upstream of GnRH to regulate reproduction (Irwig et al., 2004; Matsui et al., 2004; Pielecka-Fortuna et al., 2008).

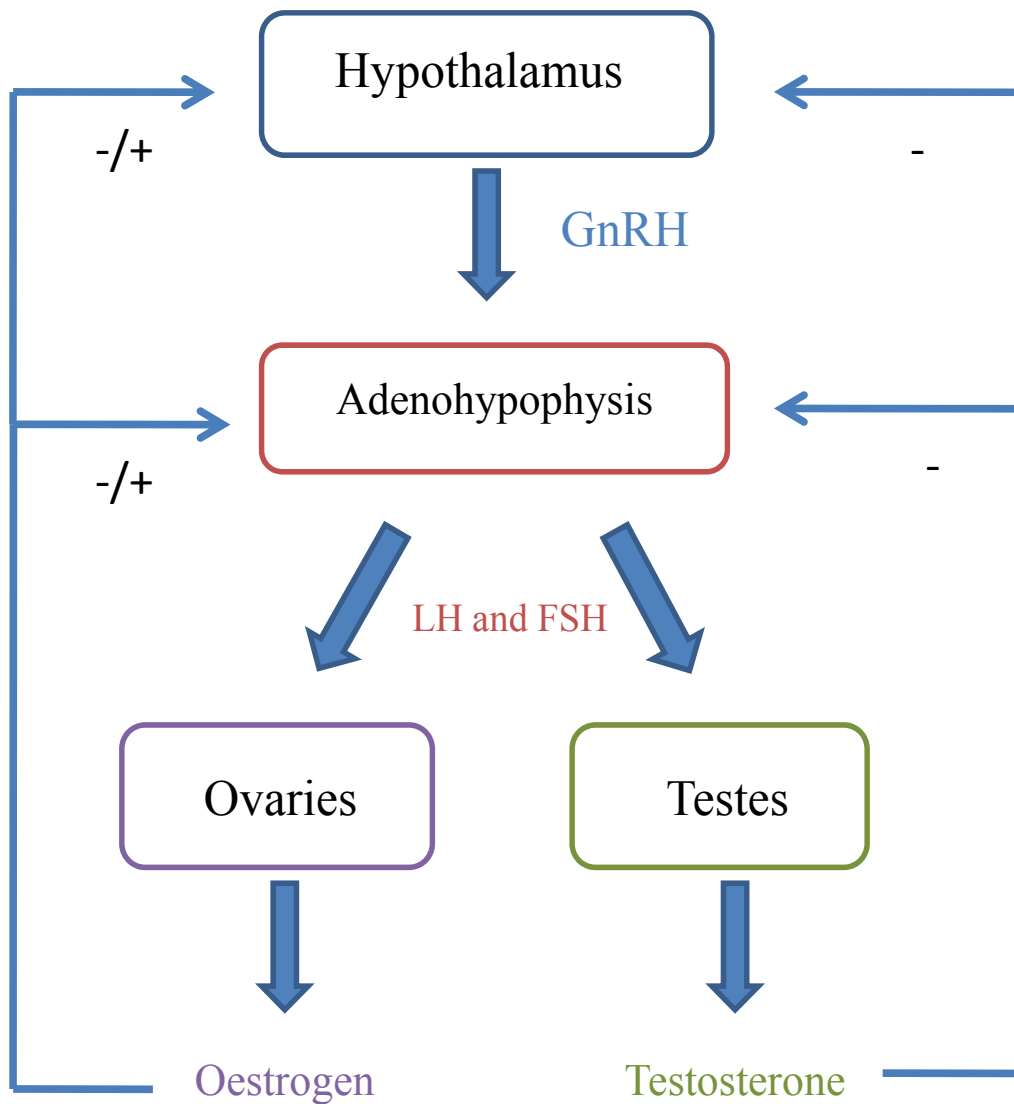


Figure 1.3: The hypothalamo-pituitary-gonadal (HPG) axis. GnRH is released by hypothalamic neurones into the portal circulation and stimulates the release of LH and FSH from the adenohypophysis. LH and FSH act at the gonads to stimulate steroidogenesis and gametogenesis. In males, testosterone exerts negative feedback at the pituitary gland and hypothalamus. In females, depending on the stage of the cycle, oestrogen exerts negative or positive feedback to inhibit or stimulate activity of the HPG axis respectively. Abbreviations: GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

1.10. Central regulators of reproduction

1.10.1. Gonadotrophin-releasing hormone

The decapeptide GnRH is the primary drive for the HPG axis in adult mammals and is crucial in initiating the onset of puberty. GnRH cell bodies are not localised within a discrete nucleus but are scattered throughout the anterior hypothalamus, in particular the preoptic and medial basal areas (Silverman et al., 1987), from where they project to the median eminence. GnRH is released in a pulsatile fashion into the pituitary portal circulation. This pulsatility is essential for normal reproductive function; exposure to continuous exogenous GnRH disrupts gonadotrophin secretion (Belchetz et al., 1978; Clayton et al., 1982). The generation of GnRH pulses is incompletely understood. Immortalised hypothalamic GnRH neurones are able to secrete GnRH in a synchronised, pulsatile fashion, demonstrating that these neurones have an intrinsic firing properties (Wetsel et al., 1992). However, it is thought that external factors also modify and coordinate GnRH pulses. Steroid hormones, leptin (Lebrethon et al., 2000) and neurotransmitters including NPY (Crowley and Kalra, 1987) and glutamate (Bourguignon et al., 1989) are known to regulate GnRH neuronal activity. Over the last decade, a critical role for the kisspeptins in GnRH release has also been described (Irwig et al., 2004).

GnRH binds to receptors on gonadotroph cells in the adenohypophysis and stimulates the pulsatile release of LH and FSH into the systemic circulation. Circulating gonadotrophins act upon the gonads. In males, LH binds to Leydig cells of the testes and regulates the synthesis of testosterone. In females, LH acts at thecal cells of the developing follicle and stimulates the production of androgens, primarily androstenedione. The mid-cycle LH surge in females also stimulates ovulation.

In males, FSH acts at Sertoli cells and regulates spermatogenesis. In females, FSH induces the aromatisation of androgens produced by thecal cells to oestrogens in neighbouring granulosa cells.

Gonadal steroids exert negative feedback at the hypothalamus and pituitary to suppress activity of the HPG axis. Additionally, in specific stages of the female reproductive cycle, oestrogen exerts positive feedback at the hypothalamus and pituitary to potentiate its own secretion. This positive feedback leads to a peak in circulating oestrogen and LH, which is crucial in initiating ovulation. However, the mechanism by which gonadal steroids exert negative and positive feedback at the hypothalamus is unclear, as GnRH neurones appear to lack oestrogen and

androgen receptors (Shivers et al., 1983). Recently, kisspeptin has emerged as a potential mediator of sex steroid feedback.

1.10.2. Kisspeptin

Kisspeptin is a 54-amino acid peptide encoded by the KiSS-1 gene. Although originally identified as a metastasis-suppressor, kisspeptin is now known to have an essential role in the control of reproduction.

In humans the KiSS-1 gene encodes a 145-amino acid product (Ohtaki et al., 2001; Muir et al., 2001) which is processed to generate kisspeptin-54, corresponding to residues 68-121 of the full length translation product (Ohtaki et al., 2001). Shorter fragments of 10, 13 and 14 amino acid residues have also been isolated (Kotani et al., 2001; Bilban et al., 2004). Collectively these peptides are termed the kisspeptins. All share a common C-terminal decapeptide which is essential for receptor binding and activation (Kotani et al., 2001).

The kisspeptins bind to and activate the G-protein coupled receptor GPR54 (Ohtaki et al., 2001; Kotani et al., 2001; Muir et al., 2001). KiSS-1 and GPR54 mRNA expression is highest within the placenta but both are also reported to be expressed in the pituitary, brain, testis, ovary, liver, pancreas and small intestine (Ohtaki et al., 2001; Kotani et al., 2001). Within the CNS, KiSS-1 is highly expressed in the hypothalamus and basal ganglia (Muir et al., 2001) and GPR54 mRNA is abundant in the hypothalamus and amygdala (Lee et al., 1999).

A role for kisspeptin and GPR54 signalling in the control of reproduction was first suggested when it was discovered that loss of function mutations within the GPR54 gene are associated with hypogonadotrophic hypogonadism, defined as impaired pubertal development and reproductive function due to defective pituitary LH and FSH secretion (Seminara et al., 2003; de Roux N. et al., 2003; Semple et al., 2005). Similarly, mice lacking either GPR54 or KiSS-1 are infertile and display abnormal sexual maturation (Seminara et al., 2003; Funes et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007).

Central and peripheral administration of kisspeptin potently increases LH and FSH release (Thompson et al., 2004; Matsui et al., 2004; Gottsch et al., 2004) and can stimulate ovulation in primed rats (Matsui et al., 2004). Strong evidence suggests that the effect of kisspeptin on the HPG axis is mediated via hypothalamic GnRH. KiSS-1 immuno-reactive fibres are located in

regions of the forebrain where GnRH neurones reside, including the medial preoptic area (MPOA), the anterior hypothalamic area and the Arc (Brailoiu et al., 2005). Over 75% of GnRH neurones co-express GPR54 mRNA (Irwig et al., 2004) and kisspeptin is able to directly depolarize and increase firing rates of GnRH neurones (Pielecka-Fortuna et al., 2008). Additionally, pre-treatment with a GnRH antagonist abolishes the effect of kisspeptin on gonadotrophin release *in vivo* (Matsui et al., 2004; Gottsch et al., 2004). In rodents, kisspeptin neuronal cell bodies located in the Arc and anteroventral periventricular nucleus (AVPV) have received particular attention as both areas are known to project heavily to forebrain areas containing GnRH neurones (Simonian et al., 1999).

Forebrain kisspeptin neurones are hypothesised to relay gonadal steroid feedback to GnRH neurones. Most kisspeptin neurones within the Arc and AVPV express receptors for oestrogen, progesterone and androgens, suggesting they are direct targets for sex steroids (Smith et al., 2006b; Smith et al., 2005b; Clarkson et al., 2008). Hypothalamic KiSS-1 mRNA expression is regulated by circulating steroids. Interestingly, in rodents this regulation is nucleus-specific. Following castration or ovariectomy, KiSS-1 mRNA expression dramatically increases in the Arc, but decreases in the AVPV (Smith et al., 2006b; Smith et al., 2005b). This suggests that the two nuclei may relay gonadal steroid hormone negative feedback (in both males and females) and positive feedback (in females only) to GnRH neurones respectively.

1.11. Energy homeostasis and reproduction are co-regulated

Although essential for propagation of the species, reproduction is not essential for the survival of an individual and so mechanisms have evolved to allow the reproductive axis to be switched off in times of energy insufficiency. Body energy stores must be adequate to permit pubertal onset and normal functioning of the HPG axis. In humans, under-nutrition and extreme exercise induce menstrual irregularities, amenorrhoea and infertility (Klentrou and Plyley, 2003; De Souza et al., 1998). In rats, food deprivation suppresses circulating LH and sex steroid levels (Badger et al., 1985; Castellano et al., 2005), delays puberty (Glass et al., 1976) and prevents normal reproductive function (Terry et al., 2005).

Leptin has long been known to influence the HPG axis. Leptin-deficient ob/ob mice are infertile and their fertility can be restored with leptin replacement (Chehab et al., 1996). Leptin increases GnRH pulse frequency *in vitro* (Lebrethon et al., 2000) and stimulates LH release *in vivo* (Finn et al., 1998). However, GnRH neurones do not express the leptin receptor ObRb (Hakansson et al., 1998) and hence the mechanism by which leptin influences the HPG axis must be indirect.

Recently, it has been suggested that leptin conveys metabolic information to the HPG axis via hypothalamic kisspeptin neurones. Kisspeptin neurones within the Arc co-express ObRb mRNA, and ob/ob mice have significantly lower hypothalamic KiSS-1 mRNA levels than wild type controls (Smith et al., 2006a). Leptin replacement rescues KiSS-1 expression in these animals (Smith et al., 2006a). Similarly, food deprivation, resulting in low circulating leptin levels, decreases hypothalamic KiSS-1 mRNA in pre-pubertal rats (Castellano et al., 2005). Central kisspeptin administration restores vaginal opening and increases circulating gonadotrophins in chronically under-fed female rats that would otherwise not progress through puberty (Castellano et al., 2005). Additionally, the LH response to exogenous kisspeptin is preserved in food-deprived animals, suggesting that even in a state of negative energy balance, kisspeptin is able to activate the HPG axis (Castellano et al., 2005). However, it has recently been found that male and female mice lacking the leptin receptor specifically on hypothalamic kisspeptin neurones progress through puberty and are fertile, suggesting that direct leptin signalling in kisspeptin neurones is not essential for normal reproductive function (Donato et al., 2010).

1.12. Aims of this thesis

In order to develop new strategies for the treatment of obesity or cachexia, it is important to characterise endogenous factors which regulate energy homeostasis and understand the pathways involved in appetite regulation. Cerebellin 1 (Cbln1) is a hypothalamic neuropeptide expressed in areas of the brain involved in appetite regulation. In chapter 2 of this thesis I describe the effects of Cbln1 on food intake.

The reward system plays a crucial role in the control of appetite, yet little is known about the mechanisms by which it modulates food intake. In chapter 3 of this thesis I have investigated the mechanisms by which reward pathways, specifically within the VTA, may affect energy homeostasis.

Reproduction and food intake are intimately linked, and the hypothalamus has a vital role in the control of both systems. Hypothalamic kisspeptin neurones are essential regulators of reproduction, and relay metabolic signals to GnRH neurones. In chapter 4 of this thesis I have investigated the role of kisspeptin signalling, specifically in the Arc, in the control of reproduction.

Chapter 2: Investigating the role of Cerebellin 1 in the regulation of energy homeostasis and neuroendocrine function

2.1. Introduction

2.1.1. Cerebellin 1

Cerebellin1 (Cbln1) is a highly conserved 16-amino acid protein (Yiangou et al., 1989) originally isolated from rat cerebellum (Slemmon et al., 1984). The amino acid sequence of Cbln1 is shown in Figure 2.1 (Slemmon et al., 1984).

S-G-S-A-K-V-A-F-S-A-I-R-S-T-N-H

Figure 2.1: Amino acid sequence of Cbln1. S: Serine; G: Glycine; A: Alanine; K: Lysine; V: Valine; F: Phenylalanine; I: Isoleucine; R: Arginine; T: Threonine; N: Asparagine; H: Histidine.

Cbln1 is widely distributed throughout the CNS and peripheral tissues (Burnet et al., 1988; Satoh et al., 1997). Pre-Cbln1 mRNA (Miura et al., 2006) and Cbln1 peptide (Burnet et al., 1988) are highly expressed within the hypothalamus, in particular in the VMN (Segal et al., 2005; Miura et al., 2006).

2.1.2. Pre-Cerebellin1

Cbln1 is a proteolytic cleavage product of Pre-Cerebellin1 (Pre-Cbln1), a 193-amino acid peptide (Urade et al., 1991). Pre-Cbln1 is a member of the Pre-Cerebellin gene family of which there are four members (Pre-Cblns1-4) (Urade et al., 1991; Pang et al., 2000). These peptides share 60-80% sequence homology and are located on different chromosomes (Pang et al., 2000).

The Pre-Cblns are part of the C1q/tumour necrosis factor superfamily which also includes adiponectin and collagen X (Kishore et al., 2004). The C-terminal two-thirds of these family members share significant homology with the globular domain of the B chain of the complement subunit C1q (Urade et al., 1991) and have a similar three-dimensional structure to tumour-necrosis factor- α (TNF- α) (Shapiro and Scherer, 1998).

The Pre-Cblns form homomeric and heteromeric, hexameric complexes *in vitro* (Pang et al., 2000; Bao et al., 2005) which can affect their secretory profiles (Iijima et al., 2007). The C1q

motif permits trimer formation and the amino terminus is essential for the formation of higher order complexes (Iijima et al., 2007; Pang et al., 2000; Bao et al., 2005).

Mice lacking Pre-Cbln1 are ataxic and have impaired motor coordination (Hirai et al., 2005). This is thought to be due to impaired matching and maintenance of synapses between parallel fibres and Purkinje cells within the cerebellum (Hirai et al., 2005). Injection of recombinant Pre-Cbln1 temporarily rescues the ataxia in these mice (Ito-Ishida et al., 2008). The phenotype of Pre-Cbln1 knockout mice is very similar to that of mice lacking the orphan glutamate receptor delta-2 subunit (GluD2) (Hirai et al., 2005) and it has recently been suggested that Pre-Cbln1 may interact with GluD2 to modulate cerebellar synaptic integrity (Matsuda et al., 2010).

2.1.3. Localisation of Pre-Cbln1 and Cbln1

Within the cerebellar cortex, Pre-Cbln1 mRNA is localised in granule cells, which form synapses with Purkinje cells via parallel fibres (Hirai et al., 2005; Miura et al., 2006). However, Cbln1 peptide is mainly located within the cell body and dendrites of Purkinje cells (Slemmon et al., 1985). Pre-Cbln1 may therefore be secreted pre-synaptically, bind to Purkinje cells and be internalised and processed to produce Cbln1 (Bao et al., 2005).

In addition to the cerebellum, Pre-Cbln1 mRNA and Cbln1 peptide has been detected in numerous regions of the CNS including the dorsal cochlear nucleus (Mugnaini and Morgan, 1987), hypothalamus (Burnet et al., 1988; Satoh et al., 1997; Miura et al., 2006), thalamus, hippocampus and spinal cord (Satoh et al., 1997; Miura et al., 2006). Within the mouse hypothalamus, the main site of Cbln1 expression is the VMN (Segal et al., 2005; Miura et al., 2006), an area important in food intake, body weight and glucose homeostasis.

Pre-Cbln1 mRNA and Cbln1 peptide has also been detected peripherally, although at lower levels than in the CNS. Cbln1 is located in numerous tissues including the thyroid gland, anterior pituitary gland, testis, ovaries, pancreas (Rucinski and Malendowicz, 2009), adrenal gland (Satoh et al., 1997), heart, and stomach (Burnet et al., 1988). Additionally, Cbln1 has been located in several human tumour tissues including pheochromocytomas and cortisol-producing adrenocortical adenomas (Satoh et al., 1997).

2.1.4. Function of Cbln1

As yet, the biological functions of Cbln1 are poorly characterised. Cbln1 is enriched in synaptosomes (Slemmon et al., 1984) and is released upon depolarisation in a calcium-dependent manner, suggesting it may have neuromodulatory properties (Burnet et al., 1988). The receptor for Cbln1 has not yet been elucidated.

Cerebellar Cbln1 levels rise in parallel with post-partum development, in particular migration of granule cells and Purkinje cell maturation (Slemmon et al., 1985; Morgan et al., 1988). Mice with mutations affecting cerebellar development, for example the ‘wheeler’, ‘reeler’ and ‘staggerer’ have reduced Cbln1 content within the cerebellum (Slemmon et al., 1988). Additionally, patients suffering from cerebellar degenerative diseases such as olivopontocerebellar atrophy and Shy-Drager syndrome have significantly reduced Cbln1 content in the cerebellum (Mizuno et al., 1995). This, together with the observed phenotype of the Pre-Cbln1 knockout mouse, suggests that Pre-Cbln1 and/or Cbln1 may have an important physiological role in cerebellar development, particularly synaptic development and maintenance.

Cbln1 stimulates catecholamine, aldosterone and corticosteroid release from human and rat adrenal glands *in vitro* (Mazzocchi et al., 1999; Albertin et al., 2000). Chronic subcutaneous administration of Cbln1 to rats increases corticosterone and aldosterone secretion independently of adrenocorticotrophic hormone (ACTH) (Hochol et al., 2001).

In addition, Cbln1 has an insulinostatic effect on pancreatic islets *in vitro*, and subcutaneous administration of Cbln1 to rats decreases plasma insulin concentrations (Strowski et al., 2009). It should be noted that Cbln1 immunoreactivity is not present in the circulation (Satoh et al., 1997) and therefore any endogenous effects of Cbln1 are likely to be mediated via local autocrine or paracrine mechanisms.

2.1.5. The effect of Cbln1 on energy homeostasis and endocrine function

The presence of Cbln1 within the hypothalamus suggests it may have an endogenous role in the control of food intake and energy expenditure. Additionally, its localisation within peripheral endocrine organs and its effects on the adrenal gland and pancreas suggest Cbln1 may have a role in endocrine function.

2.1.6. Hypothesis

I hypothesise that Cbln1 plays a role in the regulation of food intake and endocrine function.

2.1.7. Aims

To determine the role of Cbln1 in the regulation of feeding I will investigate:

- The effect of ICV Cbln1 administration on food intake in fasted and *ad libitum* fed male Wistar rats.
- The effect of ICV Cbln1 administration on behaviour in male Wistar rats.
- The effect of chronic ICV administration of Cbln1 on food intake and body weight in male Wistar rats.
- Possible mechanisms underlying the actions of Cbln1 on food intake, by determining the effect of Cbln1 on hypothalamic neuropeptide release *ex vivo*.
- The effect of nutritional status on Pre-Cbln1 mRNA expression within the rat hypothalamus.

I will also investigate the effect of ICV Cbln1 administration on plasma anterior pituitary hormones and corticosterone in male Wistar rats.

2.2. Methods

2.2.1. Materials

Cbln1, angiotensin II and neuropeptide S (NPS) were purchased from Bachem (St Helens, Merseyside, UK). BIBP 3226 trifluoroacetate (BIBP 3226) was purchased from Tocris Bioscience (Bristol, UK).

2.2.2. Animals

Male Wistar rats (specific pathogen free; Charles River, Margate, Kent, UK) weighing 250-300g were maintained in individual cages with a solid floor and wire mesh lid under controlled temperature (21-23°C) and light (12:12 light-dark cycle) conditions with *ad libitum* access to food (Rat and Mouse 1 (RM1) diet, Special Diet Service Ltd, Witham, Essex, UK) and water. Cage dimensions were 245 (width) x 415 (length) x 18.5 (depth) mm.

All animal procedures undertaken were approved by the British Home Office Animals (Scientific Procedures) Act 1986 (Project License 70/6402).

2.2.3. Intracerebroventricular cannulation and injection

Rats were anaesthetised with 2L/minute oxygen and 4% inhaled isoflurane (Abbott Laboratories Ltd, Queenborough, Kent, UK). Animals were placed in the induction chamber until they lost the pedal reflex. Once anaesthetised, animals were administered intra-peritoneal prophylactic antibiotics (amoxycillin (37.5mg/kg) and flucloxacillin (37.5mg/kg)) to prevent post-operative infection. Rats were positioned on a stereotactic frame (David Kopf Instruments, supplied by Clark Electromedical Instruments, Reading, Berkshire, UK) with the incisor bar 3mm below the interaural line. The head was cleaned with 10% w/v povidone/iodine solution (Ecolabs Ltd, Leeds, Yorkshire, UK) and a 1.5cm longitudinal incision made in the scalp. A hole was drilled using a stereotactically mounted electric drill (Department of Medical Engineering, Imperial College London, UK). Stereotactic coordinates calculated from the Rat Brain Atlas (Paxinos and Watson, 2007) were as follows: 0.8mm posterior to the bregma in the midline and 6.5mm below the surface of the skull. A permanent 22-gauge stainless steel cannula (Plastics One Inc., Roanoke, Virginia, USA) projecting to the third cerebral ventricle was implanted. Dental cement (Associated Dental Products Ltd, Swindon, Wiltshire, UK) was used to hold the cannula in

position and seal the wound; this was anchored by three stainless steel screws inserted into the skull. Animals were administered subcutaneous buprenorphine (45mg/kg, Schering-Plough Corp, Welwyn Garden City, Hertfordshire, UK) for post-operative analgesia and rehydrated with 0.9% sodium chloride (5ml/rat). A plastic-topped wire cap (Plastics One Inc.) was inserted into the cannula to prevent blockage.

After a seven day recovery period, animals were handled daily and acclimatised to the injection procedure. To ensure cannulae were correctly positioned, rats received ICV 50ng angiotensin II in a 5µl volume between 09:00 and 10:00. Following injection, animals were observed for a prompt drinking response. Those that did not respond were given a second angiotensin II injection three days later.

Peptides or vehicle were administered to conscious, freely moving rats in a volume of 5µl via a 28-gauge stainless steel injector placed into, and projecting 1mm below, the tip of the cannula. The injector was connected by polythene tubing to a Hamilton syringe (Fisher Scientific, Loughborough, Leicestershire, UK) in a Harvard infusion pump (Harvard Apparatus, Massachusetts, USA). The tubing was filled with water and an air bubble drawn up to separate the experimental compound from the water.

Each experimental group was injected by a single individual to reduce cross-contamination of peptides between injections and to permit all animals to be injected within a relatively short time period (less than 1 hour), minimising the effect of daily fluctuations in food intake. The minimum washout period between injections was 72 hours. Animals which were considered unwell, or had reduced bodyweight or low food intake, were excluded from studies.

2.2.4. The effect of ICV Cbln1 administration on food intake in fasted rats in the early light phase

As the effects of ICV Cbln1 administration on food intake were unknown, the initial study was carried out in fasted rats. Fasting induces an exaggerated re-feeding response and so the anorectic effect of an administered peptide can be easily observed.

Male Wistar rats were ICV cannulated as described in section 2.2.3 and fasted for 24 hours prior to the study. Rats were randomised into groups by body weight and ICV injected with saline or Cbln1 at doses of 3, 10 or 30nmol per rat (n=9 per group) in the early light phase (08:00-10:00).

Following injection rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection.

2.2.5. The effect of ICV Cbln1 administration on food intake in *ad libitum* fed rats in the early light phase

Rats were ICV cannulated as described in section 2.2.3. As the results from study 2.2.4 suggested that Cbln1 may induce an orexigenic effect, the second study was carried out in *ad libitum* fed rats in the early light phase. The light phase is not the usual feeding period for rats so an orexigenic effect is easier to observe.

Ad libitum fed rats were randomised into groups by body weight and ICV injected with either saline or 1, 3, 10 or 30nmol Cbln1 (n=9 per group) in the early light phase (08:00-10:00). Following injection rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection.

2.2.6. The effect of ICV Cbln1 administration on behaviour in ad libitum fed rats in the early light phase

Behaviour was monitored following ICV administration of Cbln1 to determine whether the effects of Cbln1 on food intake were secondary to behavioural changes. Rats were ICV cannulated as described in section 2.2.3. *Ad libitum* fed rats were ICV injected with saline, 10nmol Cbln1 or 30nmol Cbln1 (n=10-12 per group) in the early light phase and returned to their home cages. These doses of Cbln1 were chosen because they significantly increased food intake in study 2.2.5. NPS was used as a positive control as it is known to induce behavioural abnormalities, and was injected ICV at a dose of 10nmol per rat (Smith et al., 2006c).

Behaviour was continuously monitored for one hour by two observers blinded to the experimental treatment using methods adapted from Fray *et al* (Fray et al., 1980; Scott et al., 2005). Each rat was observed for fifteen seconds every five minutes. Each fifteen second period was sub-divided into three five second periods and the behaviour of the rat was noted in each time period. Behaviour was classified into the following categories: feeding, drinking, rearing, locomotion, grooming, burrowing, head down, sleeping and pica (Table 2.1, adapted from (Scott et al., 2005)).

Behaviour	Description
Feeding	Animal feeding at hopper or feeding from food held in paws
Drinking	Animal licking at water spout
Rearing	Animal with front paws off the ground
Locomotion	Animal moving around the cage with all four legs moving
Grooming	Animal scratching, licking or biting itself
Burrowing	Animal scratching at sawdust bedding
Head down	Animal with nose lowered
Sleeping	Animal sleeping
Pica	Animal eating non-nutritive substance

Table 2.1: Description of behaviour types observed following ICV administration of Cbln1.

2.2.7. The effect of Cbln1 on neuropeptide release from hypothalamic explants

Materials

Artificial cerebrospinal fluid (aCSF) equilibrated with 95% oxygen and 5% carbon dioxide (Appendix I)

Potassium-enriched aCSF equilibrated with 95% oxygen and 5% carbon dioxide (Appendix I)

Methods

Ad libitum fed male Wistar rats were killed by decapitation in the early light phase. The whole brain was immediately removed, mounted ventral surface uppermost onto a pre-cooled block and placed in a vibrating microtome (Microfield Scientific Ltd., Dartmouth, Devon, UK). A 1.9mm slice was taken from the basal hypothalamus and dissected free. The hypothalamic slice taken included the MPOA, SON, PVN, DMN, VMN, LH and Arc.

Hypothalamic slices were transferred to individual tubes containing 1ml aCSF. Tubes were placed on a platform in a water bath maintained at 37°C. After an initial two hour equilibration period, hypothalami were treated with 600µl aCSF for 45 minutes (basal period), followed by treatment with 600µl aCSF supplemented with 10, 100 or 1000nM Cbln1 for 45 minutes (test period, n=9-12 hypothalamic slices per group). Tissue viability was confirmed by incubating slices in 600µl aCSF containing 56mM KCl for 45 minutes. Isotonicity was maintained by substituting K⁺ for N⁺. Explants which failed to show increased peptide release in response to aCSF supplemented with KCl compared to basal levels were excluded from analysis.

Artificial CSF was collected at the end of each period and stored at -20°C until measurement of NPY, AgRP, CART and α-MSH by radioimmunoassay (RIA) (section 2.2.8.).

2.2.8. Radioimmunoassay

2.2.8.1. Principles of radioimmunoassay

Radioimmunoassay (RIA) is a technique used to detect small quantities of substances in tissue or plasma samples. The principle of RIA is based on competition between a known amount of radio-labelled antigen and unlabelled antigen in the sample for binding to a known concentration of antibody. The concentration of the antibody is limiting, so antigen binding will be finite. An increase in the concentration of unlabelled antigen will decrease the number of binding sites available for labelled antigen, and hence the amount of antibody-bound labelled antigen is inversely proportional to the quantity of unlabelled antigen. A standard curve is constructed using known concentrations of peptide to calculate the percentage of labelled peptide bound for each concentration of pure unlabelled peptide.

Antibody-bound antigen can be separated from unbound antigen by several methods. The two used in this work were separation by adsorption with charcoal, or by using a primary antibody/secondary antibody complex.

For charcoal separation, dextran was added to a charcoal suspension (Appendix I) to block large holes in the charcoal. 4mg charcoal was added to each RIA tube, where it trapped unbound antigen. Tubes were then centrifuged for 20 minutes at 1500g and the supernatant and pellet separated by aspiration.

For secondary antibody separation, the secondary antibody is derived from an animal species different from that in which the primary antibody is generated. The secondary antibody used in this work was sheep or goat anti-rabbit solid phase second antibody (Pharmacia Diagnostics, Uppsala, Sweden). Samples were pre-incubated for 60 minutes with 100 μ l of secondary antibody. Immediately prior to centrifugation, 500 μ l of 0.01% Triton-X-100 solution and 100 μ l of 10% polyethylene glycol was added to each tube. Samples were then centrifuged at 1500g at 4°C for 20 minutes. Bound and free label were separated by aspiration.

For both methods, bound and free label were counted in a gamma counter (model NE1600, Thermo Electron Corporation, Ohio, USA) and peptide concentrations in samples calculated using a non-linear plot (RIA Software, Thermo Electron Corporation).

All samples were assayed in duplicate. Non-specific binding (NSB) tubes, which did not contain antibody, were included at the beginning of each assay. Tubes containing half and twice the standard volume of radio-labelled antigen were included to check quality of the radiolabel.

Tubes with no sample (zero tubes) were interspersed throughout the assay to measure baseline drift.

All peptides used for RIAs in this work were iodinated by Professor M. Ghatei using the iodogen method (Bryant, 1982). Reaction products were purified by high performance liquid chromatography (HPLC) (Gilson 321-H1) using a C18 column (Waters, Milford, Connecticut, USA). Fractions were tested by RIA and used at 1500 counts per minute/tube (cpm/tube). Antisera were raised in rabbits immunized with each peptide conjugated to Freund's adjuvant as previously described (White, 1963).

2.2.8.2. Hypothalamic neuropeptide RIAs

All assays were performed using 0.06M phosphate buffer (Appendix I). Standard curves for each assay were prepared using synthetic peptide made up to varying concentrations in assay buffer and added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl.

2.2.8.2.1. NPY RIA

NPY peptide was purchased from Bachem (UK) Ltd. Standard curves prepared for the NPY assay were made up to 1pmol/ml in assay buffer. The assay was carried out in 0.06M phosphate buffer with 1% bovine serum albumin (BSA) and 0.02% Tween 20. Sample volume of hypothalamic aCSF was 150µl and samples were assayed in duplicate in a total volume of 350µl, containing rabbit anti-NPY antibody at a final dilution of 1:120,000 and 1500cpm/tube of iodinated NPY. The antiserum showed full cross reactivity to synthetic NPY. The assays were incubated at 4°C for 72 hours, before free and antibody bound peptide were separated using a secondary antibody. The sensitivity of the assay was 1fmol/ml. The intra- and inter-assay variation was 7% and 8% respectively.

2.2.8.2.1. AgRP RIA

AgRP was measured using a commercially developed RIA from Phoenix Pharmaceuticals Inc. (Burlingame, California, USA). The AgRP (82-131)-NH₂ assay utilises ¹²⁵I-labelled AgRP and AgRP antiserum to determine the level of AgRP in samples. Reagents were supplied and reconstituted according to the protocol. Standards, quality controls and samples (all 100µl) were added in duplicate. One hundred microlitres of primary antibody (rabbit anti-serum containing an antibody specific for AgRP) was added to all tubes except those measuring NSB. The assay was incubated for 24 hours at 4°C. Following this incubation period, 100µl of ¹²⁵I-AgRP (10000 cpm per 100µl) was added to all tubes. The assay was incubated for a further 24 hours at 4°C. Following this incubation, 100µl of goat anti-rabbit secondary antibody and normal rabbit serum were added to all tubes. The assay was incubated at room temperature for 90 minutes prior to the addition of 500µl of assay buffer.

2.2.8.2.3. Alpha-MSH RIA

Alpha-MSH peptide was purchased from Bachem (UK) Ltd. Standard curves prepared for the α -MSH assay were made up to 0.2pmol/ml in assay buffer. The assay was carried out in 0.06M phosphate buffer containing 0.3% BSA. Sample volume of hypothalamic aCSF was 100 μ l and samples were assayed in duplicate in a total volume of 400 μ l, containing a rabbit anti- α -MSH antibody at a final dilution of 1:20000 and 1500cpm/tube of iodinated α -MSH. The antiserum showed full cross reactivity to synthetic α -MSH but no cross reactivity to any other POMC product. The assays were incubated at 4°C for 72 hours, before free and antibody bound peptide were separated by charcoal adsorption of the free fraction. The sensitivity of the assay was 1fmol/ml. The intra- and inter-assay variation was 7% and 8% respectively.

2.2.8.2.4. CART RIA

CART peptide was purchased from Bachem (UK) Ltd. Standard curves prepared for the CART assay were made up to 1pmol/ml in assay buffer. The assay was carried out in 0.06M phosphate buffer containing 0.3% BSA and 0.02% Tween 20. Sample volume of hypothalamic aCSF was 20 μ l and samples were assayed in duplicate in a total volume of 350 μ l, containing a rabbit anti-CART antibody at a final dilution of 1:80000 and 1500cpm/tube of iodinated CART. The assays were incubated at 4°C for 72 hours, before free and antibody bound peptide were separated by charcoal adsorption of the free fraction. The sensitivity of the assay was 1.25fmol/tube. The intra- and inter-assay variation was 8% and 9% respectively.

2.2.9. The effect of ICV Cbln1 administration on food intake following pre-treatment with a Y1 receptor antagonist in the early light phase

My results from study 2.2.7 showed that treatment of hypothalamic explants with Cbln1 induced a significant increase in NPY release. I therefore hypothesised that the orexigenic effect of Cbln1 may be mediated via NPY. In order to investigate this further I co-administered Cbln1 with BIBP 3226, a Y1 receptor antagonist which has been shown to attenuate the orexigenic effect of NPY but have no effect on food intake when administered alone (O'Shea et al., 1997).

Rats were ICV cannulated as described in section 2.2.3. *Ad libitum* fed rats (n=10-12 per group) were given ICV injections at time 0, and 30 minutes later, in the early light phase. The following injection regimen was used: vehicle/saline, vehicle/Cbln1 (10nmol), BIBP 3226 (60nmol)/saline, BIBP 3226 (60nmol)/Cbln1 (10nmol). As BIBP 3226 is insoluble in saline, the vehicle administered for the first ICV injection was 70% ethanol, which has previously been shown to have no effect on food intake (O'Shea et al., 1997).

After the second injection rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours.

2.2.10. The effect of chronic ICV Cbln1 administration on food intake and body weight

Rats were ICV cannulated as described in section 2.2.3. *Ad libitum* fed rats (randomised by body weight, n=8-10 per group) received twice daily ICV injections (at the beginning of the light phase and just prior to the onset of the dark phase) of saline or 10nmol Cbln1 for four days. This dose of Cbln1 was chosen because it was the lowest dose to significantly increase in food intake in study 2.2.5.

Following each injection rats were returned to their home cage with a pre-determined amount of food and free access to water. Food intake and bodyweight were measured daily for the duration of the study.

2.2.11. The effect of nutritional status on hypothalamic Pre-Cbln1 mRNA expression

I decided to use *in situ* hybridisation to detect and semi-quantify Pre-Cbln1 mRNA within the hypothalamus of rats either fed *ad libitum* or fasted for 48 hours. *In situ* hybridisation relies on binding of a probe, usually radio-labelled, to target mRNA in tissue. Probe binding can then be visualised using autoradiography.

In order to do this I first constructed a probe corresponding to nucleotides 63-381 of rat Pre-Cbln1 cDNA. The cDNA fragment was produced by reverse transcription and amplified by polymerase chain reaction, then ligated into a plasmid. Following bacterial transformation, small scale and large scale amplification of plasmid DNA were performed. Plasmid DNA was purified and sent for sequence analysis, prior to use as a template for the production of a radio-labelled RNA probe.

2.2.11.1. Construction of a Pre-Cbln1 probe

2.2.11.1.1 Reverse transcription

Materials

Rat whole brain RNA (5mg/ml)

5x reverse transcriptase buffer (Promega, Madison, Wisconsin, USA)

10mM deoxynucleoside triphosphates (dNTPs) (GE Healthcare, Amersham, Bucks, UK)

Oligo dT 200ng/μl (GE Healthcare)

Reverse transcriptase (RT) 10U/μl (Promega)

Methods

The reaction was set up in a total volume of 20μl containing 20μg RNA, 1x reverse transcription buffer, 1mM dNTPs and 10mg/ml oligo dT and heated to 60°C for five minutes. The solution was left at room temperature for thirty minutes before the addition of 10U reverse transcriptase. The reaction was heated to 42°C for one hour then used in PCR.

2.2.11.1.2. Polymerase chain reaction (PCR)

Materials

10x Taq buffer (Sigma-Aldrich, Gillingham, Dorset, UK)

20µM oligonucleotide primers (Table 2.2)

10mM dNTPs (GE Healthcare)

Taq DNA polymerase (5U/µl) (Sigma-Aldrich)

Phenol/chloroform (VWR)

2M sodium acetate (NaAc) (Appendix I)

Ethanol (VWR)

5' primer	GCAGAATGAGACAGAAGCCA
3' primer	GGATGGTCTGTCTGTTGTAG
Base pairs	318
Accession number	NM_001109127
Restriction enzymes	BamH1 and EcoR1

Table 2.2: Primer sequences used in polymerase chain reaction (PCR) to detect Pre-Cbln1. The expected size of the product is in base pairs. Accession numbers refer to those published in the *Nucleotide* Sequence Database.

Methods

10µl of the reverse transcription reaction was added to a tube containing 10µl Taq buffer, 200nM oligonucleotide primers and 0.2mM dNTPs. The reaction was heated to 95°C for three minutes. Five units Taq DNA polymerase was then added and the reaction cycled twenty two times through the following program: 95°C for thirty seconds, 55°C for thirty seconds and 72°C for thirty seconds. After completion, 10µl PCR product was visualised by gel electrophoresis (see section 2.2.11.1.3).

The remaining PCR products were purified with an equal volume of phenol/chloroform, mixed well and centrifuged for two minutes at 12,300g. The aqueous phase was removed to a new tube. DNA was purified by ethanol precipitation; 1/10 sample volume of 2M sodium acetate was added, the sample mixed, then 2.5 volumes ice cold 100% ethanol added. The reaction was

stored at -20°C for one hour then centrifuged for eight minutes at 12,300g. The supernatant was removed and the pellet re-suspended in 20µl glass distilled water (GDW).

2.2.11.1.3. Agarose gel electrophoresis

Materials

Agarose, type II-A medium EEO (Sigma-Aldrich)

50x Tris-acetate-EDTA buffer (TAE) (Appendix I)

Ethidium bromide (10mg/ml) (VWR, Leicester, Leicestershire, UK)

DNA marker (1kb ladder) (Invitrogen, Paisley, UK)

Gel loading buffer (Appendix I)

Methods

A 1% (w/v) agarose gel was made by dissolving 1.5g agarose in 1x TAE using a microwave oven. Once the gel had cooled to 37°C, ethidium bromide was added to a final concentration of 0.5µg/mg.

Once the gel was set it was placed in an electrophoresis tank containing 0.5x TAE and 0.5µg/mg ethidium bromide. Ten microlitres of PCR product were run with three microlitres of gel loading buffer. Samples were electrophoresed at 120mV and visualised using UV light (300nm).

2.2.11.1.4. Restriction endonuclease digestion of PCR products and plasmid

PCR products and the plasmid into which they were inserted were cut with the same restriction endonucleases in order for them to be joined in a ligation reaction.

Materials

PCR products

pBluescript plasmid DNA (Stratagene Ltd, Amsterdam, The Netherlands)

10x restriction buffer (New England Biolabs, Hitchin, Herts, UK)

100x bovine serum albumin (BSA) (New England Biolabs)

Restriction endonucleases: BamHI and EcoRI (New England Biolabs)

Shrimp alkali phosphatase (SAP) 4U/μl (GE Healthcare)

Methods

Two separate restriction digest reactions were set up in a total volume of 100μl containing 10μg DNA (either PCR products or plasmid), 1x restriction buffer, 1x BSA, and restriction endonucleases in equal proportion to make up to 8% of total volume. The reactions were incubated at 37°C for at least one hour. Eight units of SAP was added to the pBluescript reaction 30 minutes before the end of the incubation.

Following restriction digestion, phenol/chloroform extraction and ethanol precipitation were repeated. Purified DNA was re-suspended in 20μl GDW.

2.2.11.1.5. Electroelution of DNA fragments

This procedure was performed to remove contaminating fragments and purify the fragment of interest. DNA was separated on an agarose gel and the appropriate fragment electroeluted.

Materials

1% TAE agarose gel (prepared as described in section 2.2.11.1.3)

Dialysis tubing

DNA marker (Invitrogen)

50x TAE (Appendix I)

Gel loading buffer (Appendix I)

Methods

A 1% agarose gel was prepared as described in section 2.2.11.1.3. The precipitated DNA was added to 0.3 volumes of gel loading buffer. DNA marker was dissolved 1:10 and added to 0.3 volumes gel loading buffer. Samples were electrophoresed at 120mV. After visualisation with UV illumination, the band of interest was excised and placed in a piece of dialysis tubing with 400µl 0.5x TAE. Air bubbles were excluded and the ends clipped. DNA was removed from the gel by electrophoresis at 200mV for twenty minutes then the current was reversed for forty seconds. The TAE in the tubing was removed, purified with phenol/chloroform and the DNA extracted by ethanol precipitation.

2.2.11.1.6. Ligation of PCR product into plasmid

DNA ligase is a virus-derived enzyme which catalyses the formation of phosphodiester bonds between the 5' phosphate end of one nucleotide and the 3' hydroxyl end of another.

Materials

20ng cut pBluescript plasmid

PCR product

10x ligase buffer (New England Biolabs)

T4 DNA ligase, 6U/ μ l (New England Biolabs)

Methods

Twenty nanograms of cut plasmid were added to a four-fold molar excess of insert and 1x ligase buffer. Six units of ligase enzyme were added and the volume made up to 10 μ l with GDW. The reaction was incubated overnight at 16°C then used in transformation.

2.2.11.1.7. Production of competent bacteria

In order to take up plasmid DNA, gram negative bacteria such as *E. Coli* must be rendered competent. This can be achieved by incubation in the presence of divalent cations, a method was originally described by Hanahan *et al* (Hanahan, 1983). Production of competent bacteria was carried out by Dr James Gardiner.

Materials

Lysogeny broth (LB) (Appendix I)

Tetracycline (Sigma-Aldrich)

Transformation buffer I (TFB I) (Appendix I)

Transformation buffer II (TFB II) (Appendix I)

XL1-B cells (ATCC, Middlesex, UK)

Methods

One hundred millilitres of LB supplemented with 5mg/ml tetracycline (LB_{tet}) was inoculated with a colony of XL1B cells and incubated overnight at 37°C with vigorous shaking. One millilitre of this culture was taken and incubated in LB_{tet} at 37°C with vigorous shaking, until the bacteria were in log phase growth ($OD_{550} = 0.4-0.5$). The bacteria were recovered by centrifugation for 15 minutes at 800g. Bacteria were re-suspended in 40ml ice cold TFB1 and incubated on ice for 40 minutes. The bacteria were then recovered by centrifugation, as above, re-suspended in 4ml TFB2, then incubated on ice for 4 minutes. Aliquots of 50µl were prepared, frozen on ethanol in dry ice and stored at -70°C until use.

2.2.11.1.8. Transformation of competent bacteria

Transformation refers to stable genetic change brought about by the uptake of exogenous DNA. Although able to take up DNA, competent bacteria must undergo further steps to optimise DNA entry. I used heat shock transformation to prepare this probe. Incubation on ice permits the plasmid to attach to the bacterial cell membrane, and the heat shock step allows efficient transportation of the plasmid into the bacteria. Incubation following heat shock promotes expression of resistance genes prior to antibiotic exposure.

Materials

LB (Appendix I)

E. Coli XL1-B cells

Products from ligation

LB agar plate with ampicillin

Methods

Sterile technique was used throughout this procedure. An aliquot of frozen competent XL1-B bacteria was thawed on ice and 5µl ligation reaction added. This was left on ice for twenty minutes. The mixture was then placed in a 42°C water bath for forty seconds then returned to ice for two minutes. Two hundred microlitres of LB was added and the solution incubated at 37°C for thirty minutes. An ampicillin-supplemented agar plate was warmed and 100µl LB containing the bacteria was spread across the surface and incubated at 37°C overnight.

2.2.11.1.9. Small scale preparation of plasmid DNA

Small scale culture allows several clones to be analysed simultaneously. The bacterial cell wall must first be disrupted to release intracellular contents using alkaline sodium dodecyl sulphate (SDS). Plasmid DNA is then separated from other components such as genomic DNA, RNA and protein by precipitation with NaAc and treatment with RNase. Digestion with EcoR1 and BamH1 allows clones containing the gene of interest to be identified. These are then selected for large scale amplification.

Materials

LB (Appendix I)

Ampicillin (Sigma-Aldrich)

Alkaline sodium dodecyl sulphate (SDS) (Appendix I)

5M potassium acetate (KAc) (Appendix I)

Glucose-Tris-EDTA (GTE) (Appendix I)

Propan-2-ol (VWR)

RNase A (GE Healthcare) (Appendix I)

Methods

Individual bacterial colonies were picked and inoculated in 2ml LB supplemented with 0.5mg/ml ampicillin. The reaction was left overnight at 37°C in a shaking incubator. The following morning 1.5ml of the reaction was centrifuged for three minutes at 12,300g and the pellet re-suspended in 100µl GTE. Two hundred microlitres of alkaline SDS were added and the mixture incubated on ice for five minutes. One hundred and fifty microlitres of potassium acetate was added to the reaction which was then left on ice for a further five minutes and centrifuged at 12,300g for five minutes; 350µl supernatant was removed.

Phenol chloroform extraction was carried out and the DNA precipitated by adding 200µl propan-2-ol. The reaction was left at room temperature for ten minutes and centrifuged for seven minutes at 12,300g. The pellet was re-suspended in 100µl GDW and ethanol precipitated. The reaction was centrifuged for seven minutes at 12,300g and 10µl GDW added to the pellet.

Restriction endonuclease digestion was performed to check which clones contained the correct plasmid insert. Two microlitres of purified DNA was digested with BamH1 and EcoR1 as described in section 2.2.11.1.4 with the addition of 0.5µg/µl RNase A. DNA was visualised by agarose gel electrophoresis as described in section 2.2.11.1.3. Clones containing the correct sized insert were selected for large scale purification.

2.2.11.1.10. Large scale preparation of plasmid DNA

Materials

LB (Appendix I)
Ampicillin (Sigma-Aldrich)
GTE (Appendix I)
Lysozyme (Sigma-Aldrich)
Alkaline SDS (Appendix I)
5M potassium acetate (Appendix I)
Propan-2-ol (VWR)
100x Tris-EDTA (TE) (Appendix I)

Methods

Using sterile technique, 50µl LB containing the clone of interest was added to 500ml warmed LB containing 0.5mg/ml ampicillin. The reaction was transferred to a ridged conical flask and left at 37°C overnight in a shaking incubator.

The following morning the mixture was divided between two centrifuge tubes and centrifuged at 3000g at 4°C for 8 minutes. The pellet was re-suspended in 12.5ml GTE supplemented with 2mg/ml lysozyme and left at room temperature for five minutes. 25ml alkaline SDS was added to each centrifuge tube, mixed well and left on ice for five minutes then neutralised with 19ml 5M potassium acetate. The reaction was incubated on ice for a further ten minutes, centrifuged at 9000g at 4°C for fifteen minutes then filtered through nylon gauze into clean tubes, to which 36ml room temperature propan-2-ol was added. Again tubes were incubated on ice for fifteen minutes and centrifuged for fifteen minutes at 9000g at 4°C. Supernatant was removed and the

pellet re-suspended in 5ml 1x TE. To remove any remaining RNA, 100µl 10mg/ml RNase A was added. The mixture was incubated at 37°C for thirty minutes.

Phenol/chloroform extraction was performed, followed by precipitation with propan-2-ol and 5M NaAc.

2.2.11.1.11. Caesium chloride gradient purification of plasmid DNA

This technique was used to purify plasmid DNA from other DNA components. Ethidium bromide binds to closed circular plasmid DNA, linear nucleic DNA and nicked plasmid DNA to varying degrees. This results in complexes with different buoyancies which can be separated on a caesium chloride density gradient.

Materials

N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Appendix I)

Caesium chloride (CsCl₂)

CsCl₂/isopropanol (Appendix I)

Ethidium bromide (VWR)

Methods

The DNA obtained from large scale preparation of plasmid DNA was recovered by centrifugation at 24,000g at 4°C for twenty minutes. The pellet was dissolved in 8.25ml TES, then 8.4g caesium chloride and 150µl ethidium bromide added.

The reaction was transferred to two poly-alumina centrifuge tubes which were carefully balanced, overlayed with paraffin oil, crimped and ultra-centrifuged at 20°C and 185500g overnight.

The following morning UV light was used to identify the DNA band in the caesium chloride gradient, which was removed using a hypodermic needle. Ethidium bromide was removed by repeatedly adding an equal volume of caesium chloride /isopropanol and removing the upper pink phase, until both the upper and lower phases were clear.

Ethanol precipitation was performed and DNA concentration verified using a spectrophotometer.

2.2.11.1.12. Determination of DNA concentration by spectrophotometry

DNA was diluted 1 in 100 in GDW and transferred to a cuvette. The absorbance was measured at 260nm and 280nm (UV-160 Spectrophotometer, Shimadzu, Kyoto, Japan).

As 1 optical density for DNA is equal to 50µg DNA, the equation used to calculate the final quantity of DNA was as follows:

$$\text{DNA concentration (}\mu\text{g/ml)} = (\text{A}_{260} \times \text{dilution factor}) \times 50$$

The sample was then sent for sequence analysis at the MRC CSC Genomics Core Laboratory, Imperial College London.

2.2.11.2. The effect of changes in nutritional status on hypothalamic Pre-Cb1n1 expression determined by *in situ* hybridization

In situ hybridisation was carried out on fresh frozen sections. Cryostat-cut sections are more sensitive to detection as these sections are relatively thick and thus have greater mRNA signal.

2.2.11.2.1. Preparation of slides for *in situ* hybridisation

Materials

4% formaldehyde (VWR) in 0.01M phosphate buffered saline (PBS) (Appendix I)

0.01M PBS (Appendix I)

100% acetic anhydride (VWR)

0.1M Triethanolamine pH 8.0 (TEA) (Appendix I)

70% ethanol in distilled water

Chloroform (VWR)

Methods

Male Wistar rats were either fed *ad libitum* or fasted for 48 hours (n=4-6 per group). Rats were killed by decapitation and the brains removed, mounted on cork discs and snap frozen in isopentane and liquid nitrogen. Brain slices of 12µm thickness were cut on a cryostat at -25°C (Bright Instrument Company, Huntingdon, Cambridge, UK) and mounted onto polylysine coated slides (VWR).

Slides were placed in a baked rack and fixed in 4% formaldehyde on ice for twenty minutes. Two five-minute washes in 0.01M PBS were then performed. Slides were acetylated in 0.25% (v/v) acetic anhydride in 0.1M TEA for ten minutes then rinsed twice for two minutes in 0.01M PBS. Slides were dehydrated in 70% ethanol in distilled water for three minutes, delipidated in 100% chloroform for three minutes and left to air dry.

2.2.11.2.2. Production of a radio-labelled RNA probe for in situ hybridisation

Materials

5x RNA polymerase buffer (Promega)

10x nucleotide mix: 10mM ATP, UTP and GTP (GE Healthcare)

100mM dithiothreitol (DTT)

Linearised template plasmid linearised by digestion with BamHI for anti-sense probe and EcoRI for sense probe

RNase inhibitor (30U/μl) (Invitrogen)

T3 RNA polymerase (20U/μl) (Promega)

T7 RNA polymerase (20U/μl) (Promega)

[³⁵S] RA CTP (specific activity 1250Ci (46.2TBq)/mmol) (Perkin Elmer, Waltham, Massachusetts, USA)

DNase I (7.5U/μl) (GE Healthcare)

DNase buffer (Appendix I)

5M ammonium acetate (Appendix I)

Methods

A transcription reaction was set up containing 200ng template DNA, 10mM DTT, 1x nucleotide mix, 1x polymerase buffer, 30U RNase inhibitor, 20U RNA polymerase (T3 for anti-sense strand and T7 for sense strand) and 3.75MBq [³⁵S] CTPαS in a total volume of 20μl. This reaction was incubated at 37°C for two hours. Following incubation, 7.5U DNaseI and 3μl DNase 10x buffer were added and the reaction was incubated at 37°C for a further fifteen minutes. The reaction was ethanol precipitated with 12μl 5M ammonium acetate and 80μl 100% cold ethanol at -20°C for at least ninety minutes. The mixture was then centrifuged for eight minutes, the supernatant discarded and the pellet re-suspended in 200μl GDW.

Incorporation of the nucleotide into the riboprobe was determined by analysing 1μl of the reaction on a scintillation counter.

2.2.11.2.3. Hybridisation of radio-labelled probe to slides

Materials

Hybridisation buffer: 50% (v/v) deionised formamide

600mM NaCl (Appendix I)

80mM TRIS-HCl (pH 8) (Appendix I)

4mM EDTA (Appendix I)

0.1mg/ml yeast tRNA

100mM DTT

2% (w/v) dextran sulphate (Appendix I)

20x saline sodium citrate (SSC) (Appendix I)

1x RNase buffer: 0.5M NaCl (Appendix I)

10mM TRIS (pH 8) (Appendix I)

1mM EDTA (Appendix I)

10mg/ml RNase A (Appendix I)

1M DTT

70% ethanol in distilled water

Biomax film (Kodak, Hemel Hempstead, Hertfordshire, UK)

Developer (Jet X-Ray, London, UK)

Fixer (Jet X-Ray)

Methods

Enough ^{35}S -labelled probe was added to the hybridisation buffer to give 1 million counts per slide. Seventy microlitres of probe-hybridisation mixture was pipetted onto a coverslip and the slide lowered onto the coverslip. The slides and coverslips were placed horizontally in a rack and hybridised at 60°C overnight.

Slides were washed the following morning to remove excess probe/buffer mix. Slides were soaked in 4x SSC and the coverslips removed with gentle agitation. Slides were then subjected to four five-minute washes in 4x SSC.

Slides were incubated at 37°C in 1x RNase buffer containing 100µg/ml RNase for thirty minutes. This was followed by two five minute washes in 10mM DTT/ 2x SSC, a ten minute wash in 10mM DTT/ 1x SSC and a ten minute wash in 10mM DTT/0.5x SSC at room temperature. Finally, slides were washed in 10mM DTT/0.1x SSC at 60°C for thirty minutes then rinsed briefly in 10mM DTT/0.1x SSC at room temperature.

Slides were then dehydrated in 70% ethanol and air dried. After washing and dehydration the slides were exposed to Bio-Max film. After three days' exposure the film was developed. All slides were processed together to ensure uniform hybridization, washing, and development conditions. For each rat, three anatomically matched sections were analysed. Images were analysed densitometrically using ImageJ software (Wayne Rasband, National Institute of Health, USA <http://rsb.info.nih.gov/nih-image/>). Data is expressed as optical density normalised for background (arbitrary units).

2.2.12. The effect of ICV Cbln1 administration on plasma pituitary hormones and corticosterone in *ad libitum* fed rats

Ad libitum fed male Wistar rats (n=8-10 per group per time point) were injected in the early light phase with saline or 10nmol Cbln1. Rats were killed by decapitation at either 10 or 40 minutes post injection. These time points were chosen because other hypothalamic neuropeptides which affect food intake have been shown to alter pituitary hormone release at 10 and 40 minutes post injection (Stanley et al., 1999; Kennedy et al., 2001; Smith et al., 2006c). Trunk blood was collected in plastic lithium heparin tubes containing 1000 kallikrein inactivator units of aprotinin (Nordic Pharma Ltd., Reading, UK) for analysis of TSH, LH, FSH and corticosterone, or in plastic EDTA tubes for ACTH analysis. Plasma was separated by centrifugation at 1600g and snap frozen in liquid nitrogen. Samples were stored at -20°C until analysis by RIA for TSH, LH, FSH, ACTH and corticosterone (section 2.2.13).

2.2.13. Plasma hormone RIAs

2.2.13.1. TSH RIA

The TSH assay was carried out in Kemteck buffer (Appendix I). Standards were serially diluted to construct a standard curve ranging from 0.05-25ng/ml. Sample volume was 100µl and samples were assayed in duplicate in a total volume of 400µl, containing antibody and 1200cpm/tube of iodinated TSH. Assays were incubated at 4°C for 72 hours. Free and antibody-bound TSH were separated using a goat anti-rabbit secondary antibody.

2.2.13.2. LH RIA

LH assays were carried out in Kemteck buffer (Appendix I). Standards were serially diluted to construct a standard curve ranging from 0.05-25ng/ml. Rabbit anti-LH antibody was donated by H.M Fraser (Medical Research Centre Reproductive Biology Unit, Edinburgh, UK) and used at a final dilution of 1:800,000. Sample volume was 25µl and samples were assayed in duplicate in a total volume of 400µl, containing antibody and 1200cpm/tube of iodinated LH. The assays were incubated at 4°C for 72 hours. Free and antibody-bound LH were separated using a goat anti-rabbit secondary antibody.

2.2.13.3. FSH RIA

FSH assays were carried out in Kemteck buffer (Appendix I). Standards were serially diluted to construct a standard curve ranging from 0.05-25ng/ml. Sample volume was 150µl and samples were assayed in duplicate in a total volume of 400µl, containing antibody and 1200cpm/tube of iodinated FSH. Assays were incubated at 4°C for 72 hours. Free and antibody-bound FSH were separated using a goat anti-rabbit secondary antibody.

2.2.13.4. ACTH RIA

A solid phase immunoradiometric assay (IRMA) (Euro-Diagnostica, Huntingdon, Cambridge, UK) was used for determination of levels of ACTH in plasma. Two polyclonal antibodies were used, which recognize ACTH at two different binding sites. The first was a purified radio-labelled (¹²⁵I) sheep IgG recognizing the amino-terminal of ACTH, whereas the second antibody recognized and bound non-competitively to the C-terminal of the molecule. The second antibody was immobilized and coupled to sheep anti-rabbit antibody coating the polypropylene tubes. Both antibodies reacted with the ACTH molecule and the resulting complex bound to the tube wall. Following a short incubation, the remaining ¹²⁵I labelled sheep anti-ACTH was decanted. The bound radioactivity in the tubes was proportional to the concentration of ACTH in the sample or standard.

The calibrators, reference controls and ¹²⁵I ACTH-antibody were supplied lyophilised and were reconstituted in GDW 30 minutes prior to use. Standard solutions, reference controls and samples were added in 200µl in duplicate followed by addition of 200µl ¹²⁵I ACTH-antibody. The tubes were vortexed gently and incubated overnight at room temperature. All tubes, apart from the total counts tubes, were washed by addition of 2ml wash solution into each tube and then decanted. This step was then repeated. Each tube was counted for 60 seconds in a gamma counter. The mean cpm were calculated for standard, reference controls and samples and the mean cpm then plotted against the ACTH calibrators to construct a standard curve. The standard curve was used to interpolate the ACTH concentrations for the reference preparations and the unknown plasma samples.

2.2.13.5. Corticosterone RIA

Corticosterone levels in plasma were determined by a commercial RIA kit (MP Biomedicals Inc., Illkirch, France). Calibrators, buffer and reference preparations were supplied in liquid form, ready to use. The assay was performed in a phosphosaline gelatine buffer (pH 7) containing rabbit gamma globulins in a total volume of 500µl. Samples were diluted 1:200 in buffer and diluted samples, calibrators and reference preparations were added at a volume of 100µl to numbered tubes. Labelled ¹²⁵I-corticosterone (200µl, 50,000cpm) was then added to all tubes followed by 200µl of specific corticosterone antibody. All tubes were vortexed and incubated at room temperature for 120 minutes. The free and bound fractions were then separated by addition of 500µl precipitant solution (goat anti-rabbit in TRIS buffer) followed by centrifugation at 1000g for 15 minutes. The pellet and supernatant were counted for 60 seconds in a gamma counter. Peptide concentrations in the samples were then calculated using a non-linear plot and results calculated in terms of the standard preparations.

2.2.14. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), other than data from behavioural studies which are expressed as median and inter-quartile range. Food intake studies were analysed using a one-way analysis of variance (ANOVA) with Tukey's post-hoc test (GraphPad Prism version 5 for Windows; GraphPad Software, San Diego, CA). Behavioural studies were analysed using the Kruskal-Wallis test (Systat, Evanston, IL) as data were non-parametric. Hypothalamic explant data were analysed using a paired *t*-test between the basal and test period. Body weight and food intake from the chronic study was analysed using the generalized estimating equation (Stata 9, StataCorp LP, College Station, Texas). Hypothalamic Pre-Cb1n1 expression data and plasma hormone data were analysed using an unpaired *t*-test. In all cases *p* values <0.05 were taken as significant.

2.3. Results

2.3.1. The effect of ICV Cbln1 administration on food intake in fasted rats in the early light phase

ICV administration of 30nmol Cbln1 significantly increased food intake between 1 and 2 hours post injection compared to saline injected controls (1-2 hour food intake (g): 0.45 ± 0.20 [saline], 2.51 ± 0.64 [30nmol Cbln1], $p < 0.05$ vs. saline, $n = 9$ per group) (Figure 2.2).

All doses of Cbln1 had no significant effect on food intake at any other time points.

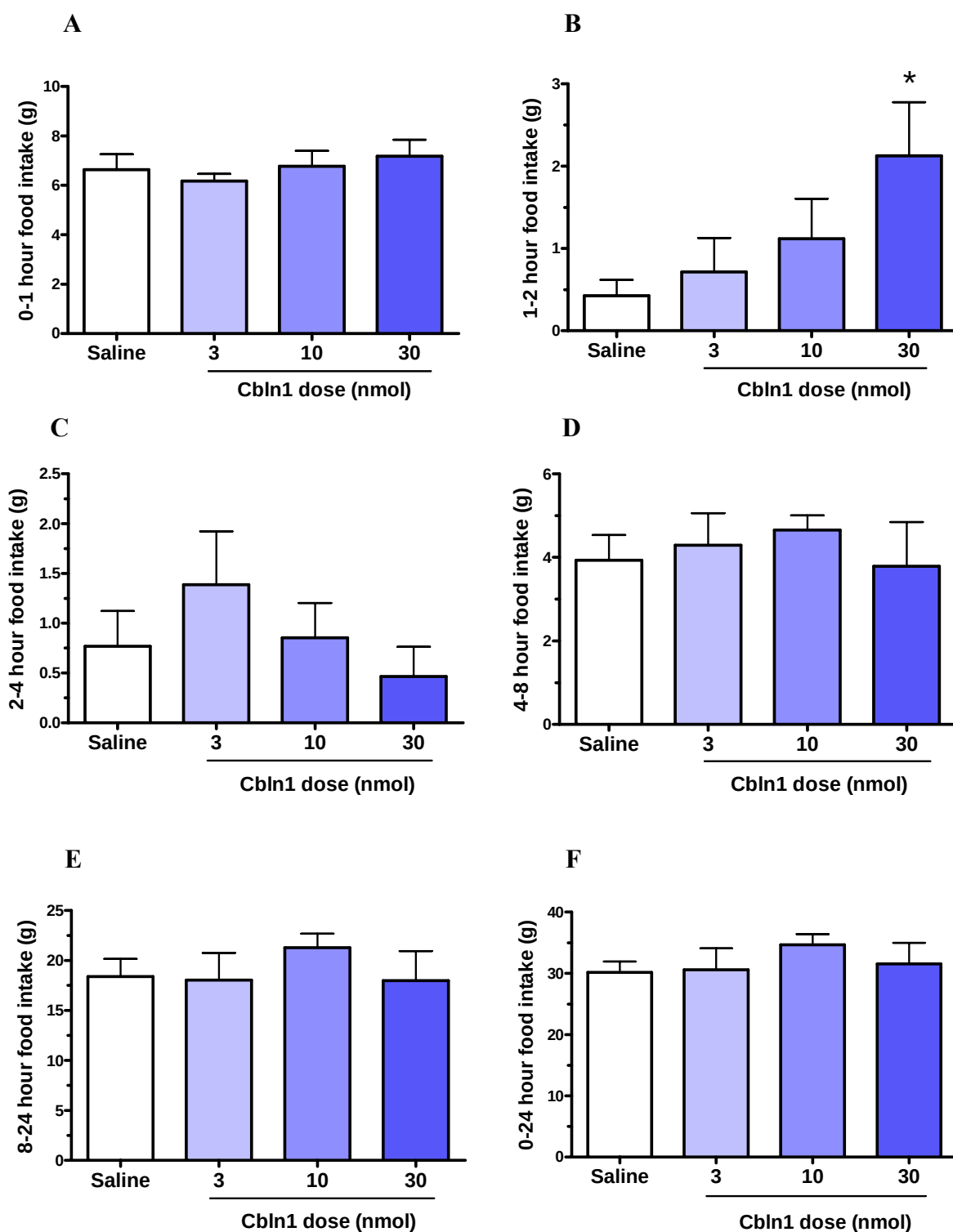


Figure 2.2: The effect of ICV Cb1r administration on food intake in 24-hour fasted rats in the early light phase. Male Wistar rats received an ICV injection of saline, 3, 10 or 30nmol Cb1r in the early light phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=9 per group). *p<0.05 vs. saline.

2.3.2. The effect of ICV Cbln1 administration on food intake in *ad libitum* fed rats in the early light phase

ICV administration of 10nmol and 30nmol Cbln1 to *ad libitum* fed rats significantly increased food intake in the first hour after injection when compared to saline injected controls (0-1 hour food intake (g): 0.37 ± 0.09 [saline], 1.41 ± 0.45 [10nmol Cbln1] $p < 0.05$ vs. saline, 1.77 ± 0.41 [30nmol Cbln1] $p < 0.05$ vs. saline, $n=9$ per group) (Figure 2.3).

Cumulative food intake was significantly increased in animals injected with 10 and 30nmol Cbln1 between 0 and 2, and 0 and 4 hours post-injection (0-2 hour food intake (g): 0.45 ± 0.11 [saline], 2.31 ± 0.50 [10nmol Cbln1] $p < 0.01$ vs. saline, 2.32 ± 0.55 [30nmol Cbln1] $p < 0.01$ vs. saline; 0-4 hour food intake (g): 0.48 ± 0.11 [saline], 2.40 ± 0.49 [10nmol Cbln1] $p < 0.01$ vs. saline, 2.75 ± 0.71 [30nmol Cbln1] $p < 0.01$ vs. saline, $n=9$ per group) (Figure 2.4).

All doses of Cbln1 had no significant effect on food intake at any other time points.

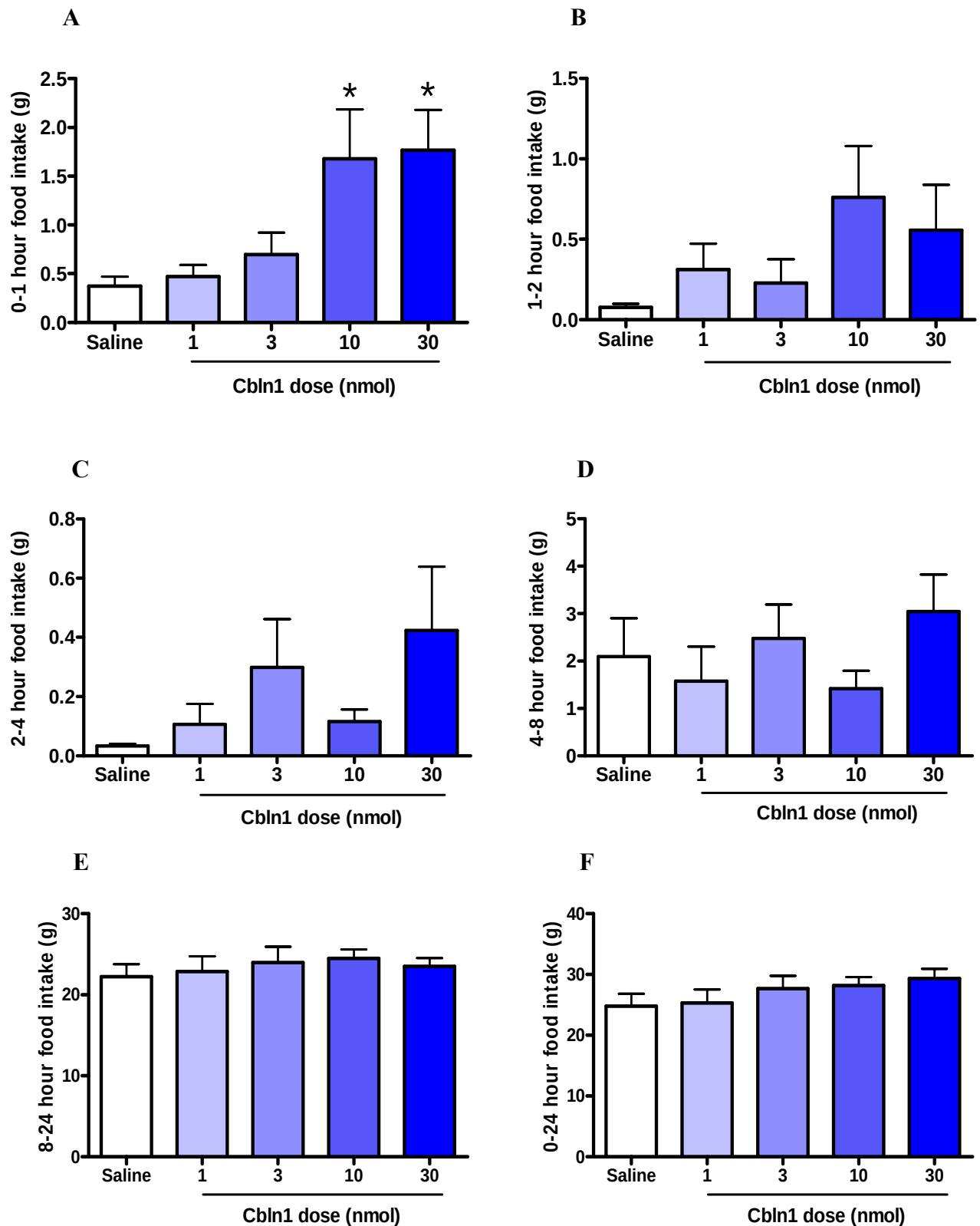


Figure 2.3: The effect of ICV Cb1n1 administration on food intake in *ad libitum* fed rats in the early light phase. Male Wistar rats received an ICV injection of saline, 1, 3, 10 or 30nmol Cb1n1 in the early light phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=9 per group). *p<0.05 vs. saline.

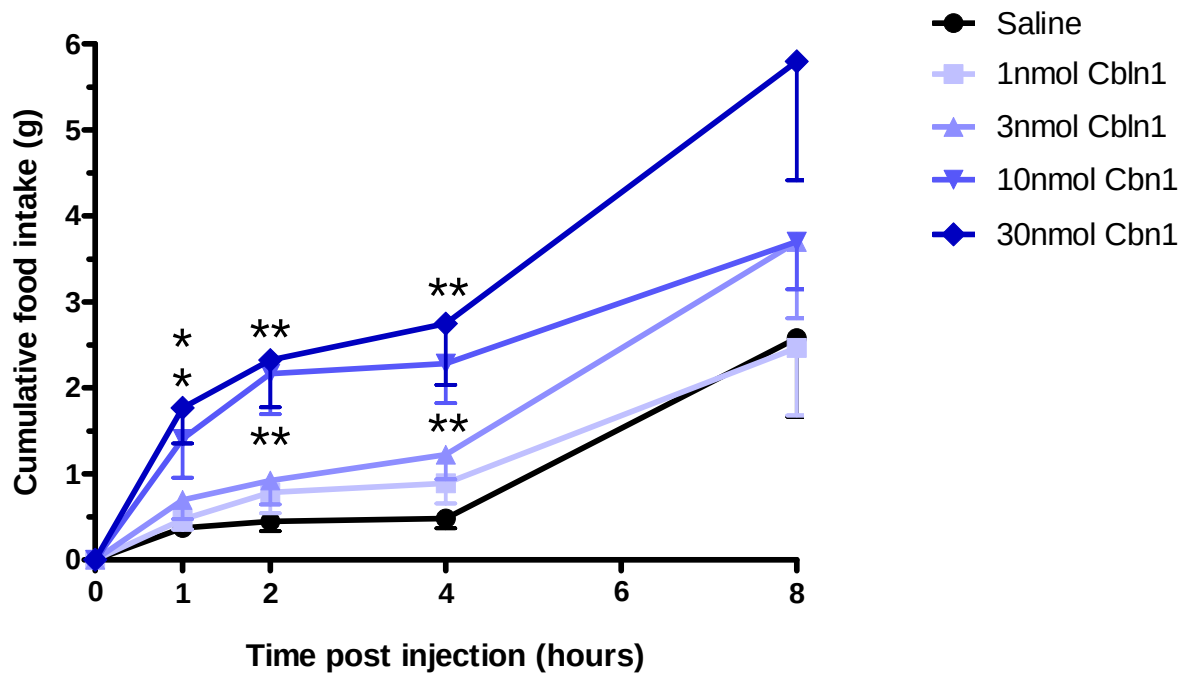


Figure 2.4: The effect of ICV Cbln1 administration on cumulative food intake in *ad libitum* fed rats in the early light phase. *Ad libitum* fed male Wistar rats received an ICV injection of saline, 1, 3, 10 or 30nmol Cbln1 in the early light phase. Food intake was measured at 1, 2, 4 and 8 hours post injection. Data is expressed as mean (g) $\pm$ SEM (n=9 per group). *p<0.05, **p<0.01 vs. saline.

2.3.3. The effect of ICV Cbln1 administration on behaviour in *ad libitum* fed rats in the early light phase

ICV administration of 10 or 30nmol Cbln1 had no significant effect on behaviour compared to saline injected controls. ICV administration of NPS significantly increased burrowing ($p<0.001$) and locomotion activity ($p<0.05$) and reduced sleeping behaviour ($p<0.001$) compared to saline treated animals (Table 2.3).

Behaviour	Saline	10nmol Cbln1	30nmol Cbln1	10nmol NPS
Feeding	1.0 (0.0-3.8)	3.0 (0.0-5.0)	3.0 (0.0-6.0)	3.0 (0.0-6.0)
Drinking	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Rearing	7.0 (1.8-11.0)	3.0 (2.0-7.0)	7.0(3.0-10.0)	5.0(5.0-13.0)
Locomotion	7.5 (2.0-8.8)	4.0 (3.0-5.5)	3.0 (2.0-4.0)	11.0 (8.0-13.0)*
Grooming	3.0 (2.3-6.0)	3.0 (2.0-8.5)	4.0 (3.0-6.0)	3.0 (0.0-5.0)
Burrowing	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (1.0-5.0)***
Head down	6.0 (3.5-6.0)	4.0 (2.5-7.0)	3.0 (0.0-8.0)	3.0 (0.0-9.0)
Sleep	12.0 (6.8-12.8)	18.0 (5.5-22.0)	12.0 (9.0-15.0)	0.0 (0.0-0.0)***
Pica	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)

Table 2.3: The effect of ICV Cbln1 administration on behaviour in *ad libitum* fed male Wistar rats during the early light phase. Behaviour was monitored for one hour post injection. Data is presented as median number of behaviours observed (inter-quartile range), n=10-12 per group; * $p<0.05$, *** $p<0.001$ vs. saline.

2.3.4. The effect of Cbln1 on neuropeptide release from hypothalamic explants

Incubation of hypothalamic explants with 10, 100 and 1000nM Cbln1 significantly increased NPY release (NPY release (% basal): 100.00 $\pm$ 16.71 [basal], 253.31 $\pm$ 60.32 [10nM Cbln1] p <0.01; 100.00 $\pm$ 12.23 [basal], 169.76 $\pm$ 34.76 [100nM Cbln1] p <0.05; 100.00 $\pm$ 14.75 [basal], 132.03 $\pm$ 19.02 [1000nM Cbln1] p <0.05, n = 9-12 slices per group) (Figure 2.5).

Treatment of hypothalamic explants with Cbln1 had no effect of the release of AgRP, CART or α -MSH (Table 2.4).

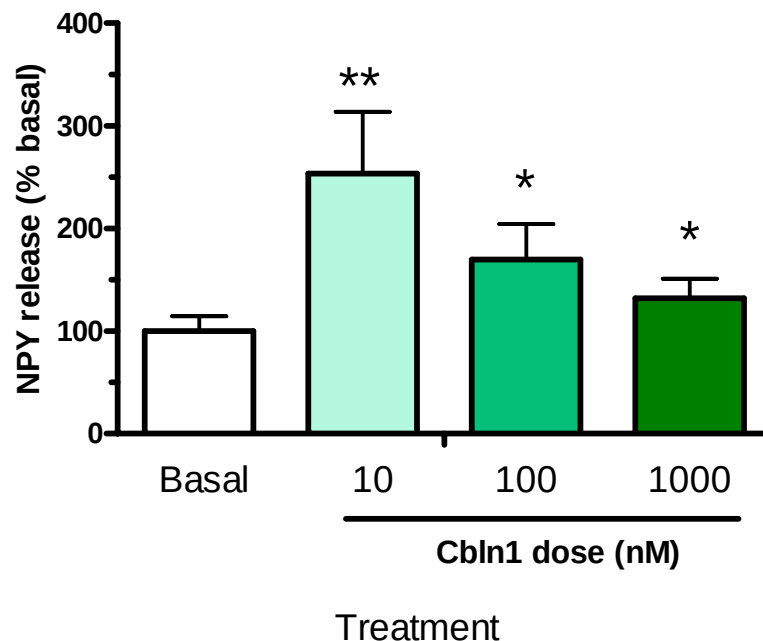


Figure 2.5: The effect of Cbln1 treatment on NPY release from hypothalamic explants. Hypothalamic explants taken from male Wistar rats were treated with 10, 100 or 1000nM Cbln1. Data is presented as mean $\pm$ SEM as a percentage of basal release. Explants were treated with artificial CSF during the basal period (n =9-12 per group). * p <0.05, ** p <0.01 vs. basal treatment.

	Dose of Cbln1 administered to hypothalamic explants					
	10nM		100nM		1000nM	
Peptide release (% basal)	Basal	Cbln1	Basal	Cbln1	Basal	Cbln1
AgRP	100.00 ± 13.27	127.52 ± 14.78	100.00 ± 10.26	112.43 ± 18.41	100.00 ± 10.29	113.29 ± 16.82
CART	100.00 ± 7.88	132.59 ± 15.71	100.00 ± 8.10	126.33 ± 12.10	100.00 ± 14.91	113.64 ± 11.56
α-MSH	100.00 ± 17.87	134.67 ± 15.69	100.00 ± 2.77	94.02 ± 8.04	100.00 ± 10.88	116.12 ± 9.86

Table 2.4: The effect of Cbln1 treatment on the release of AgRP, CART and α-MSH release from hypothalamic explants. Hypothalamic explants taken from male Wistar rats were treated with 10, 100 or 1000nM Cbln1. Data is presented as mean ± SEM as a percentage of basal release. Explants were treated with artificial CSF during the basal period (n=9-12 per group).

2.3.5. The effect of ICV Cbln1 administration on food intake following pre-treatment with a Y1 receptor antagonist in the early light phase

ICV administration of 10nmol Cbln1 significantly increased food intake compared to saline injected controls between 0 and 1 hours post-injection. BIBP 3226 at a dose of 60nmol had no significant effect on food intake. Administration of BIBP 3226 30 minutes prior to injection of 10nmol Cbln1 partially attenuated the orexigenic effect of Cbln1 (0-1hr food intake (g): 0.46 ± 0.14 [vehicle/saline]; 2.12 ± 0.59 [vehicle/Cbln1 10nmol] $p < 0.05$ vs. vehicle/saline; 1.64 ± 0.60 [BIBP 3226 60nmol/Cbln1 10nmol]; 0.33 ± 0.12 [BIBP 3226 60nmol/saline] $n = 10-12$ per group) (Figure 2.6A).

There was also a trend for an increase in 0-24 hour food intake induced by 10nmol Cbln1 which was attenuated by prior administration of BIBP 3226, although this did not reach statistical significance (Figure 2.6B).

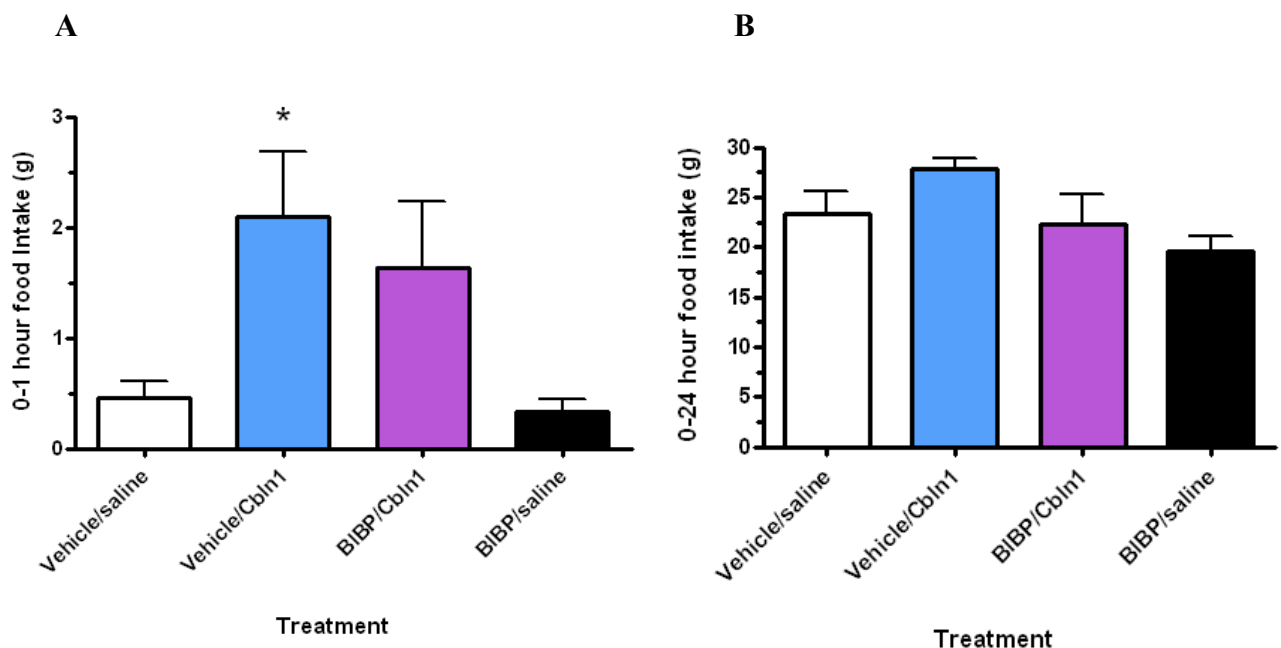


Figure 2.6: The effect of BIBP 3226 pre-treatment on Cbln1-induced food intake in *ad libitum* fed rats in the early light phase. *Ad libitum* fed male Wistar rats were injected ICV with vehicle or 60nmol BIBP 3226 in the early light phase thirty minutes prior to ICV administration of saline or 10nmol Cbln1. Food intake presented as mean $\pm$ SEM at (A) 0-1 and (B) 0-24 hours is shown ($n = 10-12$ per group). * $p < 0.05$ vs. vehicle/saline.

2.3.6. The effect of chronic ICV Cbln1 administration on food intake and body weight

Chronic ICV injection of 10nmol Cbln1 twice daily for four days had no significant effect on 24-hour food intake or body weight compared to saline treated animals (Table 2.5).

	Day 1		Day 2		Day 3		Day 4	
	Food intake	Body weight	Food intake	Body weight	Food intake	Body weight	Food intake	Body weight
Saline	20.2 ± 1.8	282.4 ± 8.2	25.2 ± 1.1	291.7 ± 7.5	23.7 ± 2.5	284.9 ± 9.1	23.6 ± 2.1	286.3 ± 9.7
Cbln1 10nmol	16.7 ± 3.7	286.7 ± 7.1	20.9 ± 2.5	289.7 ± 7.8	25.6 ± 1.0	292.7 ± 7.7	27.7 ± 1.3	301.0 ± 7.9

Table 2.5: The effect of chronic ICV Cbln1 administration on 24-hour food intake and body weight. *Ad libitum* fed male Wistar rats were administered ICV saline or 10nmol Cbln1 twice daily, at the beginning of the light phase and just prior to the onset of the dark phase, for four days (n=8-10 per group). Data are expressed as mean ± SEM.

2.3.7. Production of a Pre-Cbln1 probe

Rat whole brain tissue expressed Pre-Cbln1 as shown by RT-PCR. Although two bands were visible when compared to a 1Kb ladder, the band of interest of approximately 320 base pairs was confirmed and extracted by electroelution (Figure 2.7A). The second band represents another molecule found in the rat brain to which my primers also bound. By analysing the primer sequences and comparing the predicted PCR product sizes, I predict this band represents the rat olfactory receptor.

Pre-Cbln1 was cloned into pBluescript plasmid using the restriction enzymes BamH1 and EcoR1 (Figure 2.7B). Sequencing of plasmid DNA after large scale plasmid preparation confirmed generation of the correct probe.

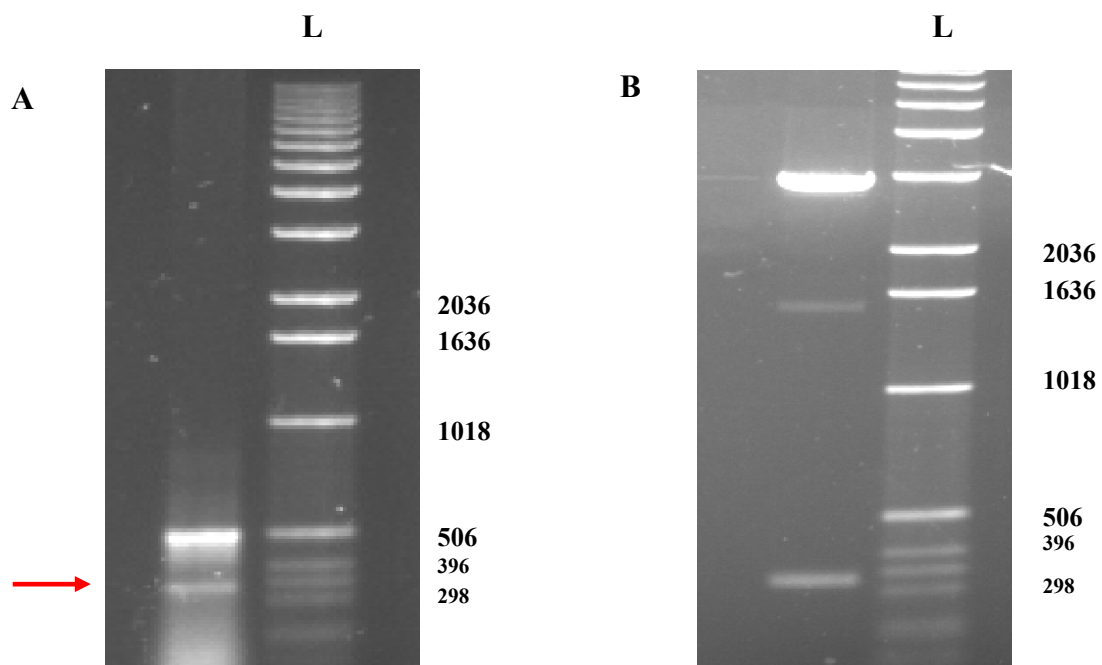


Figure 2.7: A) Pre-Cbln1 mRNA expression in rat brain as determined by reverse transcription-polymerase chain reaction (RT-PCR). L, 1-Kb ladder. The size of the expected PCR product for Pre-Cbln1 was 320 base pairs (shown by arrow). B) Pre-Cbln1 following large scale preparation of plasmid DNA in P.Bluescript and digestion with *EcoR1* and *BamHI* restriction enzymes.

2.3.8. Expression of Pre-Cbln1 mRNA

Using a radio-labelled Pre-Cbln1 anti-sense riboprobe, Pre-Cbln1 mRNA was detected in the VMN, thalamus, zona incerta, hippocampus and motor cortex (Figure 2.8A). No signal was observed when brain slices were hybridised with the corresponding Pre-Cbln1 sense probe (Figure 2.8B).

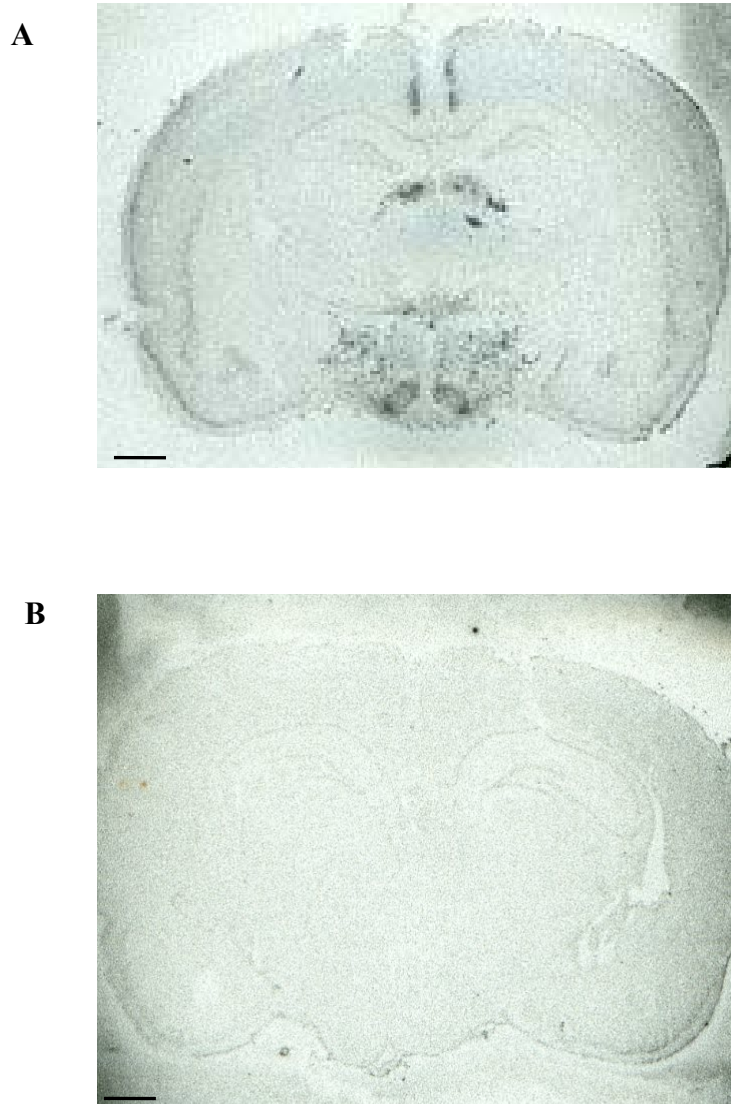


Figure 2.8: Representative microphotographs showing *in situ* hybridisation using a (A) Pre-Cbln1 anti-sense riboprobe and (B) Pre-Cbln1 sense riboprobe to confirm signal specificity. No non-specific binding was observed. Sections of 12 μ m thickness were taken approximately 3.0mm caudal to bregma. Scale bars are equivalent to 1mm.

2.3.9. The effect of nutritional status on Pre-Cbln1 mRNA expression within the VMN

Pre-Cbln1 mRNA expression in the VMN was measured by semi-quantitative *in situ* hybridisation. Pre-Cbln1 mRNA expression was significantly higher in rats fasted for 48 hours compared to *ad libitum* fed controls (Pre-Cbln1 mRNA expression (arbitrary units): 3.40 ± 0.09 (fed) vs. 3.97 ± 0.19 (48-hour fasted) $p < 0.05$, $n = 4-6$ per group) (Figures 2.9 and 2.10).

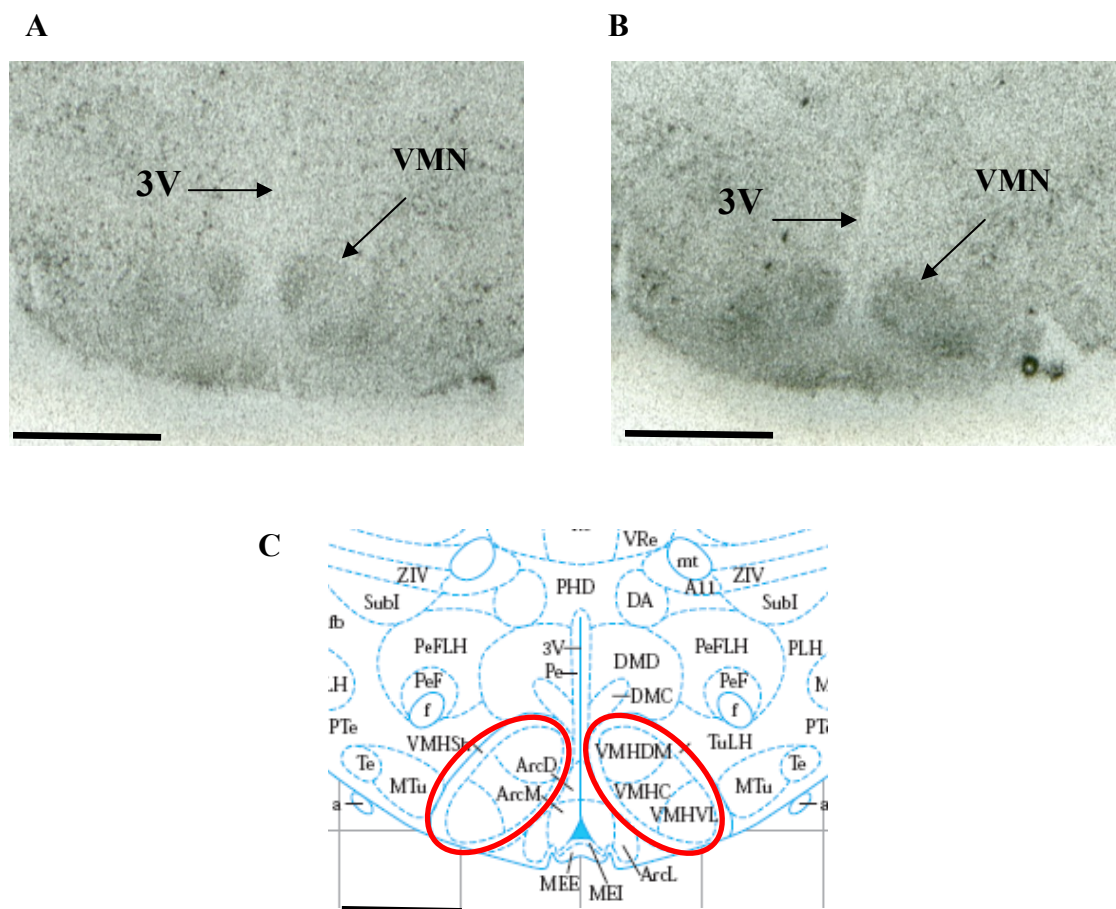


Figure 2.9: Microphotographs of semi-quantitative *in situ* hybridisation for VMN Pre-Cbln1 mRNA in (A) *ad libitum* fed and (B) 48-hour fasted male Wistar rats (12µm coronal sections, -3.0mm caudal to bregma). (C) Sections were compared to 'The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 2007). A coronal section at -3.0mm from bregma is shown. The VMN is outlined in red. Scale bars are equivalent to 1mm. Abbreviations: 3V, third cerebral ventricle; VMN, ventral medial nucleus of the hypothalamus.

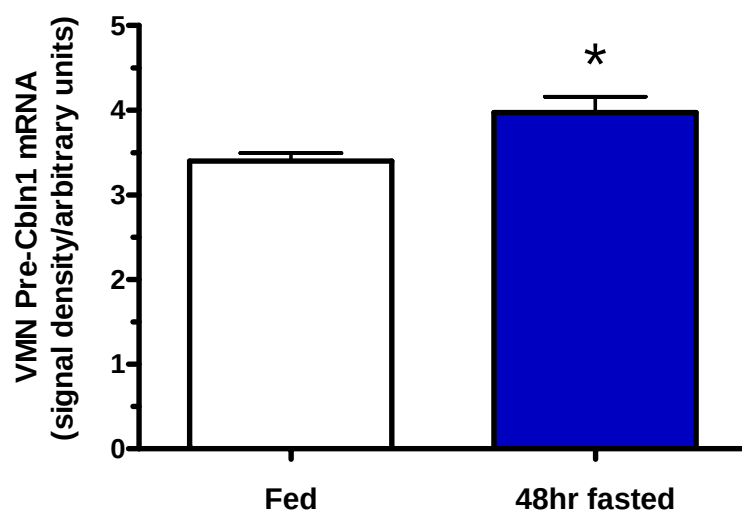


Figure 2.10: Pre-Cb1n1 mRNA expression as semi-quantified using Image J Software. Data is presented as mean $\pm$ SEM arbitrary units, * $p < 0.05$ vs. fed animals (n=4-6 per group).

2.3.10. The effect of ICV Cbln1 administration on plasma pituitary hormones and corticosterone in *ad libitum* fed rats

2.3.10.1. Thyroid-stimulating hormone

ICV administration of 10nmol Cbln1 significantly decreased plasma thyroid-stimulating hormone (TSH) levels at 10 minutes post-injection compared to saline-injected controls (plasma TSH (ng/ml): 5.36 ± 0.50 [saline], 3.77 ± 0.28 [Cbln1 10nmol] $p < 0.05$, $n = 10-12$ per group) (Figure 2.11). No differences were observed at 40 minutes post-injection.

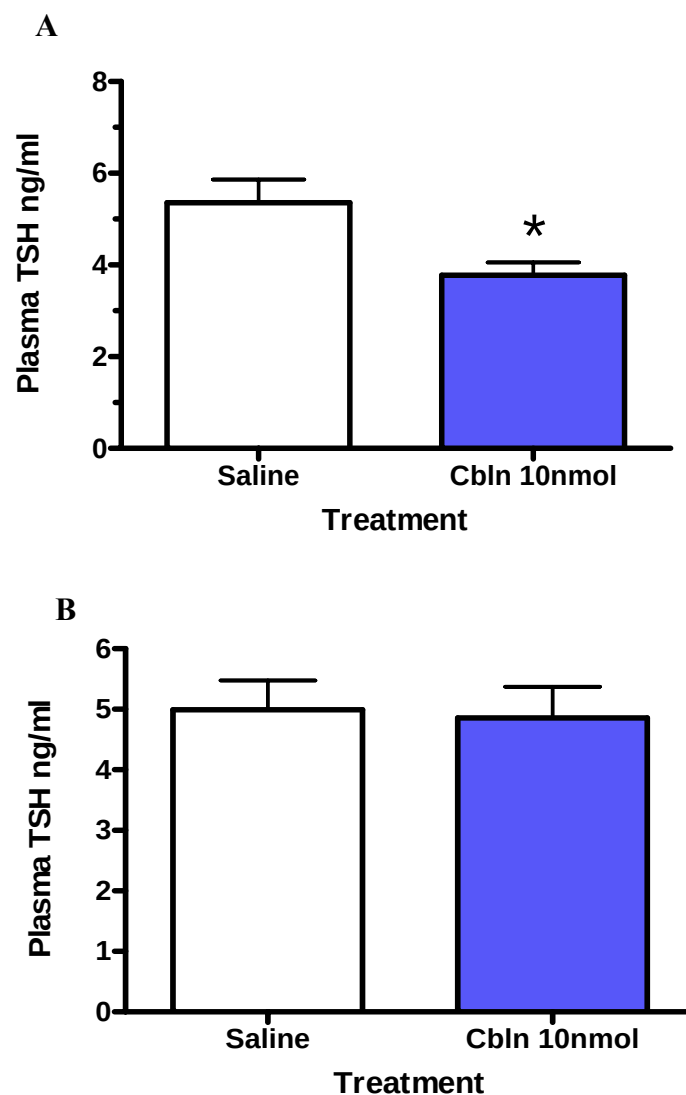


Figure 2.11: The effect of ICV saline or 10nmol Cbln1 on plasma thyroid-stimulating hormone (TSH) levels in male Wistar rats. Plasma was collected at (A) 10 or (B) 40 minutes post-injection. Plasma TSH was measured by radioimmunoassay ($n = 8-10$ per group per time point, $*p < 0.05$ vs. saline).

2.3.10.2. Luteinizing hormone and follicle-stimulating hormone

ICV injection of 10nmol Cbln1 had no effect on plasma luteinizing hormone (LH) levels at 10 minutes post injection. However, at 40 minutes Cbln1 significantly increased circulating LH compared to saline controls (plasma LH (ng/ml): 0.86 ± 0.10 [saline], 1.32 ± 0.16 [Cbln1 10nmol] $p < 0.05$, $n = 10-12$ per group) (Figure 2.12A and B).

Similarly, although ICV 10nmol Cbln1 had no effect on circulating follicle-stimulating hormone (FSH) levels at 10 minutes post-injection, it induced a significant increase at 40 minutes compared to controls (plasma FSH (ng/ml): 8.92 ± 0.56 [saline]; 10.66 ± 0.59 [Cbln1 10nmol] $p < 0.05$, $n = 10-12$ per group) (Figure 2.12C and D).

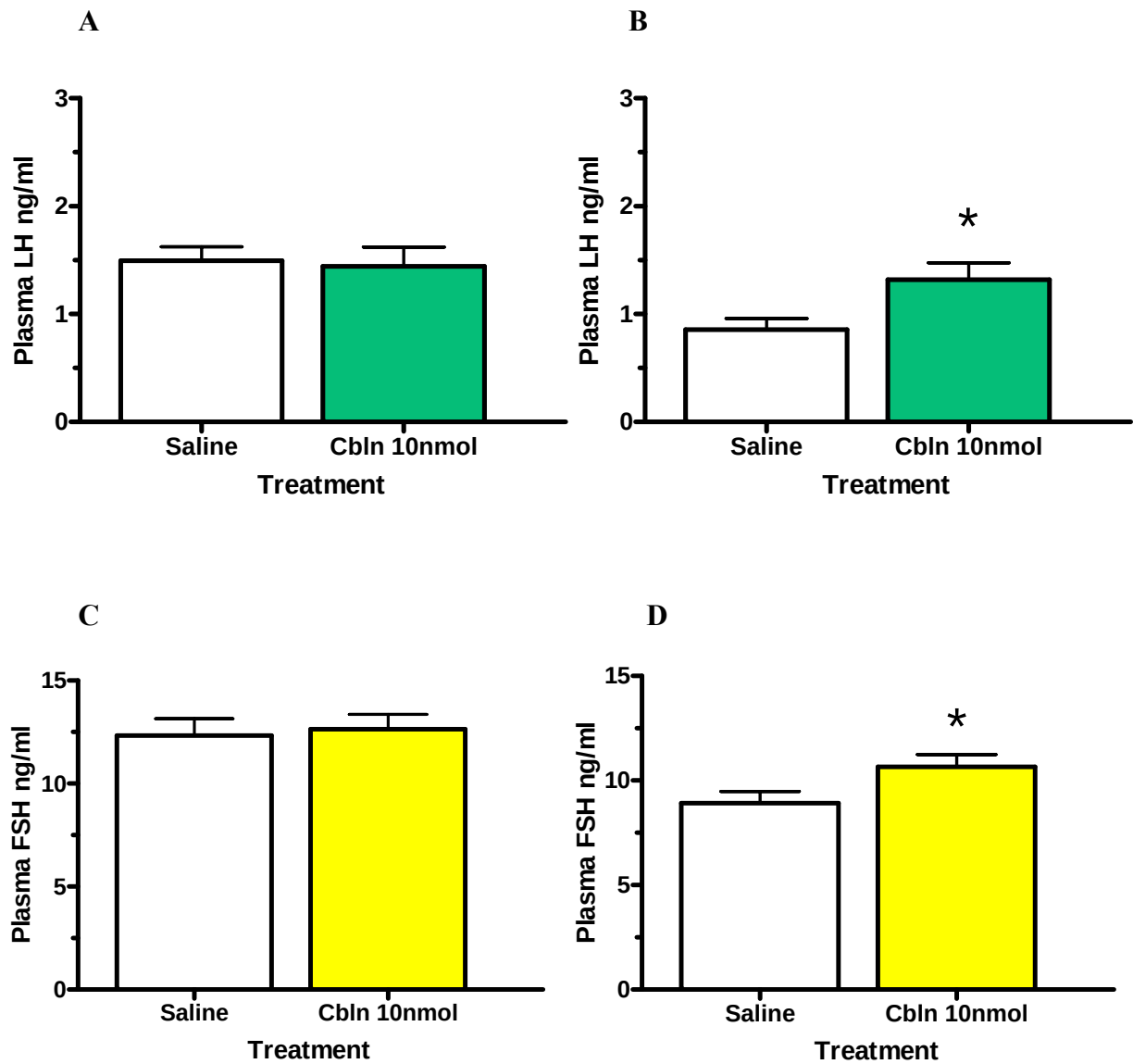


Figure 2.12: The effect of ICV saline or 10nmol Cbln1 on plasma (A and B) luteinizing hormone (LH) and (C and D) follicle-stimulating hormone (FSH) levels in male Wistar rats. Plasma was collected at (A and C) 10 or (B and D) 40 minutes post-injection. Plasma LH and FSH were measured by radioimmunoassay (n=8-10 per group per time point, *p<0.05 vs. saline).

2.3.10.3. ACTH and corticosterone

ICV administration of 10nmol Cbln1 had no effect on circulating ACTH or corticosterone at either 10 or 40 minutes post injection (Figure 2.13A and B).

There was a non-significant reduction in circulating corticosterone 40 minutes post-injection in animals administered 10nmol Cbln1 ICV (plasma corticosterone 40 minutes (ng/ml): 121.40 ± 29.51 [saline], 52.30 ± 14.74 [Cbln1 10nmol], $n=10-12$ per group) (Figure 2.13C and D).

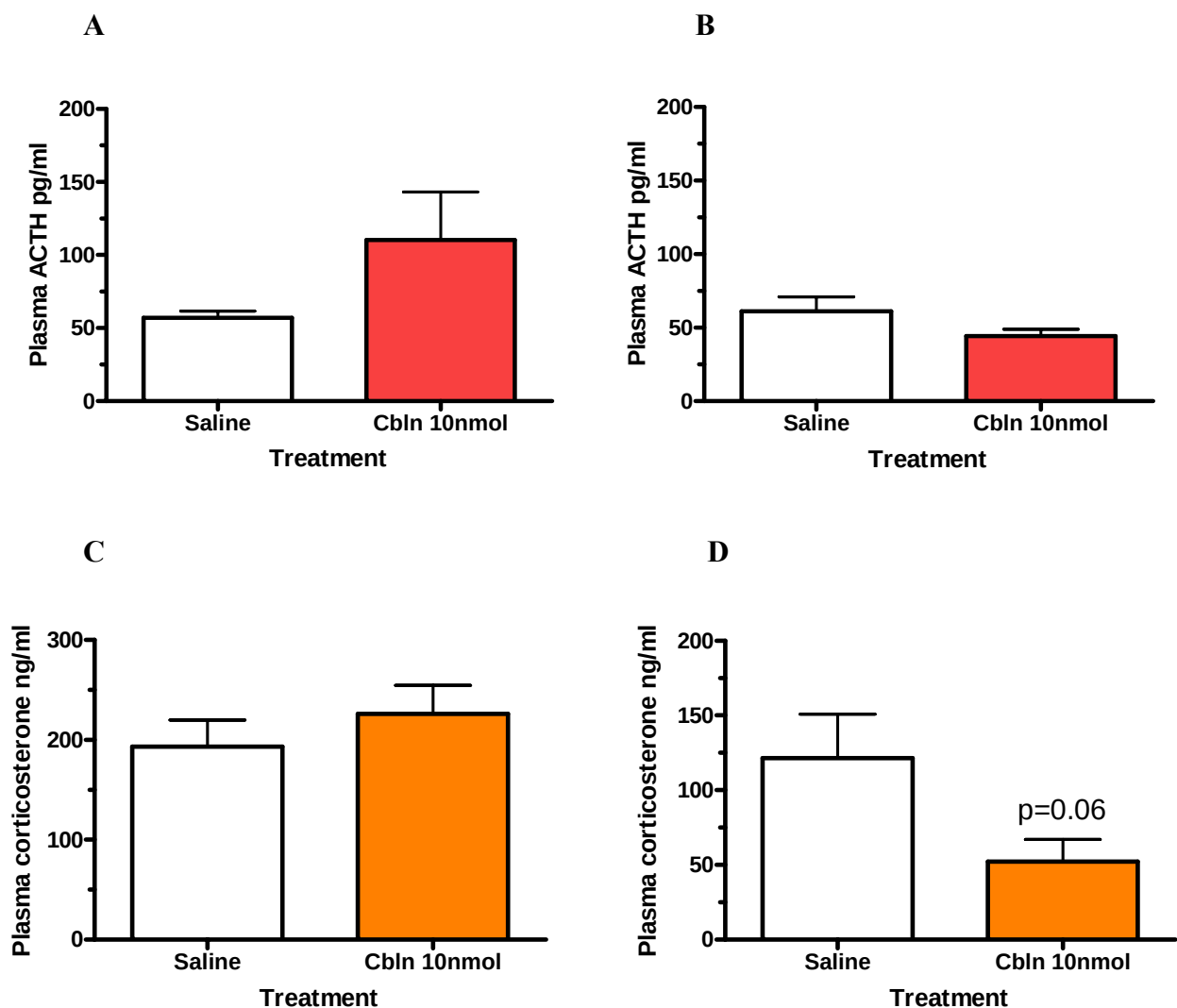


Figure 2.13: The effect of ICV saline or 10nmol Cbln1 on plasma (A and B) adrenocorticotrophic hormone (ACTH) and (C and D) corticosterone levels in male Wistar rats. Plasma was collected at (A and C) 10 or (B and D) 40 minutes post-injection. Plasma ACTH and corticosterone were measured by radioimmunoassay ($n=8-10$ per group per time point).

2.4. Discussion

I have identified a novel orexigenic role for the 16-amino acid peptide Cbln1, which may partially be mediated by hypothalamic NPY. When administered ICV to satiated rats in the early light phase, Cbln1 at doses of 10nmol and 30nmol induced a statistically significant increase in food intake which lasted up to 4 hours post injection. Although not statistically significant, there was also a trend towards an increase in food intake between 0 and 24 hours in animals administered 10 and 30nmol Cbln1. As rats are nocturnal, the light phase is not the usual feeding period and so stimulation of hunger circuits is more apparent in this experimental paradigm. The effect of Cbln1 on food intake was rapid, which indicates that the site of action for Cbln1 may be close to the third ventricle. However, the receptor for Cbln1 is unknown and so it is not possible to determine whether this assumption is correct.

The orexigenic effect of Cbln1 was relatively weak compared to hypothalamic neuropeptides such as NPY and AgRP, which are able to stimulate food intake at doses as low as 0.5nmol and 1nmol respectively (Levine and Morley, 1984; Rossi et al., 1998). Indeed, the effect of Cbln1 on food intake was much less potent when administered to fasted rats; 30nmol Cbln1 only elicited an increase in food intake between 1 and 2 hours post-injection. It is often difficult to observe the effect of an orexigenic peptide post-fasting because the drive to feed is extremely high in all animals and masks any additional stimulatory effects on feeding.

Although Cbln1 had an acute effect on food intake, it did not affect 24-hour food intake or body weight when administered twice daily for four days. This is in contrast to NPY, which induced a dramatic increase in food intake and body weight gain when injected into the PVN three times daily for ten days (Stanley et al., 1986). It is therefore unlikely that Cbln1 plays a major role in long term maintenance of body weight and the development of obesity. However, other hypothalamic neuropeptides which acutely stimulate feeding, such as MCH, have no effect on body weight or 24-hour food intake when administered ICV twice daily for several days (Rossi et al., 1997).

Mice lacking Pre-Cbln1 have not been reported to have any alterations in food intake or body weight compared to their wild type littermates (Hirai et al., 2005). However, these mice had grossly impaired motor function which may have masked any changes in energy homeostasis. Additionally, transgenic models can be complicated by developmental compensation, as demonstrated by the NPY knock-out mouse which unexpectedly has a normal body weight and food intake (Erickson et al., 1996). It would be interesting to reduce endogenous hypothalamic

Cbln1 levels using an antibody or anti-sense RNA, to determine whether Cbln1 has a physiological role in the control of energy homeostasis. Additionally, if the receptor by which Cbln1 mediates its effects on feeding could be isolated, an antagonist could be administered to further investigate the role of Cbln1 in appetite regulation.

When studying food intake it is important to determine the effect of an administered substance on behaviour, as non-specific effects of the substance may alter feeding. For example, adverse effects such as nausea and pain may reduce food intake. An increase in grooming behaviour is often associated with activation of stress pathways (Dunn et al., 1987). Alternatively, stimulation of arousal pathways, demonstrated by an increase in locomotor activity, may increase food intake (Ida et al., 1999; Hagan et al., 1999). ICV administration of both 10 and 30nmol Cbln1 had no effect on behaviour compared to saline controls. This suggests that the orexigenic effect of Cbln1 is not secondary to non-specific behavioural changes and may therefore be due to activation of hypothalamic pathways involved in the control of energy homeostasis.

An *ex vivo* hypothalamic explant system was used to investigate whether Cbln1 acts via known hypothalamic regulators of energy homeostasis to increase food intake. Incubation of hypothalamic slices in 10, 100 and 1000nM Cbln1 induced a significant increase in NPY release. As incubation in 10nM Cbln1 induced the largest response, Cbln1 may have a threshold, rather than a dose-response, effect on NPY release. Alternatively, receptor desensitization may occur at high doses of Cbln1, leading to a blunted response.

Cbln1 had no effect on the release of AgRP, α -MSH or CART release from hypothalamic explants. This is surprising, as NPY is co-localised with AgRP within the Arc, and NPY/AgRP neurones inhibit adjacent POMC/CART neurones via release of GABA. I would therefore expect Cbln1 to increase AgRP release and reduce α -MSH or CART release from hypothalamic explants. However, there are numerous NPY-expressing neurones within the hypothalamus, and not all express AgRP (Broberger et al., 1998). Additionally, only a sub-population inhibit neighbouring anorectic neurones (Cowley et al., 2001). Orexigenic substances have previously been reported to increase NPY release from hypothalamic explants but have no effect on AgRP (Seth et al., 2003).

Within the hypothalamus, Cbln1 is primarily located within the VMN (Segal et al., 2005). Although the primary output of the VMN is anorexigenic, excitatory projections from the VMN exist which terminate on NPY neurones in the Arc (Sternson et al., 2005). Cbln1 may therefore be expressed by these neurones and mediate its effects on food intake via arcuate NPY.

I hypothesised that Cbln1 may act upstream of NPY to stimulate feeding. To test this *in vivo*, I administered an NPY Y1 receptor antagonist, BIBP 3226, 30 minutes prior to administration of Cbln1. BIBP 3226 is a non-peptide antagonist at the Y1 receptor and has been shown to attenuate the effect of NPY on feeding, but have no effect when administered alone at the dose used in this study (O'Shea et al., 1997).

Pre-administration of BIBP 3226 partially attenuated the orexigenic effect of Cbln1 between 0 and 1 hours post-injection; this trend was also observed between 0 and 24 hours, suggesting Cbln1 may act upstream of the Y1 receptor. However, as blockade of NPY Y1 receptors did not completely abolish the orexigenic effect of Cbln1 acutely, it is likely to act through additional mechanisms to stimulate feeding.

Brain-derived neurotrophic factor (BDNF), a neurotrophin essential for brain development, is highly expressed within the VMN (Xu et al., 2003). BDNF is involved in energy homeostasis and is thought to act downstream of the melanocortin system to reduce food intake (Xu et al., 2003). Cbln1 may therefore inhibit local BDNF production to stimulate feeding. Dopamine and serotonin are also expressed within the VMN and have been implicated in the control of food intake (Davidowa et al., 2002; Hikiji et al., 2004). Cbln1 may modulate the release or activity of these neurotransmitters to influence energy homeostasis.

I chose to use semi-quantitative *in situ* hybridisation to determine the effect of nutritional status on Pre-Cbln1 mRNA expression because this technique allows changes within specific hypothalamic nuclei to be detected. Within the VMN, the primary hypothalamic location of Pre-Cbln1, mRNA expression was significantly higher in rats fasted for 48 hours compared to *ad libitum* fed controls. This suggests that Pre-Cbln1 expression is regulated by nutritional status. Many other endogenous hypothalamic neuropeptides important in the control of energy homeostasis are regulated by energy status. Fasting increases NPY mRNA expression within the Arc (White and Kershaw, 1990) which is thought to be mediated by a reduction in circulating leptin levels (Schwartz et al., 1998). It would be interesting to investigate if Pre-Cbln1 expression is also negatively regulated by leptin, as the leptin receptor is abundantly expressed within the VMN (Elmqvist et al., 1998b) and peripheral leptin administration has been shown to induce Fos-like immuno-reactivity, an indirect marker of neuronal activation, in this nucleus (Elmqvist et al., 1997).

As peptide and mRNA levels are often poorly correlated (Gygi et al., 1999) it would be interesting to perform punch biopsies of the VMN and extract and quantify Cbln1 peptide, to determine whether Cbln1 protein expression is also altered by acute food restriction.

The hypothalamo-pituitary-thyroid axis plays a vital role in the control of energy expenditure. TRH, produced in the PVN, acts at the anterior pituitary gland to stimulate the release of TSH into the circulation. TSH binds to receptors on the thyroid gland to stimulate the synthesis and release of the thyroid hormones, T3 and T4. Thyroid hormones increase basal metabolic rate and oxygen consumption and hence stimulate energy expenditure (Yen, 2001).

ICV Cbln1 administration significantly lowered circulating TSH levels at 10 minutes post-injection. It is possible that Cbln1 may mediate this effect via hypothalamic NPY. NPY-expressing neurones within the Arc terminate on TRH neurones in the PVN (Toni et al., 1990) and administration of NPY has been shown to lower circulating TSH, T3 and T4 levels and suppress hypothalamic pro-TRH mRNA expression (Fekete et al., 2001). Cbln1 may therefore act via NPY to induce a state of hypothyroidism, potentially mediated by TRH, to reduce energy expenditure and promote weight gain.

Starvation suppresses the HPT axis as an adaptive response to conserve energy. A reduction in circulating leptin during fasting increases hypothalamic NPY/AgRP and reduces POMC/CART signalling, causing a reduction in TRH expression (Sarkar and Lechan, 2003). As hypothalamic Pre-Cbln1 mRNA expression was up-regulated by a 48-hour fast, this further supports the hypothesis that Cbln1 is an endogenous factor which stimulates feeding and lowers energy expenditure to promote a state of positive energy balance.

Although ICV administration of Cbln1 significantly increased circulating LH and FSH 40 minutes post-injection, these results must be treated with caution as levels of both LH and FSH dropped between 10 and 40 minutes in saline injected animals. This experiment requires repeating and it would be interesting to perform a dose-response study to confirm the effect of Cbln1 on the reproductive axis.

However, many hypothalamic neuropeptides involved in the control of energy homeostasis also influence reproductive function. For example, NPY and galanin both increase feeding and paradoxically stimulate the HPG axis in gonadally intact animals (Sahu et al., 1987; Kyrkouli et al., 1986; Levine and Morley, 1984). If my results regarding the effect of Cbln1 on LH and FSH were to be confirmed, they may further support the hypothesis that Cbln1 acts upstream of NPY, as ICV NPY administration also increases circulating LH levels (Kalra and Crowley, 1984).

NPY is hypothesised to act at hypothalamic GnRH neurones to aid the pre-ovulatory GnRH surge (Bauer-Dantoin et al., 1991). It would be interesting to investigate whether Cbln1 also increases GnRH release from hypothalamic explants *ex vivo*.

Peripheral Cbln1 administration has been demonstrated to increase circulating corticosterone independently of ACTH release in rats (Hochol et al., 2001). I therefore investigated the effect of ICV Cbln1 on circulating ACTH and corticosterone. ICV Cbln1 administration had no effect on plasma levels of ACTH or corticosterone. This suggests that Cbln1 does not act at the PVN to stimulate CRH release and supports the hypothesis that Cbln1 exerts its effects at the adrenal gland via an autocrine or paracrine mechanism, which may be dependent on catecholamine release (Mazzocchi et al., 1999; Hochol et al., 2001). Interestingly, 10nmol Cbln1 lowered circulating corticosterone at 40 minutes post-injection, although this did not reach significance.

In conclusion, I have shown that Cbln1 acutely stimulates food intake when administered ICV to male rats. Additionally, ICV Cbln1 administration lowered circulating TSH, suggesting it may reduce energy expenditure and promote a state of positive energy balance. It is possible that Cbln1 exerts its effects on feeding and the HPT axis via hypothalamic NPY. Similarly to other orexigenic neuropeptides, hypothalamic Pre-Cbln1 mRNA expression was increased following a 48 hour fast, suggesting it may be regulated by leptin and hence have an endogenous role in the control of energy homeostasis.

Chapter 3: Investigating the role of VTA melanocortin signalling in the regulation of food intake

3.1. Introduction

3.1.1. Homeostatic and non-homeostatic control of food intake

Hypothalamic circuits involved in the homeostatic regulation of food intake and energy expenditure are effective in defending an organism against starvation, but are relatively ineffective at limiting food intake in an environment in which there is an excess of readily available, palatable, energy dense food (Berthoud, 2006). Homeostatic circuits can thus be easily over-powered by non-homeostatic signals such as the palatability and rewarding value of food. Such non-homeostatic cues are processed by cortico-limbic structures, including the ventral tegmental area (VTA) and nucleus accumbens (NAc). Projections from limbic areas to the hypothalamus may permit reward signals to over-ride homeostatic circuits, and hence promote food intake even in the fed state (Berthoud, 2006). Conversely, hypothalamic neurones project to reward centres and may modulate reward- and motivation-based food intake.

3.1.2. Anatomy of the mesocorticolimbic pathway

The mesocorticolimbic pathway is composed of dopamine neurones which project primarily from the VTA to the ventral striatum (which includes the NAc and olfactory tubercle), and to other limbic structures including the amygdala, hippocampus, and pre-frontal cortex.

This pathway is important in mediating the rewarding and reinforcing effects of stimuli including natural rewards, such as food and social contact, and drugs of abuse. It also modulates goal-directed and motivational behaviour in response to such stimuli.

3.1.2.1. The VTA

The VTA is located in the ventral midbrain between the caudal hypothalamus and brainstem. It is termed an area, rather than a nucleus, due to its heterogeneous structure and the lack of clear borders distinguishing it from the neighbouring substantia nigra (Ikemoto, 2007).

The rodent VTA can be sub-divided into four zones: the paranigral nucleus (PN), the parabrachial pigmented area (PBP), the parafasciculus retroflexus area and the ventral tegmental tail (VTT) (Halliday and Tork, 1986; Ikemoto, 2007). The PN and PBP, which make up the middle two-thirds of the VTA, are rich in dopamine-expressing cells and stain heavily for tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. In contrast, the parafasciculus retroflexus area and VTT contain only a low number of dopamine-expressing neurones (Ikemoto, 2007).

Dopamine neurones comprise approximately 60-70% of the VTA; most of the remaining 30-40% of neurones are GABAergic (Swanson, 1982; Olson and Nestler, 2007). Additionally, a small number of VTA neurones are glutamatergic (Lavin et al., 2005). The majority of VTA GABAergic neurones are local interneurones, which are interspersed with, and terminate on, dopamine-expressing neurones (Bayer and Pickel, 1991; Olson and Nestler, 2007). These interneurones tonically inhibit the activity of VTA dopamine neurones (Matthews and German, 1984; David et al., 1997). A small number of VTA GABAergic neurones project to limbic areas including the pre-frontal cortex and NAc (Carr and Sesack, 2000; Van Bockstaele and Pickel, 1995).

The VTA sends efferent fibres to forebrain areas including the ventral striatum, amygdala, pre-frontal and insular cortices, septal region, hypothalamus and thalamus (Swanson, 1982) (Figure 3.1). The VTA also projects through the midbrain to the periaqueductal grey, the parabrachial nucleus, the locus coeruleus and the raphe nuclei (Swanson, 1982).

The VTA receives afferent inputs from many of the limbic areas to which it projects, including the NAc (Nauta et al., 1978) and pre-frontal cortex (Leonard, 1969). It also receives projections from hypothalamic nuclei including the VMN and the LH (Saper et al., 1979), and from brainstem regions including the tegmental pedunculopontine nucleus (TPP), an area which mediates dopamine-independent aspects of reward, and the laterodorsal tegmental nucleus (Oakman et al., 1995) (Figure 3.1).

3.1.2.2. The nucleus accumbens

The NAc lies in the anterior and ventral striatum and has two subdivisions; the core and the shell. The shell lies medial and ventral to the core and is thought to be particularly important in mediating reward (Di Chiara, 2002). The NAc is primarily composed of GABAergic neurones which project widely to other limbic and non-limbic structures, but also contains GABAergic interneurones and cholinergic neuronal cell bodies (Meredith, 1999).

In addition to receiving afferent projections from the VTA, the NAc receives glutamatergic inputs from regions including the hippocampus, amygdala and prefrontal cortex (O'Donnell and Grace, 1995) (Figure 3.1). The NAc sends efferent fibres to numerous structures including the ventral pallidum, substantia nigra, VTA, preoptic region, and thalamus (Nauta et al., 1978). The shell, but not the core, of the NAc also has reciprocal projections with the LH (Heimer et al., 1991) and this connection is thought to be important in integrating homeostatic and hedonistic control of food intake (Stratford and Kelley, 1999).

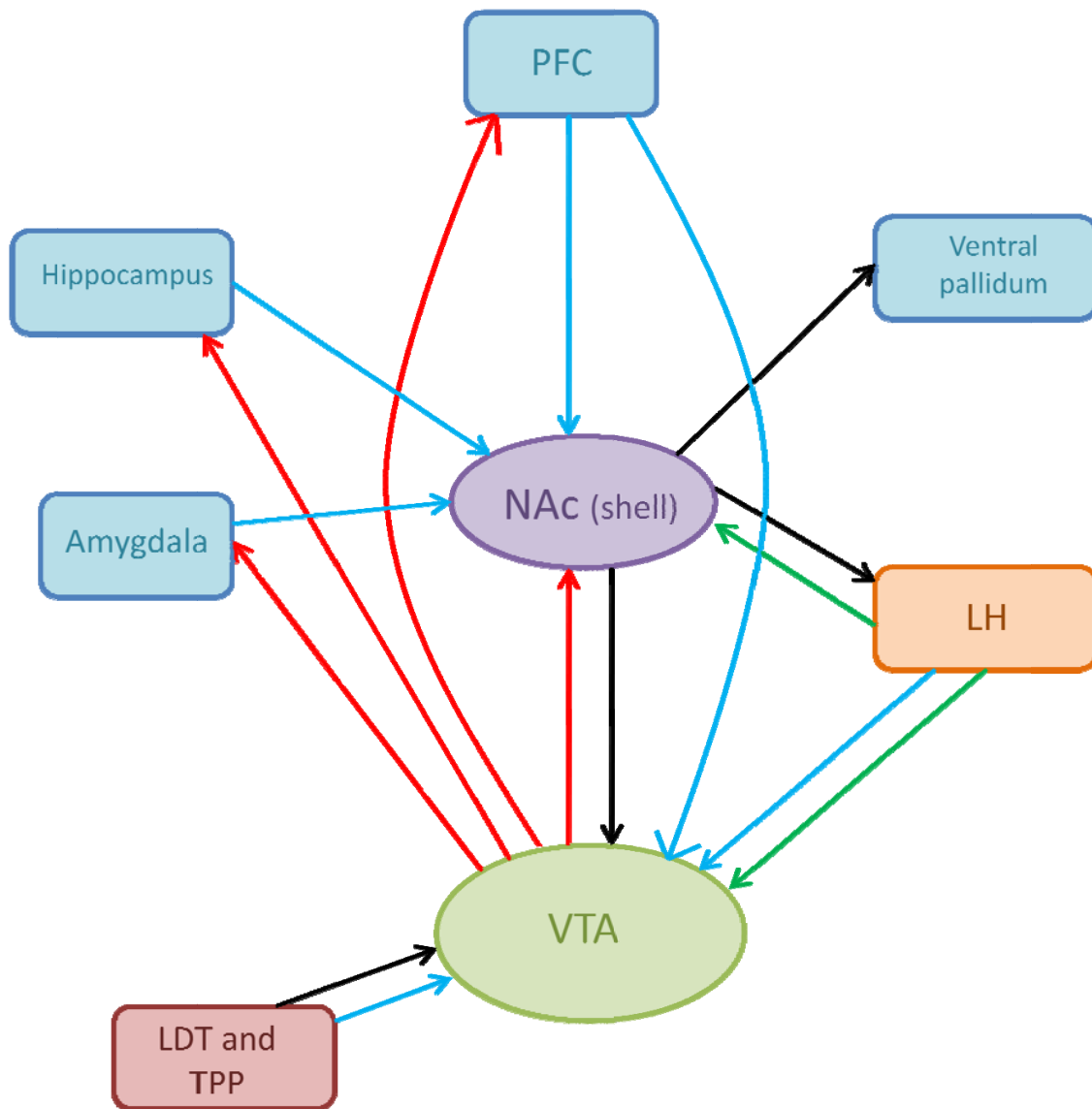


Figure 3.1: Simplified schematic diagram showing connections between areas of the reward system, highlighting inputs to the ventral tegmental area (VTA) and nucleus accumbens (NAc). Dopaminergic projections are shown in red, glutamatergic projections in blue, GABAergic projections in black and orexigenic projections in green. Abbreviations: LDT, laterodorsal tegmental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; TPP, tegmental pedunculopontine nucleus.

3.1.3. Dopamine signalling

The amino acid tyrosine is the initial substrate in dopamine biosynthesis. Tyrosine is taken up by dopamine neurones, and is converted to levodopa (L-DOPA) by the cytosolic enzyme tyrosine hydroxylase (TH). L-DOPA is then converted to dopamine by aromatic amino acid decarboxylase (AADC), also known as dopa decarboxylase. Dopamine is transported from the cytoplasm into storage vesicles, which fuse with the neuronal membrane and release dopamine into the synapse in response to a pre-synaptic action potential (Elsworth and Roth, 1997) (Figure 3.2).

Tonic dopamine release is maintained by slow, irregular firing of dopamine neurones (Overton and Clark, 1997). Midbrain dopamine neurones fire action potentials in bursts in response to sensory stimuli, including those associated with reward. This results in large quantities of dopamine being released into target areas such as the NAc (Overton and Clark, 1997; Gonon, 1988).

Dopamine receptors are G-protein coupled receptors. There are at least five types of dopamine receptors in the CNS, which can be subdivided according to whether they increase (D1-like) or decrease (D2-like) adenylate cyclase activity (Kebabian, 1978; Seeman and Van Tol, 1994) (Figure 3.2). The D2 receptor gene is alternatively spliced to generate two isoforms; a long form (D2L), which is located post-synaptically, and a short form (D2S), which is a pre-synaptic autoreceptor (Usiello et al., 2000).

D2 receptors play a critical role in reward and reinforcement. Peripheral pre-treatment with a D2 receptor agonist enhances the reinforcing effects of cocaine in rats (Caine et al., 2000), and mice lacking the D2 receptor show reduced sensitivity to the rewarding properties of morphine (Maldonado et al., 1997) and cocaine (Welter et al., 2007).

The dopamine transporter (DAT), located on dopamine nerve terminals, pumps extracellular dopamine back into the neuron and hence switches off the dopamine signal at the synapse (Figure 3.2). Dopamine is then recycled or degraded by the enzymes monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT), which are located within dopamine neurones, and in neighbouring glial cells and astrocytes.

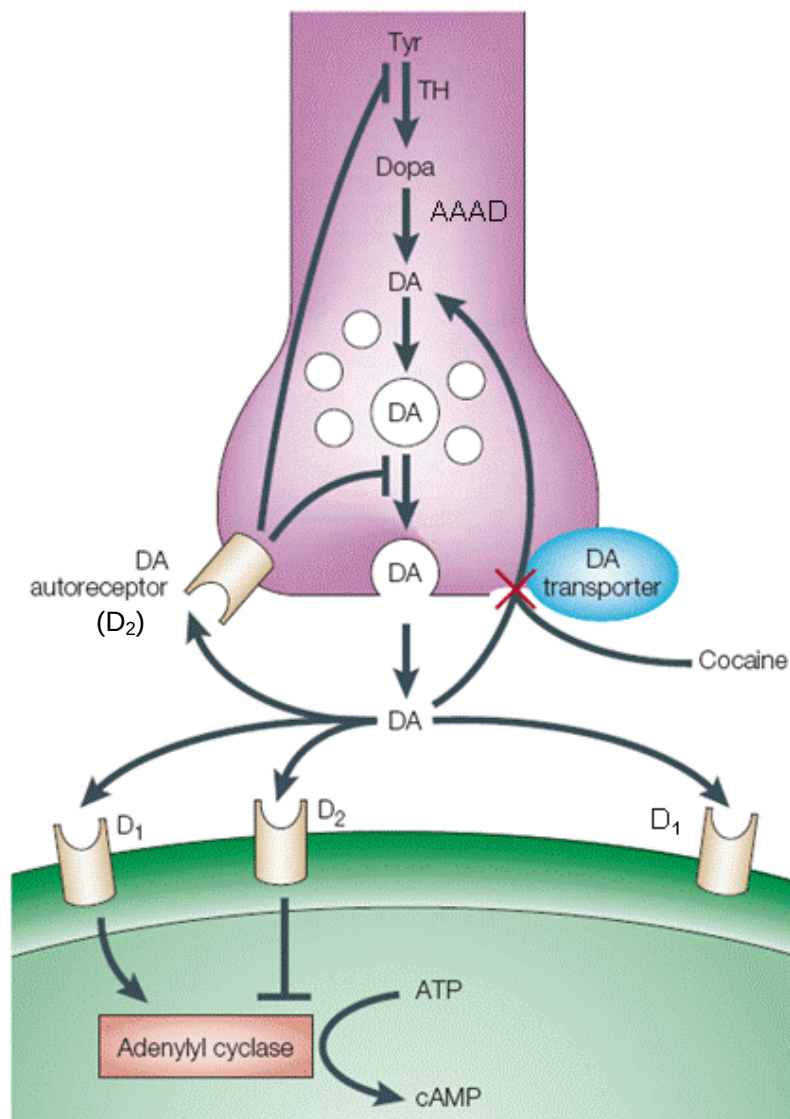


Figure 3.2: The dopamine synapse. Tyrosine (Tyr) is converted to L-DOPA by tyrosine hydroxylase (TH), and then to dopamine (DA) by aromatic amino acid decarboxylase (AAAD). Dopamine is packaged into vesicles which fuse with the neuronal membrane upon stimulation by an action potential. Dopamine binds to and activates D1-like (D₁) and D2-like (D₂) receptors, which stimulate or inhibit adenylate cyclase activity respectively. Dopamine is cleared from the synaptic cleft by the dopamine transporter; this transporter is inactivated by cocaine. Abbreviations: ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate. Adapted from (Kreek et al., 2002).

3.1.4. The role of the VTA in motivation, reward and addiction

The VTA, NAc and related limbic structures are critical in mediating the rewarding and motivational properties of natural rewards and addictive drugs. It should be noted that ‘wanting’ and ‘liking’ a stimulus are considered to be separate components of reward (Berridge, 1996). The mesocorticolimbic dopamine pathway primarily mediates the ‘wanting’, or incentive salience, of a reward, but not necessarily the ‘liking’ (Berridge, 1996; Pecina et al., 2003). This is hypothesised to be primarily controlled by opioid circuits and endocannabinoids (Pecina and Berridge, 2000; Mahler et al., 2007).

Drugs of abuse and naturally rewarding behaviours stimulate the firing of VTA dopamine neurones and increase extracellular dopamine levels within the NAc (Ljungberg et al., 1992; Pettit and Justice, Jr., 1989; Di Chiara and Imperato, 1988). Amphetamines and cocaine increase dopamine levels directly by increasing dopamine release and inhibiting dopamine re-uptake, respectively (Bradberry and Roth, 1989; Ritz et al., 1987; Hurd et al., 1992). In contrast, opiates increase NAc dopamine levels indirectly by inhibiting GABAergic interneurons and hence disinhibiting VTA dopamine neurones (Bergevin et al., 2002). Lesioning the VTA dramatically reduces motivational behaviour in rodents (Ungerstedt, 1971). Additionally, destruction of the NAc prevents cocaine self-administration (Roberts et al., 1980), and inhibits the rewarding effects of opiates, in rats (Kelsey et al., 1989).

Reward is linked to a reduction in NAc output. Administration of morphine (Hakan and Henriksen, 1989) or cocaine (Peoples and West, 1996) decreases neuronal activity in the NAc. The major output of the NAc is GABAergic and thus inhibitory. Rewarding stimuli decrease NAc firing and therefore disinhibit downstream structures, such as the ventral pallidum. In support of this theory, a sucrose reward increases the activity of ventral pallidal neurones in rats (Tindell et al., 2004).

Drug addiction is a highly complex process that is not fully understood. Repetitive stimulation of reward circuits induced by chronic drug use induces neuroadaptations within the mesolimbic dopamine pathway. Addicted subjects have decreased dopamine function, and are less sensitive to non-drug related stimuli including natural reinforcers. However, dopamine transmission in response to the addicted drug is increased, which is postulated to cause drug craving (Volkow et al., 2004; Nestler, 2005). It is possible that excessive stimulation of reward circuits by over-consumption of palatable food may induce similar adaptations, and play a role in the development of obesity.

3.1.5. The role of mesolimbic dopamine in the control of food intake

Midbrain dopamine neurones have long been implicated in the control of food intake. Feeding is a naturally rewarding behaviour, and, similarly to administration of drugs of abuse, is associated with an increase in extracellular dopamine levels within the NAc (Hernandez and Hoebel, 1988). Peripheral administration of a dopamine receptor antagonist attenuates lever-pressing behaviour for a food reward in fasted rats (Phillips and Nikaido, 1975) and blocks feeding induced by electrical stimulation of the LH (Wise et al., 1978). Additionally, dopamine depletion within the midbrain induced by the neurotoxin 6-hydroxydopamine (6-OHDA) induces hypophagia and hypodipsia in mice (Ungerstedt, 1971). However, the role of dopamine in general motivation and movement must be considered when evaluating this model.

A hypophagic, lean phenotype is also observed in mice unable to synthesise dopamine specifically within neurones due to TH inactivation. L-DOPA administration restores feeding and growth in these animals (Zhou and Palmiter, 1995). Conversely, mice with increased synaptic dopamine due to deletion of the DAT gene are hyperphagic, hyperdipsic and show increased behavioural motivation to obtain a sweet reward compared to wild type littermates (Pecina et al., 2003).

In humans, positron emission tomography (PET) scanning has demonstrated that obese individuals have significantly lower striatal D2 receptor availability compared to non-obese controls, and that D2 receptor availability is inversely correlated with body mass index (BMI) in obese subjects (Wang et al., 2001). A similar reduction in D2 receptors has also been identified in cocaine (Volkow et al., 1993) and opiate (Wang et al., 1997a) addicts, suggesting that drug addiction and over-eating may either be caused by, or exert, similar changes in the mesolimbic dopamine system.

Two hypotheses have been suggested to explain these findings. D2 receptors may be down-regulated following over-stimulation induced by excessive feeding or drug-taking. Alternatively, subjects with lower striatal D2 receptor availability may be more susceptible to addictive behaviour, including over-eating (Wang et al., 2001). Recent work has demonstrated that overconsumption of palatable food decreases the responsiveness of brain reward circuits and reduces striatal D2 receptor density in rats (Johnson and Kenny, 2010). Additionally, D2 receptor availability increases in female subjects following weight loss induced by Roux-en-Y gastric bypass surgery (Steele et al., 2010). The evidence therefore favours the hypothesis that reduced dopamine receptor density is a consequence, rather than a cause, of obesity.

3.1.6. Changes in nutritional status alter the mesolimbic dopamine system

Caloric deficit is generally thought to increase reward-related behaviour, and the evolutionary advantage of the rewarding value of food increasing during periods of food deprivation is clear. Conditioned place preference (CPP) is a technique used to determine reward-induced learning. Rats form a CPP to food more effectively when food restricted than when fed (Swerdlow et al., 1983). This effect can be abolished by peripheral pre-treatment with a dopamine receptor antagonist (Spyraki et al., 1982). Unsurprisingly, rats will also work harder for food when food-deprived (Figlewicz et al., 2006). Food restriction increases drug self-administration (Carroll et al., 1979; Carroll and Meisch, 1981; Bell et al., 1997) and drug-induced locomotion (Campbell and Fibiger, 1971), suggesting functional overlap in CNS circuits which control food intake and drug reward.

Chronic food restriction increases TH and DAT mRNA and protein levels in the rat VTA (Lindblom et al., 2006), and has been reported to reduce basal extracellular dopamine levels in the NAc (Pothos et al., 1995). It has been proposed that in states of negative energy balance, dopamine synthesis and re-uptake increases, resulting in an accumulation of pre-synaptic dopamine and low extracellular levels. This leads to sensitization of post-synaptic dopamine receptors (Carr et al., 2003) and enables higher than usual quantities of dopamine to be released upon stimulation, leading to initiation of reward-seeking behaviour (Pothos et al., 1998; Lindblom et al., 2006).

It is currently unclear which energy homeostasis-regulating factors influence the dopamine reward system. However, recent evidence suggests that peripheral hormones may be involved.

3.1.7. The role of peripheral hormones involved in energy homeostasis in the VTA

Recent work has suggested that peripheral hormones including leptin, insulin, and ghrelin signal not only to the hypothalamus and brainstem, but also to limbic structures involved in reward, to modulate food intake.

3.1.7.1. Leptin

A large body of evidence suggests that midbrain dopamine neurones are direct targets for leptin. Approximately 60% of VTA dopamine neurones express both ObRb and signal transducer and activator of transcription 3 (STAT3), an intracellular signalling molecule which is phosphorylated (pSTAT3) upon leptin binding to its receptor (Hommel et al., 2006; Fulton et al., 2006). Peripheral and intra-VTA leptin administration increases VTA pSTAT3 protein expression, suggesting that leptin signals directly to VTA neurones (Hommel et al., 2006; Fulton et al., 2006). Similarly to food-restricted animals, leptin deficient ob/ob mice have impaired dopamine release within the NAc compared to wild-type controls (Fulton et al., 2006).

It has been suggested that leptin may both modulate reward-seeking behaviour and influence feeding by acting directly on VTA dopamine neurones. Leptin administration into the VTA decreases food intake in rats, and peripheral leptin treatment reduces dopamine cell firing within the VTA (Hommel et al., 2006). Mice lacking the leptin receptor specifically within the VTA consume more sucrose and palatable high fat food than wild-type controls (Hommel et al., 2006). Peripheral leptin administration reverses CPP in food deprived rats (Figlewicz et al., 2001), and ICV leptin treatment reduces lever pressing behaviour to obtain a sucrose reward (Figlewicz et al., 2006). Leptin may therefore inhibit the firing of VTA dopamine neurones to decrease food intake and blunt the rewarding properties of food.

3.1.7.2. Insulin

Similarly to leptin, insulin is hypothesised to reduce the rewarding properties of food. Insulin receptors are expressed on dopamine neurones within the VTA (Figlewicz et al., 2003), suggesting insulin may have a direct action on reward circuits. ICV administration of insulin abolishes the CPP associated with a food reward in fed rats (Figlewicz et al., 2004), and, like leptin administration, reduces lever-pressing behaviour to obtain sucrose (Figlewicz et al., 2006). Central administration of insulin attenuates consumption of palatable food induced by ICV injection of an opioid receptor agonist (Sipols et al., 2002), and enhances the action of a D2 receptor antagonist to attenuate sucrose ingestion (Sipols et al., 2000).

Electrical stimulation of certain areas of the brain, including the LH, is thought to artificially activate reward circuitry and hence has reinforcing and rewarding properties. Rats will actively seek out, and perform tasks in order to receive, electrical stimulation of the LH (Margules and Olds, 1962). Lateral hypothalamic self-stimulation (LHSS) is therefore a useful technique to assess the responsiveness of brain reward systems. The threshold for LHSS is lowered in insulin-deficient diabetic and food-restricted rats, implying there is an increase in sensitivity to reward when circulating insulin and/or leptin levels are low (Carr et al., 2000). This threshold increases to baseline levels following central insulin (Carr et al., 2000) or leptin (Fulton et al., 2000) administration.

3.1.7.3. Ghrelin

The orexigenic hormone ghrelin is also hypothesised to target the mesolimbic dopamine system. Ghrelin increases the firing rate of VTA dopamine neurones, and increases dopamine turnover in the NAc (Abizaid et al., 2006). Injection of ghrelin directly into the VTA stimulates feeding (Naleid et al., 2005; Abizaid et al., 2006). Additionally, administration of a ghrelin receptor antagonist into the VTA blocks feeding induced by peripheral ghrelin injection and by fasting (Abizaid et al., 2006). This suggests ghrelin may enhance reward signals and therefore have opposing actions to leptin and insulin.

3.1.8. The role of hypothalamic neuropeptides involved in energy homeostasis in the VTA

Hypothalamic neuropeptides involved the control of energy homeostasis may also influence food intake by modulating reward pathways.

3.1.8.1. Orexins

Orexin neurones located within the LH project heavily to the VTA, where they terminate in close proximity to TH-expressing neurones (Fadel and Deutch, 2002; Marcus et al., 2001). Orexin 1 and 2 receptor immunoreactivity is colocalised with TH within the VTA (Narita et al., 2006). Orexin A increases calcium influx in isolated VTA dopamine neurones (Uramura et al., 2001), and both orexin A and B increase the firing frequency of VTA dopamine neurones (Korotkova et al., 2003). Additionally, intra-VTA administration of either orexin A and B increases dopamine levels within the NAc (Narita et al., 2006).

Orexin signalling is involved in mediating the rewarding effects of morphine. Morphine administration fails to increase extracellular dopamine in the NAc, and does not induce formation of a CPP, in mice lacking the prepro-orexin gene (Narita et al., 2006). The orexins also have a role in mediating reward-based food intake. Administration of a μ -opioid receptor agonist into the NAc stimulates consumption of a palatable high-fat diet in rats (Zhang et al., 1998). Pre-administration of an orexin 1 receptor antagonist into the VTA inhibits this orexigenic effect (Zheng et al., 2007). This suggests that orexin signalling from the LH to the VTA may regulate activation of the mesolimbic dopamine system by opioid receptors, and thus influence both food intake and responsiveness to morphine.

3.1.8.2. NPY

NPY immunoreactive neuronal cell bodies and axons are widely distributed throughout the mammalian brain, and the NAc is one of the areas within the CNS in which NPY immunoreactivity is highest (de Quidt and Emson, 1986). Administration of NPY into the NAc induces a CPP in rats, which can be prevented by pre-administration of a dopamine receptor antagonist (Josselyn and Beninger, 1993). This suggests that NPY signalling within the NAc may have rewarding and reinforcing properties, and this effect may be mediated by dopamine. Additionally, ICV administration of NPY increases heroin self-administration, and re-establishes extinguished heroin-seeking behaviour, in rats (Maric et al., 2008). NPY may also have a role in mediating the rewarding effect of food, as central administration of a Y1 receptor antagonist inhibits high fat feeding induced by injection of a μ -opioid receptor agonist into the NAc (Zheng et al., 2010).

3.1.8.3. The melanocortin system

POMC and AgRP immunoreactive fibres project widely to numerous sites within the CNS, including the VTA and NAc shell (Bagnol et al., 1999). The MC4R (Mountjoy et al., 1994) and MC3R (Roselli-Reh fuss et al., 1993) are expressed within the VTA and NAc.

There is evidence to suggest that the melanocortin system may have a role in mediating reward-related behaviours, although studies investigating drug reward have produced conflicting results. ICV injection of α -MSH has been shown to antagonise the addictive properties of opiates in mice (Contreras and Takemori, 1984). However, a more recent study reported that infusion of a melanocortin receptor antagonist into the NAc blocked the reinforcing effects of cocaine (Hsu et al., 2005).

It has also been suggested that the melanocortin system may interact with reward pathways to influence feeding. Mice lacking the MC4R are hyperphagic and show increased motivation to obtain food compared to wild-type controls (Vaughan et al., 2006). Additionally, animals will work harder to obtain a high-fat reward, but not sucrose, following ICV administration of AgRP, suggesting that AgRP may specifically increase the rewarding value of high-fat foods (Tracy et al., 2008).

Fos protein is coded for by the immediate early gene *c-fos*, and is used as an indirect marker of neuronal activation. ICV administration of AgRP induces fos-like immunoreactivity in midbrain

dopamine neurones (Davis et al., 2010), the NAc shell, and the lateral hypothalamus (Hagan et al., 2001). Additionally, ICV AgRP administration increases dopamine turnover within the forebrain (Davis et al., 2010). This suggests that AgRP may influence mesolimbic dopamine pathways and activate areas involved in reward, whilst stimulating food intake. However, little is known about the role of melanocortin signalling within the VTA in the control of food intake, and whether melanocortins directly influence VTA neurones to regulate food intake.

3.1.9. Hypothesis

Administration of melanocortin receptor agonists and antagonists directly into the VTA will influence feeding behaviour.

3.1.10. Aims

I aim to investigate the role of the VTA melanocortin system in the control of food intake. In order to do this I will determine:

- The effect of intra-VTA administration of NDP-MSH, AgRP and a selective MC4R antagonist on food intake in male Wistar rats.
- The effect of intra-VTA NDP-MSH administration on behaviour in male Wistar rats.
- Possible mechanisms by which the VTA melanocortin system may influence food intake.

3.2. Methods

3.2.1. Materials

AgRP (86-132) was supplied by The Peptide Institute (Osaka, Japan). *Cis*-(Z)-Flupenthixol dihydrochloride, a dopamine receptor antagonist, and bicuculline methiodide, a GABA_A receptor antagonist, were supplied by Sigma-Aldrich. All other peptides used were supplied by Bachem.

3.2.2. Animals

Adult male Wistar rats (Charles River) were maintained in individual cages as described in section 2.2.2.

3.2.3. Verification of intra-VTA cannula placement

3.2.3.1. Ink injections

Prior to intra-VTA cannulation, suitable stereotactic coordinates were identified using ‘The Rat Brain in Stereotactic Co-ordinates’ (Paxinos and Watson, 2007) and verified by injection of 1µl 10% dilution of India ink in water.

Male Wistar rats (n=6) were terminally anaesthetised with intraperitoneal 200mg/kg Pentobarbitone (Animalcare Ltd., Dunnington, Yorkshire, UK). Rats were positioned on a stereotactic frame (David Kopf Instruments, supplied by Clark Electromedical Instruments) with the incisor bar 3mm below the interaural line. A 1.5cm longitudinal incision was made in the scalp. A hole was drilled using a stereotactically mounted electric drill (Department of Medical Engineering, Imperial College London, UK). A 26-gauge cannula (Plastics One Inc.) was directed to the dopamine-rich PBP nucleus of the VTA and therefore implanted 6mm posterior to the bregma, 0.5mm lateral from the midline and 8.5mm below the surface of the skull. Each animal was injected unilaterally with ink via a 33-gauge steel injector (Plastics One Inc.) inserted into, and projecting 1mm beyond the tip of, the guide cannula.

Following injection, animals were removed from the frame and brains were rapidly dissected. Brains were fixed in a 4% formaldehyde solution (Appendix I) at 4°C overnight, then dehydrated in a 30% sucrose solution at room temperature for five days.

Brains were snap frozen in isopentane, and 30µm coronal slices taken using a sled microtome (Bright Instrument Company). Sections containing ink were stored in antifreeze (Appendix I) at -20°C. Slices were mounted onto polylysine coated slides (VWR) then counter-stained using cresyl violet (Section 3.2.3.2).

3.2.3.2 Cresyl violet staining of ink injected rat brains

Materials

Acetic acid (VWR)

Ethanol (VWR)

Cresyl violet stain (Appendix I)

Xylene (VWR)

DPX (Thermo Fisher Scientific, Epsom, Surrey, UK)

Method

Once mounted onto slides, brain sections containing India ink were allowed to dry for at least four hours. Slides were fixed in a solution of 5% acetic acid in 100% ethanol, and then stained in cresyl violet for 15 minutes. Slides were washed twice in GDW, dehydrated in 70% ethanol and delipidated in xylene overnight. The following morning, slides were mounted with DPX and coverslips. Animals with ink more than 0.2mm outside the VTA when viewed under the microscope (Nikon, Eclipse 50i) were classed as outliers.

3.2.4. VTA cannulation and injection

Cannulation surgery was carried out as described in section 2.2.3 except that for VTA cannulation, a 26-gauge stainless steel cannula (Plastics One Inc.) and different cannulation co-ordinates were used (see section 3.2.3).

Peptides or vehicle were administered to conscious, freely moving rats in a volume of 1µl via a 33-gauge stainless steel injector placed into, and projecting 1mm below, the tip of the cannula.

3.2.5. The effect of intra-VTA NDP-MSH administration on food intake in *ad libitum* fed rats in the early dark phase

Nle⁴, D-Phe⁷- α -MSH (NDP-MSH) is a potent, degradation resistant, synthetic analogue of α -MSH (Sawyer et al., 1980). *Ad libitum* fed rats were randomised into groups by body weight and injected into the VTA with either saline or 0.1, 0.3 or 1nmol NDP-MSH (n=7 per group) in the early dark phase (19:30-20:30). Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.6. The effect of intra-VTA administration of lower doses of NDP-MSH on food intake in *ad libitum* fed rats in the early dark phase

The doses of NDP-MSH administered in study 3.2.5 elicited a profound reduction in food intake. I therefore repeated the study using ten-fold lower doses, to determine the minimum effective dose of intra-VTA NDP-MSH.

Ad libitum fed rats were randomised into groups by body weight and injected into the VTA with either saline or 0.01, 0.03 or 0.1nmol NDP-MSH (n=7 per group) in the early dark phase. Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.7. The effect of intra-VTA NDP-MSH administration on food intake in fasted rats in the early light phase

The effect of intra-VTA NDP-MSH administration on fasting-induced re-feeding was studied to investigate whether the responsiveness of the VTA to melanocortins is altered by changes in nutritional status.

Overnight-fasted rats were randomised into groups by body weight and injected into the VTA with either saline or 0.03, 0.1 or 1nmol NDP-MSH (n=7 per group) in the early light phase. Following injection, rats were returned to their home cage with a pre-determined amount of food

and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.8. The effect of intra-VTA NDP-MSH administration on behaviour in *ad libitum* fed rats in the early dark phase

Behaviour was monitored following intra-VTA administration of NDP-MSH to determine whether the effects on food intake were secondary to behavioural changes.

Ad libitum fed rats were injected into the VTA with saline or 0.03nmol NDP-MSH (n=8 per group) in the early dark phase and returned to their home cages. This dose of NDP-MSH was chosen because it was the lowest to significantly reduce food intake in study 3.2.6.

Behaviour was continuously monitored for thirty minutes by two observers blinded to the experimental treatment using methods adapted from Fray *et al* (Fray et al., 1980; Scott et al., 2005). Each rat was observed for fifteen seconds every five minutes. Each fifteen second period was sub-divided into three five second periods and the behaviour of the rat was noted in each time period. Behaviour was classified into the following categories: feeding, drinking, rearing, locomotion, grooming, burrowing, head down, sleeping and pica.

3.2.9. The effect of intra-VTA NDP-MSH administration on food intake following intra-peritoneal pre-treatment with a GABA_A receptor antagonist in the early dark phase

I hypothesised that the inhibitory effect of intra-VTA NDP-MSH administration on food intake may be mediated by stimulation of endogenous VTA GABAergic neurones, which tonically inhibit dopamine neurones. It has been suggested that this inhibition is mediated by GABA_A receptors (Sugita et al., 1992). I therefore used the GABA_A receptor antagonist bicuculline to determine whether the anorexigenic effect of intra-VTA NDP-MSH can be attenuated by GABA_A receptor antagonism. Bicuculline methiodide is a stable, water-soluble form of bicuculline.

Ad libitum fed rats were randomised by body weight and injected intra-peritoneally (IP) with either saline or 3mg/kg bicuculline methiodide fifteen minutes prior to onset of the dark phase.

This dose of bicuculline methiodide was chosen because it has previously been shown to not affect food intake in rats (Zarrindast et al., 1989).

Fifteen minutes later, rats were injected into the VTA with either saline or 0.03nmol NDP-MSH (n=6-7 per group). This dose of NDP was chosen because it was the lowest to significantly reduce food intake in study 3.2.6.

Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.10. The effect of intra-VTA AgRP administration on food intake in *ad libitum* fed rats in the early light phase

Ad libitum fed rats were randomised into groups by body weight and injected into the VTA with either saline or 0.1, 0.3 or 1nmol AgRP (n=9-11 per group) in the early light phase. Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.11. The effect of intra-VTA AgRP administration on food intake following intra-peritoneal pre-treatment with a dopamine receptor antagonist in the early light phase

In order to determine whether VTA dopamine neurones mediate the orexigenic effect of intra-VTA AgRP administration, I treated rats with a non-specific dopamine receptor antagonist prior to intra-VTA AgRP administration.

Ad libitum fed rats were randomised by body weight and injected IP with either saline or 0.15mg/kg *Cis*-(Z)-Flupenthixol dihydrochloride, a non-specific dopamine receptor antagonist, in the early light phase. This dose has previously been shown not to induce motor impairments in rats (Cousins et al., 1994). Fifteen minutes later, rats were injected into the VTA with either saline or 0.3nmol AgRP (n=10 per group). This dose of AgRP was chosen because it robustly increased food intake in study 3.2.10.

Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.12. The effect of intra-VTA administration of JKC 363, an MC4R antagonist, on food intake in *ad libitum* fed rats in the early light phase

Ad libitum fed rats were randomised into groups by body weight and injected into the VTA with either saline or 0.1, 0.3 or 1nmol JKC 363 (n=9 per group) in the early light phase. Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.13. The effect of intra-VTA JKC 363 administration on food intake following intra-peritoneal pre-treatment with a dopamine receptor antagonist in the early light phase

I treated rats with a dopamine receptor antagonist prior to intra-VTA JKC 363 administration to determine whether VTA dopamine neurones act downstream of the MC4R to stimulate food intake.

Ad libitum fed rats were randomised by body weight and injected IP with either saline or 0.15mg/kg *Cis*-(Z)-Flupenthixol dihydrochloride in the early light phase. Fifteen minutes later, rats were injected into the VTA with either saline or 1nmol JKC 363 (n=7-8 per group). This dose of JKC 363 was chosen because it significantly increased 0-1 hour food intake in study 3.2.12.

Following injection, rats were returned to their home cage with a pre-determined amount of food and free access to water. Food was re-weighed at 1, 2, 4, 8 and 24 hours post-injection. Body weight was measured at 24 hours post-injection.

3.2.14. Statistics

All data are presented as mean $\pm$ standard error of the mean (SEM), other than data from behavioural studies which are expressed as median and inter-quartile range. Food intake studies were analysed using a one-way analysis of variance (ANOVA) with Tukey's post-hoc test (GraphPad Prism version 5 for Windows; GraphPad Software, San Diego, CA). Behavioural studies were analysed using the Kruskal-Wallis test (Systat, Evanston, IL) as data were non-parametric. In all cases p values <0.05 were taken as significant.

3.3. Results

3.3.1. Verification of intra-VTA cannula placement

Prior to intra-VTA cannulation, correct coordinates were confirmed with ink injections. Ink was observed unilaterally in the PBP of the VTA in 83% of animals (n=6). A representative photograph is shown in Figure 3.3A.

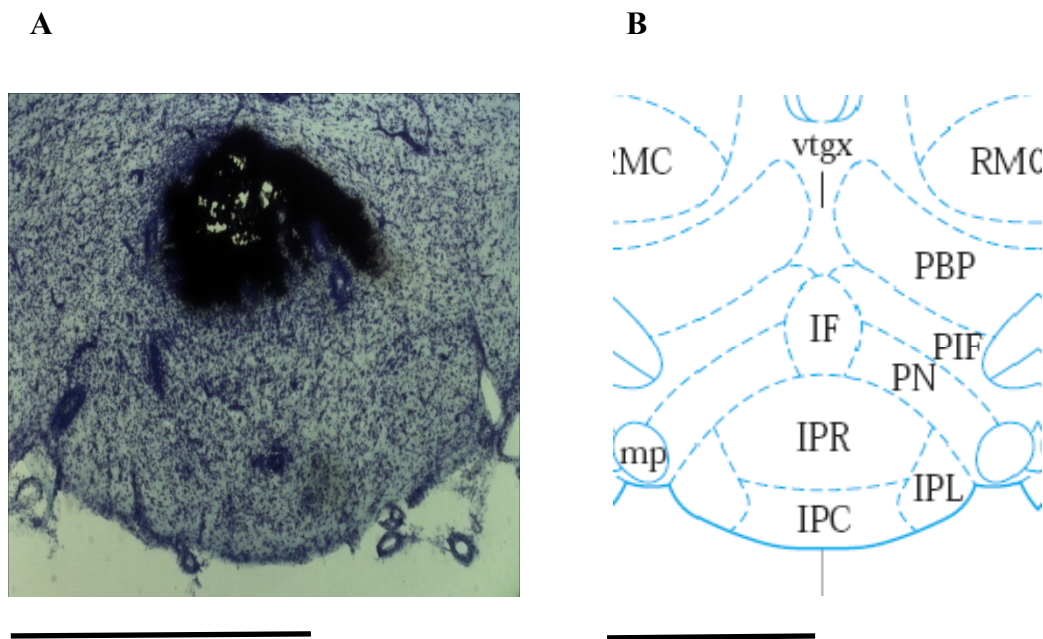


Figure 3.3: Confirmation of the parabrachial pigmented nucleus (PBP) coordinates using ink injections. Rats were injected unilaterally with India ink (coordinates 6mm posterior to the bregma, 0.5mm lateral from the midline and 9.5mm below the surface of the skull). Brain sections were stained with cresyl violet (**A**), and compared to ‘The Rat Brain in Stereotaxic Coordinates’ (Paxinos and Watson, 2007) (**B**). A coronal section at -6mm from bregma is shown. Scale bar is equivalent to 1mm. Abbreviations: IF, interfascicular nucleus; IPC, interpeduncular nucleus, caudal part; IPL, interpeduncular nucleus, lateral part; IPR, interpeduncular nucleus, rostral part; PIF, parainterfascicular nucleus of the VTA; PN, paranigral nucleus of the VTA; vtgx, VTA decussation.

3.3.2. The effect of intra-VTA NDP-MSH administration on food intake in *ad libitum* fed rats in the early dark phase

Intra-VTA administration of 0.1, 0.3 and 1.0nmol NDP-MSH to *ad libitum* fed rats in the early dark phase significantly reduced food intake in the first hour after injection when compared to saline injected controls (0-1 hour food intake (g): 2.29 ± 0.34 [saline]; 0.41 ± 0.15 [0.1nmol NDP-MSH] $p < 0.001$ vs. saline; 0.38 ± 0.07 [0.3nmol NDP-MSH] $p < 0.001$ vs. saline; 0.17 ± 0.02 [1nmol NDP-MSH] $p < 0.001$ vs. saline, $n=7$ per group) (Figure 3.4A). This reduction was maintained at 1-2 hours post-injection (1-2 hour food intake (g) 2.79 ± 0.25 [saline]; 1.03 ± 0.35 [0.1nmol NDP-MSH] $p < 0.01$ vs. saline; 0.43 ± 0.13 [0.3nmol NDP-MSH] $p < 0.001$ vs. saline; 0.78 ± 0.34 [1nmol NDP-MSH] $p < 0.001$ vs. saline, $n=7$ per group) (Figure 3.4B).

All doses of NDP-MSH also induced a significant reduction in food intake between 2 and 4 hours post injection (2-4 hour food intake (g) 4.63 ± 0.65 [saline]; 0.92 ± 0.34 [0.1nmol NDP-MSH] $p < 0.001$ vs. saline; 0.76 ± 0.27 [0.3nmol NDP-MSH] $p < 0.001$ vs. saline; 1.96 ± 0.65 [1nmol NDP-MSH] $p < 0.01$ vs. saline, $n=7$ per group) (Figure 3.4C).

All doses of NDP-MSH had no significant effect on food intake at any other time points.

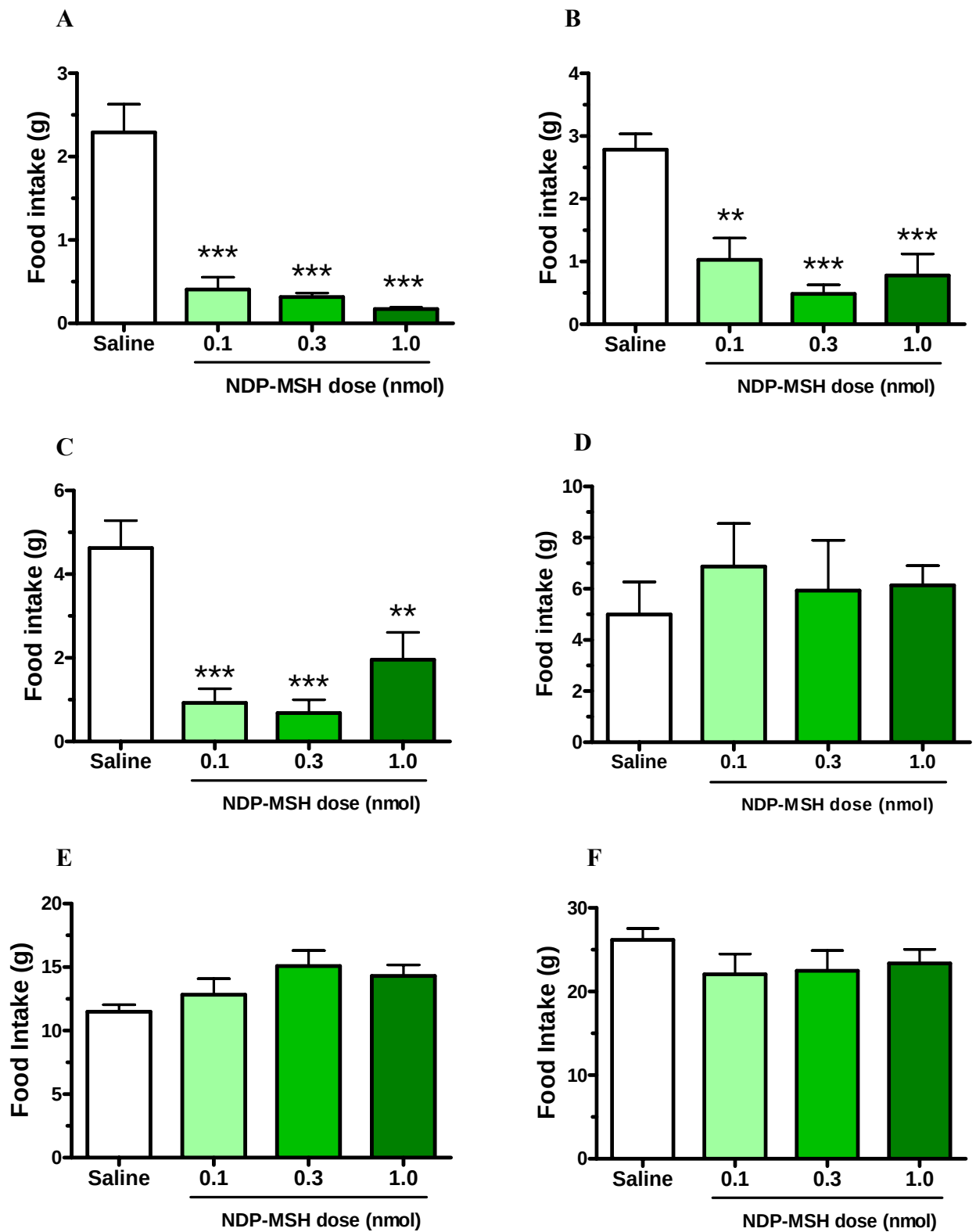


Figure 3.4: The effect of intra-VTA NDP-MSH administration on food intake in *ad libitum* fed rats in the early dark phase. Male Wistar rats received an intra-VTA injection of saline, 0.1, 0.3 or 1.0nmol NDP-MSH in the early dark phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=7 per group). **p<0.01, ***p<0.001 vs. saline.

3.3.3. The effect of intra-VTA administration of lower doses of NDP-MSH on food intake in *ad libitum* fed rats in the early dark phase

Intra-VTA administration of 0.03 and 0.1nmol NDP-MSH to *ad libitum* fed rats in the early dark phase significantly reduced food intake 0-1 hours post-injection compared to saline injected controls (0-1 hour food intake (g): 2.16 ± 0.23 [saline]; 0.93 ± 0.42 [0.03nmol NDP-MSH] $p < 0.05$ vs. saline; 0.51 ± 0.21 [0.1nmol NDP-MSH] $p < 0.01$ vs. saline, $n=7$ per group) (Figure 3.5A).

These doses of NDP-MSH also significantly reduced food intake 2-4 hours post-injection (2-4 hour food intake (g): 4.02 ± 0.47 [saline]; 1.46 ± 0.80 [0.03nmol NDP-MSH] $p < 0.05$ vs. saline; 1.28 ± 0.84 [0.1nmol NDP-MSH] $p < 0.05$ vs. saline, $n=7$ per group) (Figure 3.5C).

Additionally, intra-VTA administration of 0.03nmol NDP-MSH significantly reduced food intake between 4 and 8, and 0 and 24 hours post-injection (4-8 hour food intake (g): 8.22 ± 0.76 [saline] vs. 3.38 ± 1.24 [0.03nmol NDP-MSH] $p < 0.01$ (Figure 3.5D); 0-24 hour food intake (g): 27.41 ± 0.81 [saline] vs. 18.24 ± 2.71 [0.03nmol NDP-MSH] $p < 0.01$, $n=7$ per group (Figure 3.5F)).

All doses of NDP-MSH had no significant effect on food intake at any other time points.

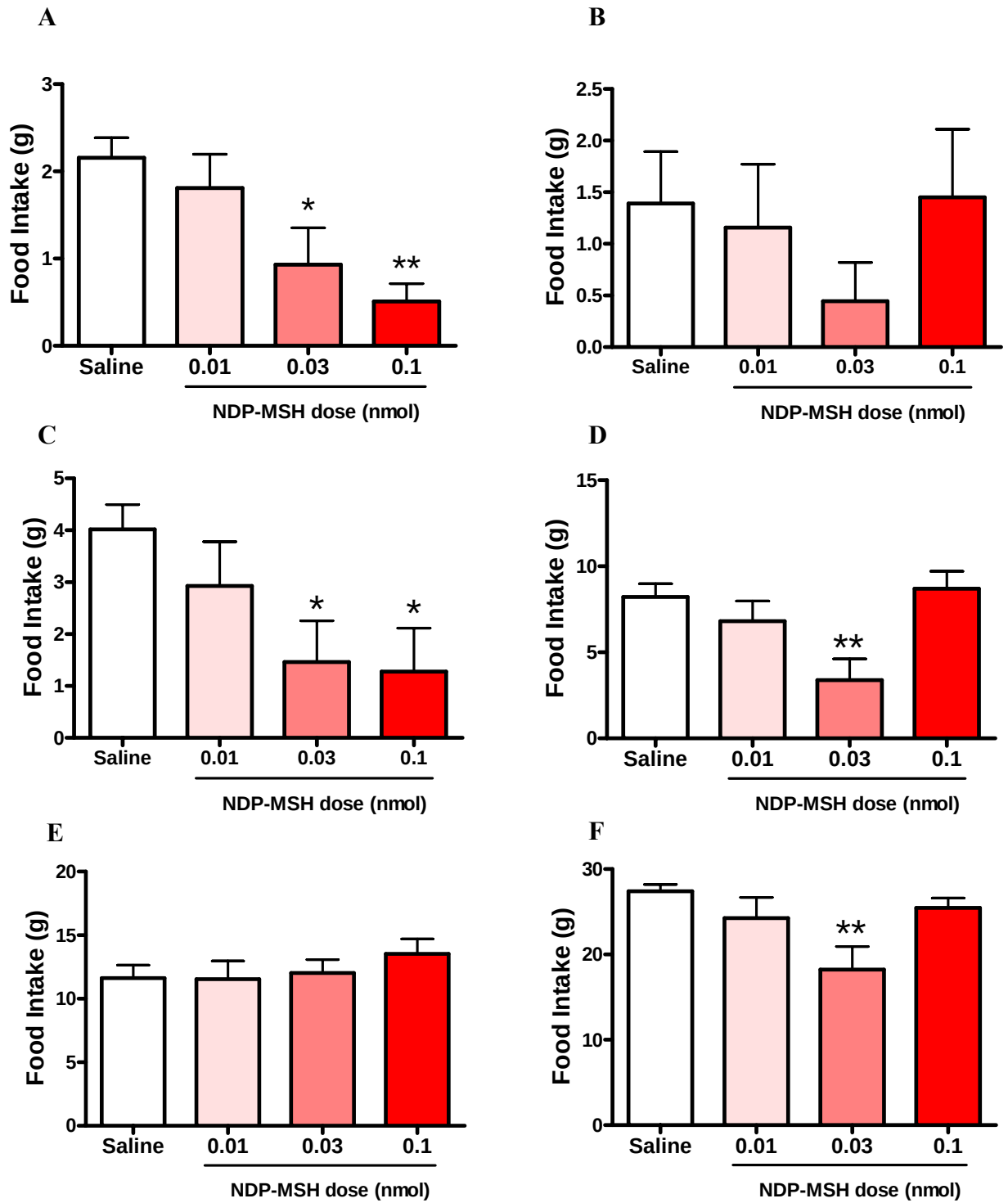


Figure 3.5: The effect of intra-VTA NDP-MSH administration on food intake in *ad libitum* fed rats in the early dark phase. Male Wistar rats received an intra-VTA injection of saline, 0.01, 0.03 or 0.1nmol NDP-MSH in the early dark phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=7 per group). *p<0.05, **p<0.01 vs. saline.

3.3.4. The effect of intra-VTA NDP-MSH administration on food intake in fasted rats in the early light phase

Intra-VTA administration of 0.03, 0.1 and 1nmol NDP-MSH to overnight fasted rats in the early light phase significantly reduced food intake between 2 and 4 hours post-injection (2-4 hour food intake (g): 3.80 ± 1.06 [saline]; 1.19 ± 0.47 [0.03nmol NDP-MSH] $p < 0.05$ vs. saline; 0.62 ± 0.51 [0.1nmol NDP-MSH] $p < 0.05$ vs. saline; 0.80 ± 0.43 [1nmol NDP-MSH] $p < 0.05$ vs. saline, $n=7$ per group) (Figure 3.6C).

All doses of NDP-MSH had no significant effect on food intake at any other time points.

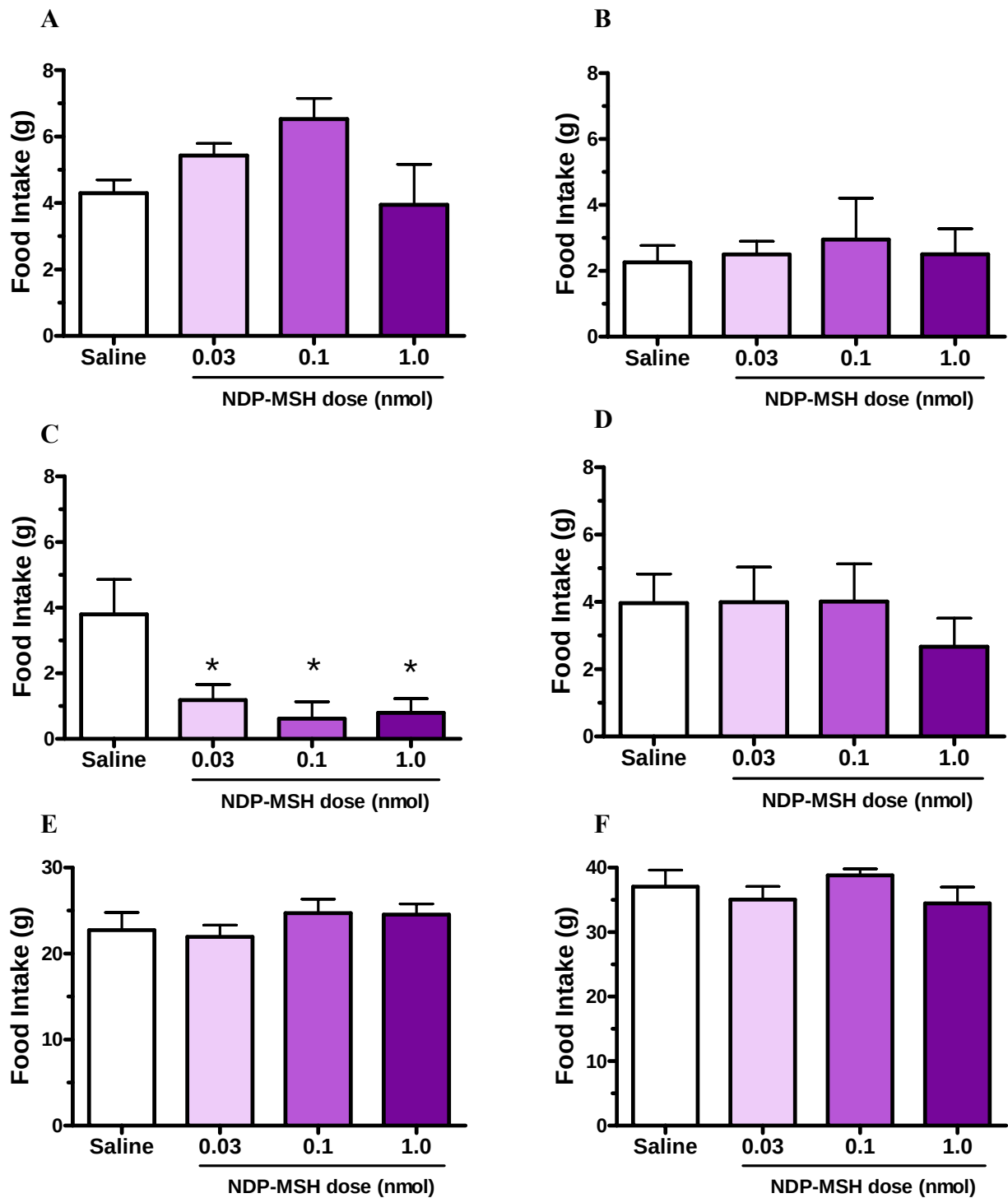


Figure 3.6: The effect of intra-VTA NDP-MSH administration on food intake in fasted rats in the early light phase. Male Wistar rats received an intra-VTA injection of saline, 0.03, 0.1 or 1nmol NDP-MSH in the early dark phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=7 per group). *p<0.05 vs. saline.

3.3.5. The effect of intra-VTA NDP-MSH administration on behaviour in *ad libitum* fed rats in the early dark phase

Intra-VTA administration of 0.03nmol NDP-MSH to *ad libitum* fed male Wistar rats in the early dark phase significantly reduced head down behaviour compared to saline injected controls ($p<0.05$, $n=8$ per group) (Table 3.1).

Behaviour	Saline	0.03nmol NDP-MSH
Feeding	3.0 (1.5-3.5)	1.0 (0.5-4.5)
Drinking	0.0 (0.0-2.5)	0.0 (0.0-1.5)
Rearing	4.0 (3.5-5.5)	3.5 (2.0-5.5)
Locomotion	2.5 (2.0-3.0)	2.5 (1.5-3.5)
Grooming	4.5 (3.5-6.5)	6.5 (5.0-10.0)
Burrowing	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Head down	2.5 (1.0-3.0)	1.0 (0.5-1.0)*
Sleep	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Pica	0.0 (0.0-0.0)	0.0 (0.0-0.0)

Table 3.1: The effect of intra-VTA NDP-MSH administration on behaviour in *ad libitum* fed rats during the early dark phase. Behaviour was monitored for thirty minutes post-injection. Data is presented as median number of behaviours observed (inter-quartile range), $n=8$ per group; * $p<0.05$ vs. saline.

3.3.6. The effect of intra-VTA NDP-MSH administration on food intake following intra-peritoneal pre-treatment with a GABA_A receptor antagonist in the early dark phase

Intra-VTA administration of 0.03nmol NDP-MSH significantly reduced food intake between 0 and 2 hours post-injection in *ad libitum* fed rats compared to saline injected controls. IP administration of 3mg/kg bicuculline-methiodide had no significant effect on food intake. Administration of 3mg/kg bicuculline-methiodide fifteen minutes prior to injection of 0.03nmol NDP-MSH partially attenuated the anorexigenic effect of NDP-MSH (0-2hr food intake (g): 4.23 ± 0.56 [saline/saline]; 4.05 ± 0.37 [bicuculline/saline]; 1.92 ± 0.62 [saline/NDP-MSH 0.03nmol] $p < 0.05$ vs. saline/saline; 3.27 ± 0.64 [bicuculline/NDP-MSH 0.03nmol] $n = 6-7$ per group) (Figure 3.7).

No significant differences in food intake were observed between groups at any other time points.

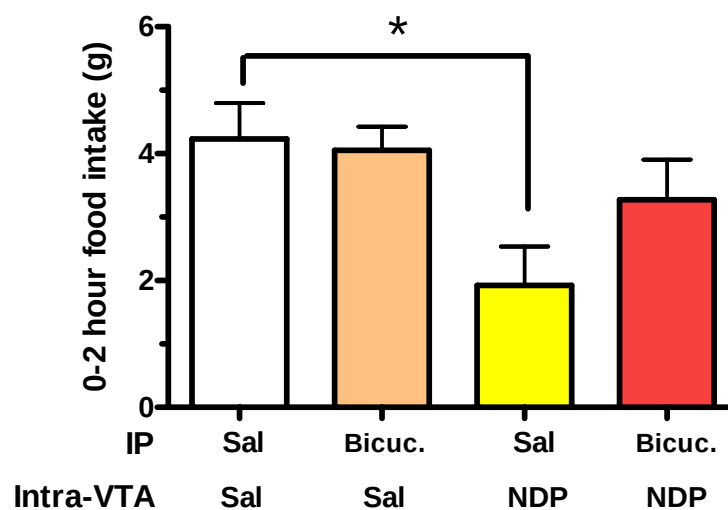


Figure 3.7: The effect of intra-VTA NDP-MSH administration on food intake following intra-peritoneal pre-treatment with a GABA_A receptor antagonist in the early dark phase. *Ad libitum* fed male Wistar rats were injected IP with saline or 3mg/kg bicuculline methiodide in the early dark phase fifteen minutes prior to intra-VTA administration of saline or 0.03nmol NDP-MSH. Food intake, presented as mean $\pm$ SEM, at 0-2 hours post injection is shown ($n = 6-7$ per group). * $p < 0.05$ vs. saline/saline.

3.3.7. The effect of intra-VTA AgRP administration on food intake and body weight in *ad libitum* fed rats in the early light phase

Administration of 1nmol AgRP intra-VTA to *ad libitum* fed rats in the early light phase significantly increased food intake between 1 and 2 hours post-injection (1-2 hour food intake (g): 0.89 ± 0.30 [saline] vs. 2.81 ± 0.65 [1nmol AgRP] $p < 0.05$, $n=9-11$ per group) (Figure 3.8B).

All doses of AgRP significantly increased food intake between 4 and 8 hours following intra-VTA injection (4-8 hour food intake (g): 0.71 ± 0.35 [saline]; 2.55 ± 0.34 [0.1nmol AgRP] $p < 0.05$ vs. saline; 3.17 ± 0.60 [0.3nmol AgRP] $p < 0.01$ vs. saline; 3.06 ± 0.78 [1nmol AgRP] $p < 0.05$ vs. saline, $n=9-11$ per group) (Figure 3.8D).

Cumulative food intake was significantly increased between 0 and 2 hours following intra-VTA administration of 1nmol AgRP (0-2 hour food intake (g): 2.03 ± 0.50 [saline] vs. 4.94 ± 1.21 [1nmol AgRP] $p < 0.05$, $n=9-11$ per group) (Figure 3.9). Intra-VTA injection of 0.1 and 1nmol significantly increased cumulative food intake between 0 and 4 hours compared to saline controls (0-4 hour food intake (g): 2.89 ± 0.62 [saline]; 5.25 ± 0.62 [0.1nmol AgRP] $p < 0.05$ vs. saline; 6.28 ± 0.99 [1nmol AgRP] $p < 0.01$ vs. saline, $n=9-11$ per group) (Figure 3.9). Additionally, all doses of AgRP induced a significant rise in food intake between 0 and 8 hours after injection (0-8 hour food intake (g): 3.60 ± 0.89 [saline]; 7.81 ± 0.62 [0.1nmol AgRP] $p < 0.001$ vs. saline; 7.03 ± 1.42 [0.3nmol AgRP] $p < 0.01$ vs. saline; 9.34 ± 0.68 [1nmol AgRP] $p < 0.001$ vs. saline, $n=9-11$ per group) (Figure 3.9). The highest dose of AgRP administered, 1nmol, also significantly increased 0-24 hour food intake (0-24 hour food intake (g): 26.61 ± 2.93 [saline] vs. 38.05 ± 1.45 [1nmol AgRP] $p < 0.01$, $n=9-11$ per group) (Figure 3.8F).

There was a dose-dependent increase in body weight change at 24 hours following intra-VTA AgRP administration (0-24 hour body weight change (g): -9.9 ± 3.2 [saline]; 3.5 ± 2.6 [0.1nmol AgRP] $p < 0.01$ vs. saline; 5.2 ± 2.3 [0.3nmol AgRP] $p < 0.001$ vs. saline; 9.3 ± 1.7 [1nmol AgRP] $p < 0.001$ vs. saline, $n=9-11$ per group) (Figure 3.10).

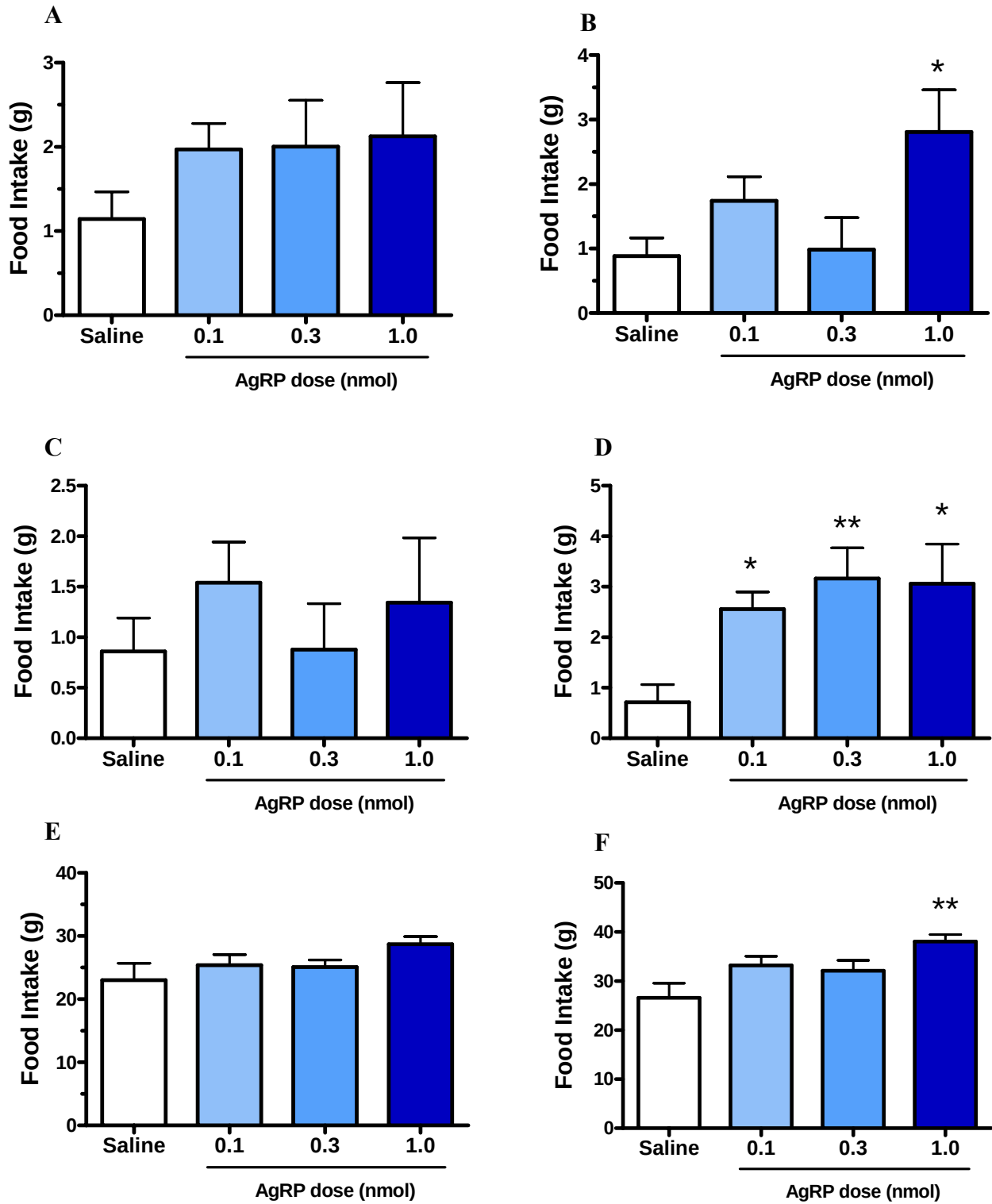


Figure 3.8: The effect of intra-VTA AgRP administration on food intake in *ad libitum* fed rats in the early light phase. Male Wistar rats received an intra-VTA injection of saline, 0.1, 0.3 or 1nmol AgRP in the early light phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=9-11 per group). * p <0.05, ** p <0.01 vs. saline.

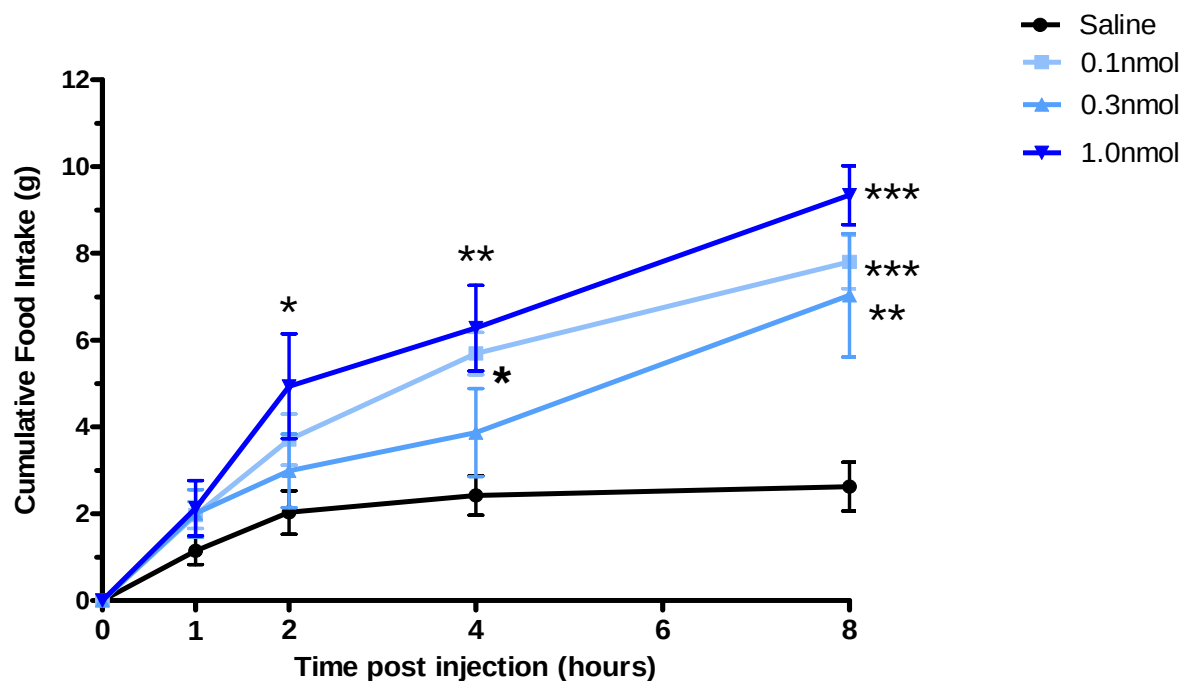


Figure 3.9: The effect of intra-VTA AgRP administration on cumulative food intake in *ad libitum* fed rats in the early light phase. Male Wistar rats received an intra-VTA injection of saline, 0.1, 0.3 or 1nmol AgRP in the early light phase. Food intake expressed as mean (g) $\pm$ SEM at 0, 1, 2, 4 and 8 hours post injection is shown (n=9-11 per group). * p <0.05, ** p <0.01, *** p <0.001 vs. saline.

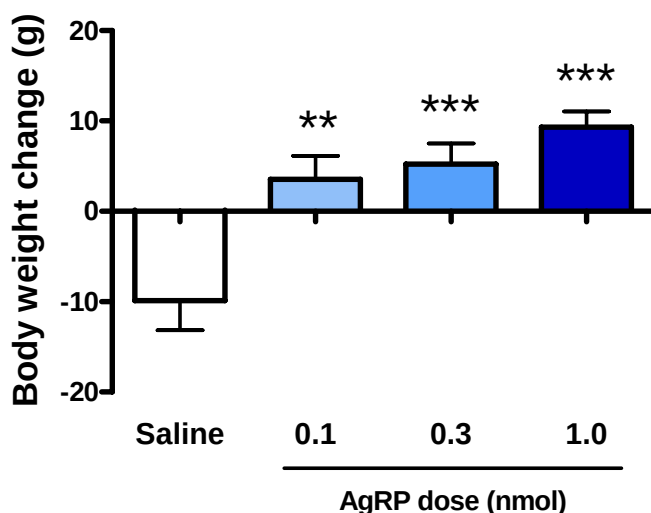


Figure 3.10: The effect of intra-VTA AgRP administration on 24-hour body weight change in *ad libitum* fed rats in the early light phase. Male Wistar rats received an intra-VTA injection of saline, 0.1, 0.3 or 1nmol AgRP in the early light phase. Body weight change expressed as mean (g) $\pm$ SEM at 24 hours post injection is shown (n=9-11 per group). ** p <0.01, *** p <0.001 vs. saline.

3.3.8. The effect of intra-VTA AgRP administration on food intake following intra-peritoneal pre-treatment with a dopamine receptor antagonist in the early light phase

Intra-VTA administration of 0.3nmol AgRP significantly increased food intake between 4 and 8 hours post-injection in *ad libitum* fed rats compared to saline injected controls. Administration of 0.15mg/kg dopamine receptor antagonist fifteen minutes prior to injection of 0.3nmol AgRP attenuated the orexigenic effect of AgRP (4-8hr food intake (g): 1.36 ± 0.40 [saline/saline]; 1.40 ± 0.34 [dopamine receptor antagonist /saline]; 4.33 ± 0.76 [saline/AgRP 0.3nmol] $p < 0.01$ vs. saline/saline and dopamine receptor antagonist/saline; 3.10 ± 0.48 [dopamine receptor antagonist/AgRP 0.3nmol] $n=10$ per group) (Figure 3.11).

A similar trend was observed in cumulative food intake between 0 and 8 hours post-injection. Intra-VTA administration of 0.3nmol AgRP significantly increased food intake compared to saline injected controls, and this effect was partly attenuated by pre-administration of 0.15mg/kg dopamine receptor antagonist (0-8hr food intake (g): 4.25 ± 0.42 [saline/saline]; 3.61 ± 0.72 [dopamine receptor antagonist/saline]; 9.06 ± 0.10 [saline/AgRP 0.3nmol] $p < 0.01$ vs. saline/saline, $p < 0.001$ vs. dopamine receptor antagonist/saline; 7.20 ± 1.23 [dopamine receptor antagonist/AgRP 0.3nmol] $n=10$ per group) (data not shown).

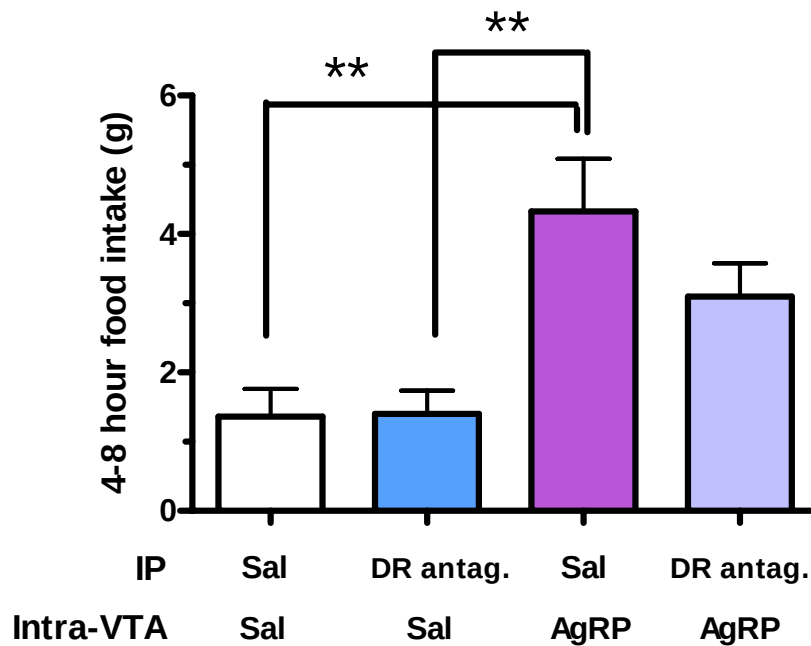


Figure 3.11: The effect of pre-treatment with a dopamine receptor antagonist on AgRP-induced food intake in *ad libitum* fed rats in the early light phase. *Ad libitum* fed male Wistar rats were injected IP with saline or 0.15mg/kg dopamine receptor antagonist in the early light phase fifteen minutes prior to intra-VTA administration of saline or 0.3nmol AgRP. Food intake, presented as mean $\pm$ SEM, at 4-8 hours post-injection is shown (n=10 per group). **p<0.01 vs. saline/saline and dopamine receptor antagonist/saline.

3.3.9. The effect of intra-VTA administration of JKC 363, an MC4R antagonist, on food intake in *ad libitum* fed rats in the early light phase

Intra-VTA administration of 0.3 and 1nmol JKC 363 significantly increased food intake in a dose-dependent manner in the first hour following injection compared to saline controls (0-1 hour food intake (g): 1.42 ± 0.28 [saline]; 3.03 ± 0.62 [0.3nmol JKC] $p < 0.05$ vs. saline; 3.09 ± 0.33 [1nmol JKC] $p < 0.05$ vs. saline, $n=9$ per group) (Figure 3.12A).

JKC 363 administered at the lowest dose of 0.1nmol significantly increased food intake between 4 and 8 hours (4-8 hour food intake (g): 1.01 ± 0.21 [saline]; 2.67 ± 0.35 [0.1nmol JKC] $p < 0.05$ vs. saline, $n=9$ per group) (Figure 3.12D).

Additionally, cumulative food intake was significantly increased between 0 and 2, and 0 and 4 hours following injection of 1nmol JKC 363 (0-2 hour food intake (g): 2.17 ± 0.57 [saline]; 4.58 ± 0.56 [1nmol JKC] $p < 0.05$ vs. saline; 0-4 hour food intake (g): 2.89 ± 0.65 [saline]; 5.83 ± 0.81 [1nmol JKC] $p < 0.05$ vs. saline, $n=9$ per group) (Figure 3.13). All doses of JKC 363 significantly increased food intake between 0 and 8 hours post-injection (0-8 hour food intake (g): 3.90 ± 0.52 [saline]; 7.00 ± 0.76 [0.1nmol JKC] $p < 0.05$ vs. saline; 7.16 ± 0.58 [0.3nmol JKC] $p < 0.05$ vs. saline; 8.14 ± 1.12 [1nmol JKC] $p < 0.01$ vs. saline, $n=9$ per group) (Figure 3.13).

All doses of JKC 363 had no significant effect on food intake at any other time points.

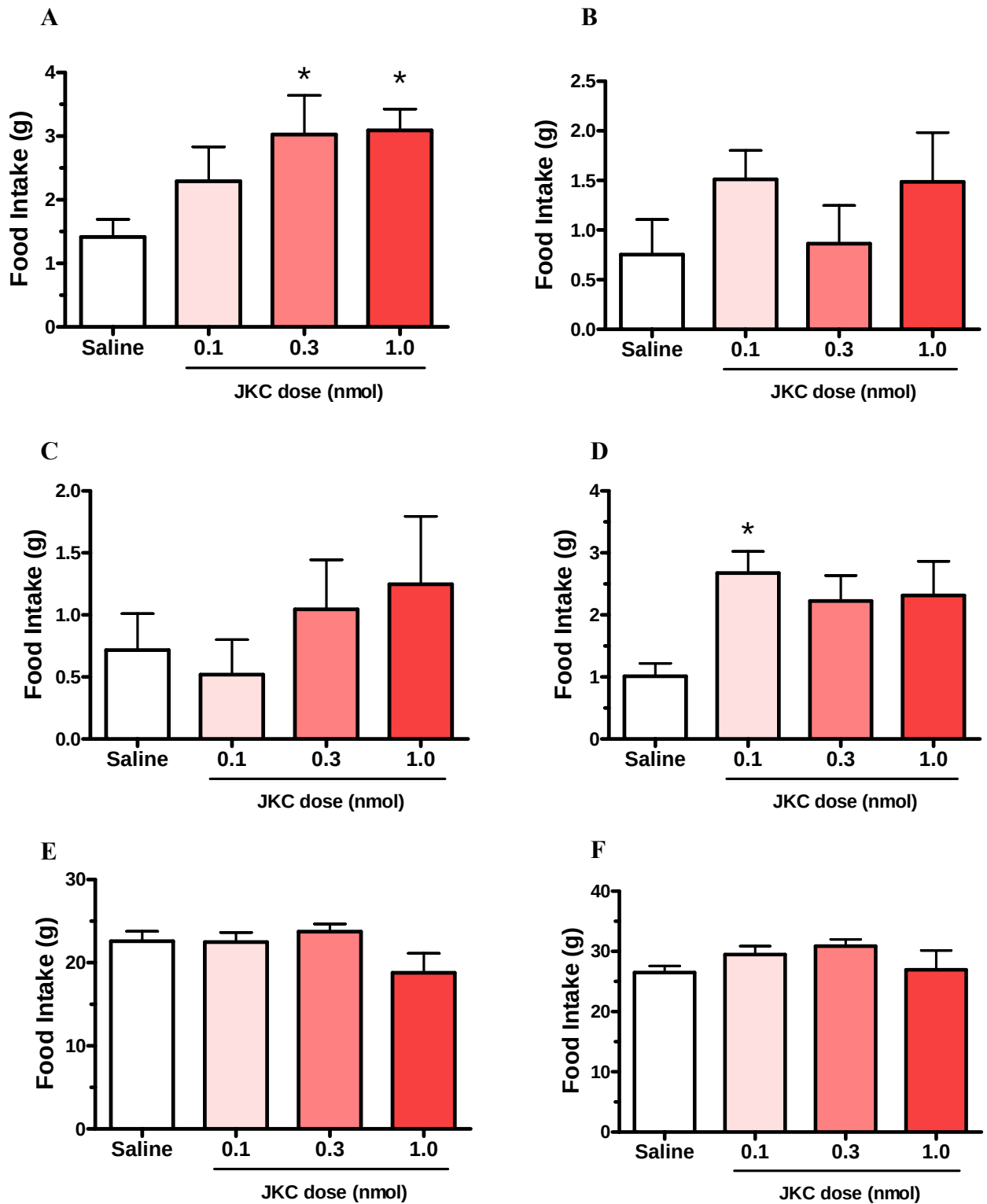


Figure 3.12: The effect of intra-VTA JKC 363 administration on food intake in *ad libitum* fed rats in the early light phase. Male Wistar rats received an intra-VTA injection of saline, 0.1, 0.3 or 1nmol JKC 363 in the early light phase. Food intake expressed as mean (g) $\pm$ SEM at (A) 0-1, (B) 1-2, (C) 2-4, (D) 4-8, (E) 8-24 and (F) 0-24 hours post injection is shown (n=9 per group). *p<0.05 vs. saline.

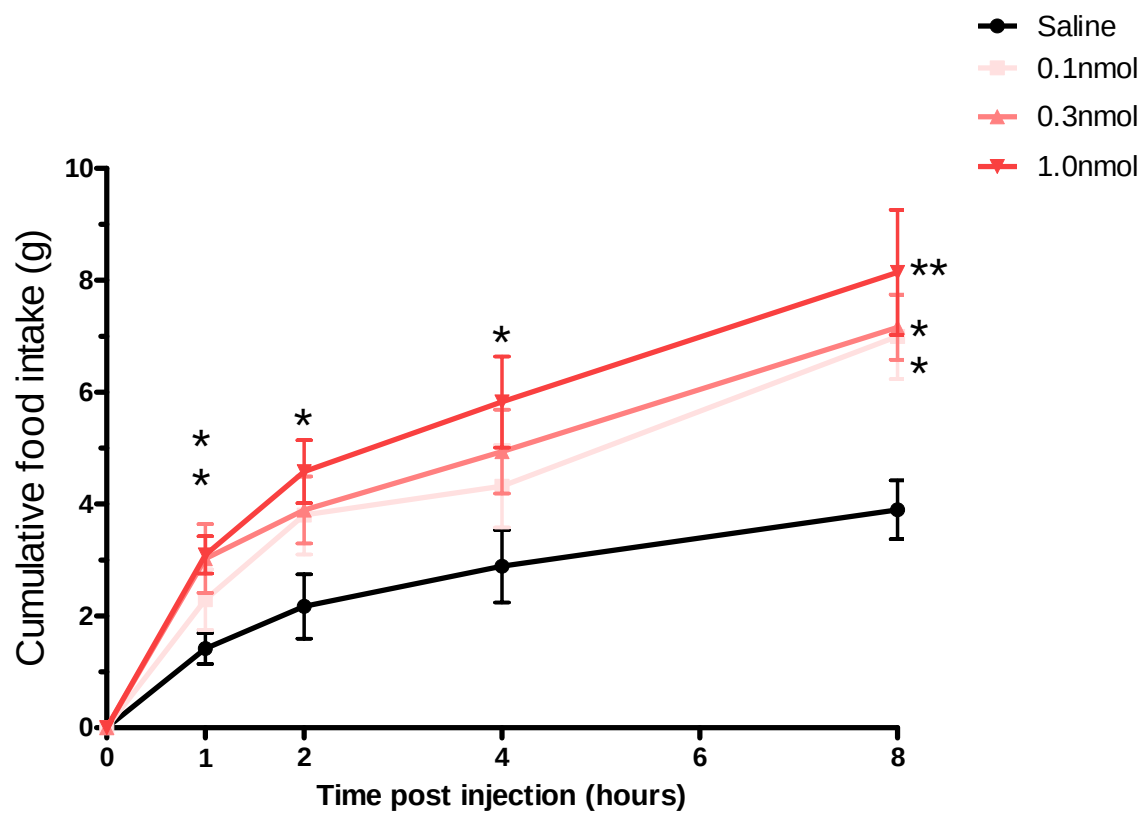


Figure 3.13: The effect of intra-VTA JKC 363 administration on cumulative food intake in *ad libitum* fed rats in the early light phase. Male Wistar rats received an intra-VTA injection of saline, 0.1, 0.3 or 1nmol JKC 363 in the early light phase. Food intake expressed as mean (g) $\pm$ SEM at 0, 1, 2, 4 and 8 hours post injection is shown (n=9 per group). *p<0.05, **p<0.01 vs. saline.

3.3.10. The effect of intra-VTA JKC 363 administration on food intake following intra-peritoneal pre-treatment with a dopamine receptor antagonist in the early light phase

Intra-VTA administration of 1nmol JKC 363 significantly increased food intake between 1 and 2 hours post-injection in *ad libitum* fed rats compared to saline injected controls. Administration of 0.15mg/kg dopamine receptor antagonist fifteen minutes prior to injection of 1nmol JKC 363 significantly attenuated the orexigenic effect of JKC 363 (1-2hr food intake (g): 0.93 ± 0.25 [saline/saline]; 0.64 ± 0.24 [dopamine receptor antagonist/saline]; 3.09 ± 0.35 [saline/JKC 1nmol] $p < 0.001$ vs. saline/saline, dopamine receptor antagonist/saline and dopamine receptor antagonist/JKC; 0.81 ± 0.43 [dopamine receptor antagonist/JKC 1nmol] $n=7-8$ per group) (Figure 3.14A).

JKC 363 also significantly increased food intake between 2 and 4 hours post-injection, and this effect was partly attenuated by pre-treatment with a dopamine receptor antagonist (2-4hr food intake (g): 0.23 ± 0.18 [saline/saline]; 0.43 ± 0.23 [dopamine receptor antagonist/saline]; 2.02 ± 0.88 [saline/JKC 1nmol] $p < 0.05$ vs. saline/saline; 0.53 ± 0.29 [dopamine receptor antagonist/JKC 1nmol] $n=7-8$ per group) (Figure 3.14B).

Cumulative food intake was significantly increased following administration of JKC 363 between 0 and 2, 0 and 4, and 0 and 8 hours compared to saline injected controls. This effect was significantly attenuated by pre-treatment with a dopamine receptor antagonist (0-2hr food intake (g): 2.04 ± 0.47 [saline/saline]; 1.98 ± 0.45 [dopamine receptor antagonist/saline]; 5.65 ± 0.62 [saline/JKC 1nmol] $p < 0.01$ vs. saline/saline and dopamine receptor antagonist/saline, $p < 0.05$ vs. dopamine receptor antagonist/JKC; 2.74 ± 1.03 [dopamine receptor antagonist/JKC 1nmol]; 0-4hr food intake (g): 2.33 ± 0.54 [saline/saline]; 2.41 ± 0.52 [dopamine receptor antagonist/saline]; 7.67 ± 1.47 [saline/JKC 1nmol] $p < 0.01$ vs. saline/saline and dopamine receptor antagonist/JKC, $p < 0.05$ vs. dopamine receptor antagonist/saline; 3.71 ± 1.07 [dopamine receptor antagonist/JKC 1nmol]; 0-8hr food intake (g): 3.87 ± 0.87 [saline/saline]; 4.10 ± 0.94 [dopamine receptor antagonist/saline]; 9.49 ± 1.15 [saline/JKC 1nmol] $p < 0.01$ vs. saline/saline and dopamine receptor antagonist/saline, $p < 0.05$ vs. dopamine receptor antagonist/JKC; 5.27 ± 0.75 [dopamine receptor antagonist/JKC 1nmol], $n=7-8$ per group) (data not shown).

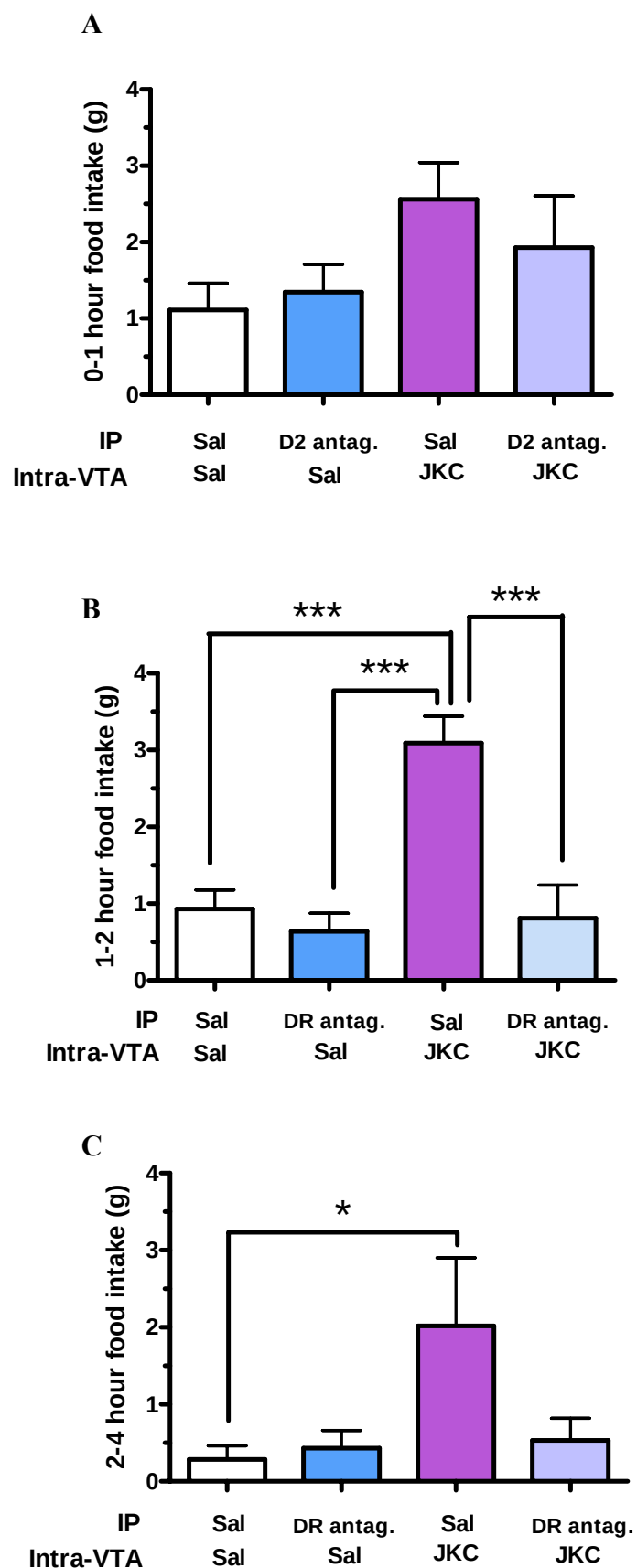


Figure 3.14: The effect of pre-treatment with a dopamine receptor antagonist on JKC 363-induced food intake in *ad libitum* fed rats in the early light phase. *Ad libitum* fed male Wistar rats were injected IP with saline or 0.15mg/kg dopamine receptor antagonist in the early light phase fifteen minutes prior to intra-VTA administration of saline or 1nmol JKC 363. Food intake, presented as mean $\pm$ SEM, at (A) 0-1, (B) 1-2 and (C) 2-4 hours post-injection is shown (n=7-8 per group). *p<0.05 and ***p<0.001.

3.4. Discussion

The VTA is part of the mesolimbic dopamine system, and is important in mediating the rewarding aspects of numerous substances, including food. The melanocortin system is classically thought to act within the hypothalamus to regulate energy homeostasis. However, melanocortin-expressing neurones extend to extra-hypothalamic regions including the VTA, and may influence dopamine reward pathways to modulate food intake.

The VTA contains POMC and AgRP immunoreactive fibres (Bagnol et al., 1999), and both the MC4R (Mountjoy et al., 1994) and MC3R (Roselli-Rehfuss et al., 1993) are expressed within the VTA. Although there is evidence to suggest that the melanocortin system may influence the rewarding properties of drugs of abuse (Contreras and Takemori, 1984; Hsu et al., 2005), few studies have focused on the effects of melanocortin receptor signalling within the VTA on feeding. My results suggest the presence of an endogenous melanocortin tone within the VTA which reduces food intake.

Intra-VTA administration of NDP-MSH (0.03, 0.1, 0.3 and 1.0nmol) significantly reduced food intake for up to 4 hours post-injection when administered to *ad libitum* fed rats in the early dark phase. The reduction in food intake was almost immediate, and the highest dose administered (1.0nmol) reduced food intake to approximately 10% of that of saline-injected controls in the first hour post-injection.

In contrast, injection of NDP-MSH to fasted rats in the early light phase had a delayed effect on food intake, which was significant only between 2 and 4 hours. No difference in food intake was observed at any other time points after injection compared to saline controls. It is interesting to compare the effect of NDP-MSH on food intake when administered into the VTA and the hypothalamus. When injected into discrete hypothalamic nuclei, including the PVN and DMN, 0.1nmol NDP-MSH significantly reduced food intake to approximately 50% of controls between 0 and 1 hours in fasted rats (Kim et al., 2000a). The same dose of NDP-MSH had no effect when injected into the VTA under similar fasted conditions. However, when administered to *ad libitum* fed rats, 0.1nmol NDP-MSH reduced food intake to approximately 20% of controls in the first hour after injection. This suggests that the VTA may be more sensitive to the anorectic effect of NDP-MSH than the hypothalamus, but only under free-feeding conditions. In the fasted state, homeostatic signals acting upon and within the hypothalamus may override signals from reward pathways to ensure that feeding behaviour is maintained.

The effect of intra-VTA NDP-MSH administration on behaviour was investigated to determine whether changes in food intake were secondary to changes in behaviour. NDP-MSH (0.03nmol) significantly reduced head-down behaviour in the first thirty minutes following injection compared to controls, suggesting an increase in activity and wakefulness. Intra-VTA administration of α -MSH has previously been reported to induce grooming behaviour in rats (Klusa et al., 1998). However, I did not observe any differences in grooming between groups. This may be because I used a much lower dose than previous studies (0.03nmol compared to 3nmol).

It is interesting that NDP-MSH reduced both head-down behaviour and food intake. Other neuropeptides reported to increase wakefulness, such as the orexins, stimulate feeding when administered ICV (Ida et al., 1999). It is unlikely that NDP-MSH reduced food intake secondary to nausea or malaise, as these symptoms are associated with an increase in head-down and hunching behaviour in rodents (Holmes et al., 2009). In addition, blockade of melanocortin receptor signalling by intra-VTA AgRP administration increased food intake, which suggests the presence of an endogenous melanocortin agonist tone in the VTA, and that the effects of NDP-MSH administration on food intake are due to the activation of a physiological circuit.

Within the VTA, GABAergic interneurons tonically inhibit the activity of dopamine neurones, mainly via GABA_A receptors (Matthews and German, 1984; Sugita et al., 1992). As an increase in dopamine transmission within the mesolimbic pathway is associated with feeding (Hernandez and Hoebel, 1988), it is likely that intra-VTA NDP-MSH exerts its anorexigenic effects either via direct inhibition of dopamine neurones, or via stimulation of GABAergic neurones and hence indirect inhibition of dopamine neuronal activity. In order to determine if GABAergic neurones signal downstream of melanocortin receptors in the VTA, I administered the GABA_A receptor antagonist bicuculline prior to intra-VTA injection of NDP-MSH.

Bicuculline partly attenuated the effect of NDP-MSH on feeding, although food intake was not completely restored to that observed in control animals. This suggests that GABA signalling may be important in mediating the anorectic effect of intra-VTA NDP-MSH. However, it is likely that other pathways also contribute. In addition to dopamine neurones and GABAergic interneurons, the VTA contains GABAergic and glutamatergic neuronal cell bodies which project to the NAc and pre-frontal cortex (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000; Lavin et al., 2005). These may also have a role in mediating the effects of intra-VTA NDP-MSH on food intake.

AgRP (0.1, 0.3 and 1.0nmol) administered into the VTA elicited a potent increase in food intake which was significant between 4 and 8, and 0 and 8 hours following injection. Additionally, the highest dose (1.0nmol) significantly increased food intake between 1 and 2, and 0 and 24 hours. AgRP is documented to have a delayed, but long-lasting effect on food intake. When administered into the PVN, AgRP significantly increased food intake at 4 hours, and this effect was maintained at 24 hours post-injection (Kim et al., 2000a). This time-course is similar to that observed in my studies. This delayed yet long-lasting effect may be due to the structure of AgRP. AgRP is very stable within the brain, and its structure has been suggested to render it relatively resistant to degradation by peptidases (Kastin et al., 2000; Lu et al., 2001). It has also been shown that the AgRP molecule is prone to aggregation, which may slow its diffusion time to its target sites, resulting in a delayed onset of action (Kastin et al., 2000; Kim et al., 2002). Alternatively, AgRP may alter the properties of target neurones, or activate downstream circuits (Hagan et al., 2001), to elicit its long-lasting orexigenic effects.

Intra-VTA administration of AgRP at all doses also significantly increased body weight at 24 hours post-injection compared to controls. This is likely due to an increase in water intake in AgRP-injected animals. Administration of AgRP into the third ventricle has previously been reported to increase water intake in proportion to food intake (Clegg et al., 2003).

I hypothesised that dopamine neurones may mediate the effects of intra-VTA AgRP administration on food intake. ICV administration of AgRP has been reported to increase Fos expression in midbrain dopamine neurones, and increase dopamine turnover in the forebrain (Davis et al., 2010). Feeding is associated with an increase in dopamine release in the NAc (Hernandez and Hoebel, 1988).

In order to investigate this I administered a dopamine receptor antagonist prior to intra-VTA injection of AgRP. Dopamine receptor antagonists have been reported to induce motor impairments, which may have a secondary effect on food intake. However, care was taken to select a dose of dopamine receptor antagonist which has previously been shown not to affect motor function (Cousins et al., 1994).

Pre-administration of a dopamine receptor antagonist partially attenuated the orexigenic effect of intra-VTA AgRP, suggesting that AgRP may partly increase food intake by activating VTA dopamine neurones. As the orexigenic effect of AgRP was not completely suppressed, the dose of antagonist administered may not have been sufficient to induce complete receptor antagonism.

Alternatively, other pathways may mediate the effect of AgRP on food intake, as discussed previously.

AgRP is an antagonist at both the MC3R and the MC4R. In order to determine which melanocortin receptor mediates the effect of intra-VTA AgRP on feeding, I administered JKC 363, a selective MC4R antagonist, into the VTA. The two highest doses of JKC 363 administered (0.3 and 1nmol) significantly increased food intake in the first hour post injection, and the lowest dose (0.1nmol) significantly increased food intake between 4 and 8 hours. This time course of action is different to that of AgRP, which had no acute effect. A similar discrepancy between the time course of AgRP and JKC 363 has been reported when both compounds were injected into the third ventricle (Kim et al., 2002). It is possible that the delayed action of AgRP is due to MC4R-independent pathways, or may reflect pharmacokinetic differences between the two compounds. It would be interesting to administer a selective MC3R antagonist intra-VTA and compare the effect on feeding to that of JKC 363. However, selective MC3R antagonists are difficult to obtain; most have some effects at the MC4R.

The increase in feeding induced by JKC 363 was completely abolished by pre-treatment with a dopamine receptor antagonist. This is in contrast to AgRP, where pre-treatment with a dopamine receptor antagonist only partially attenuated the orexigenic effect of AgRP. This suggests that dopamine neurones may predominantly mediate the effects of MC4R signalling, whereas the MC3R may act via alternative pathways, as discussed above.

My results tentatively suggest that melanocortin signalling within the VTA may stimulate GABAergic neurones, causing suppression of dopamine neuronal firing. Antagonism of melanocortin receptor signalling by AgRP may disinhibit dopamine neurones, leading to increased neuronal firing, increased dopamine release within the NAc and increased food intake.

More work is required to elucidate the mechanisms by which the VTA melanocortin system influences food intake. Co-localisation studies are necessary to determine whether the MC3R and MC4R are located on GABAergic and/or dopamine neurones in the VTA. It would be interesting to examine the effect of intra-VTA NDP-MSH and AgRP administration on dopamine release within the NAc using microdialysis, and the effect of AgRP on dopamine neuronal cell firing using electrophysiological techniques. Additionally, I would like to examine the pattern of fos-like immunoreactivity in response to intra-VTA AgRP administration. If staining was observed in areas downstream of the VTA, for example the NAc and pre-frontal

cortex, it would suggest that reward pathways are involved in mediating the orexigenic effect of AgRP.

It would also be interesting to determine the effect of intra-VTA NDP-MSH and AgRP administration on reward-related behaviours, for example sucrose self-administration and CPP. ICV administration of AgRP has been shown to enhance a CPP for high fat diet in rats (Davis et al., 2010), and increase motivational behaviour to obtain a high-fat reward (Tracy et al., 2008). However, no studies have focused on the effect of AgRP or NDP-MSH administration directly into the VTA or NAc on food reward.

In summary, my results suggest that melanocortin signalling within the VTA may play a physiological role in the regulation of food intake in rats.

Chapter 4: Investigating the role of arcuate kisspeptin signalling in the control of reproduction

4.1. Introduction

4.1.1. The hypothalamo-pituitary-gonadal axis

Fertility and energy homeostasis are intimately linked, and the hypothalamus plays a key role in integrating metabolic signals to ensure body fat reserves are sufficient to support reproduction. Conversely, gonadal steroid hormones have been shown to influence food intake and body weight (Bull et al., 1974; Asarian and Geary, 2006; Terao et al., 2004).

Fertility in mammals is maintained by the hypothalamo-pituitary-gonadal (HPG) axis. GnRH is released in a pulsatile pattern from the hypothalamus into the pituitary portal circulation, and stimulates the pulsatile release of LH and FSH from gonadotrophs in the anterior pituitary gland. LH and FSH circulate peripherally to stimulate the gonads, and ultimately control gonadal steroidogenesis and gametogenesis. Gonadal steroid feedback to the pituitary and hypothalamus ensures this system is tightly controlled. Recently, kisspeptin has been identified as an upstream regulator of GnRH, which is postulated to mediate the feedback of sex steroids and other signals onto GnRH neurones (d'Anglemont de Tassigny and Colledge, 2010).

4.1.2. The female reproductive cycle

4.1.2.1. The human menstrual cycle

Reproductive capacity in female mammals is maintained by repetitive cycles of follicle maturation and ovulation. In humans, the menstrual cycle is approximately 28 days long. During the follicular phase of the cycle, ovarian follicles develop and their surrounding cells secrete oestrogen in response to stimulation by LH and FSH. Oestrogen exerts negative feedback at the hypothalamus and pituitary gland, which suppresses FSH and LH release. By the late follicular phase, a single dominant follicle is selected and the remaining follicles undergo atresia.

At this point in the cycle, oestrogen switches from a negative to a positive feedback effect at the hypothalamus and pituitary gland. This causes an increase in GnRH and LH pulse frequency, ultimately leading to a surge in LH release which is responsible for inducing final maturation of the follicle, and ovulation. Ovulation generally occurs on day 14 of the cycle.

In the post-ovulatory luteal phase, the corpus luteum secretes progesterone and oestrogen, which exert negative feedback on LH and FSH release. The corpus luteum degenerates as gonadotrophin levels fall, leading to a reduction in sex steroid production and shedding of the

endometrial lining. Gonadotrophin levels then begin to rise, stimulating the development of the next follicle (Laycock J and Wise P, 1996).

4.1.2.2. The rodent oestrous cycle

In rats, the reproductive cycle is known as the oestrous cycle. This cycle is much shorter than the menstrual cycle in humans, lasting on average for four days. It can be divided into four stages: proestrous, oestrous, metestrous (or diestrous 1) and diestrous (or diestrous 2) (Mandl, 1951).

Hormonal changes in the rat oestrous cycle are similar to those that occur in humans. Figure 4.1 demonstrates levels of oestrogen, progesterone, LH and FSH in each stage of the rat and human reproductive cycle. The LH surge occurs in the afternoon of proestrous, which induces the final stages of ovarian follicular maturation. High circulating oestrogen levels at the time of the LH surge induce sexual receptivity in rodents (Nequin and Schwartz, 1971), and ovulation follows the LH surge by 10 to 12 hours. Progesterone levels begin to increase during proestrous, and peak around the time of ovulation. Unlike humans, a fully functioning corpus luteum does not develop unless mating occurs during oestrous. Vaginal stimulation induces prolactin release, which maintains the corpus luteum and prolongs diestrous. If mating is fertile, the fertilised ovum implants into the endometrium. However, if mating does not result in a fertilised ovum, the female enters a state of pseudopregnancy which can last for up to 10 days before normal cycles resume.

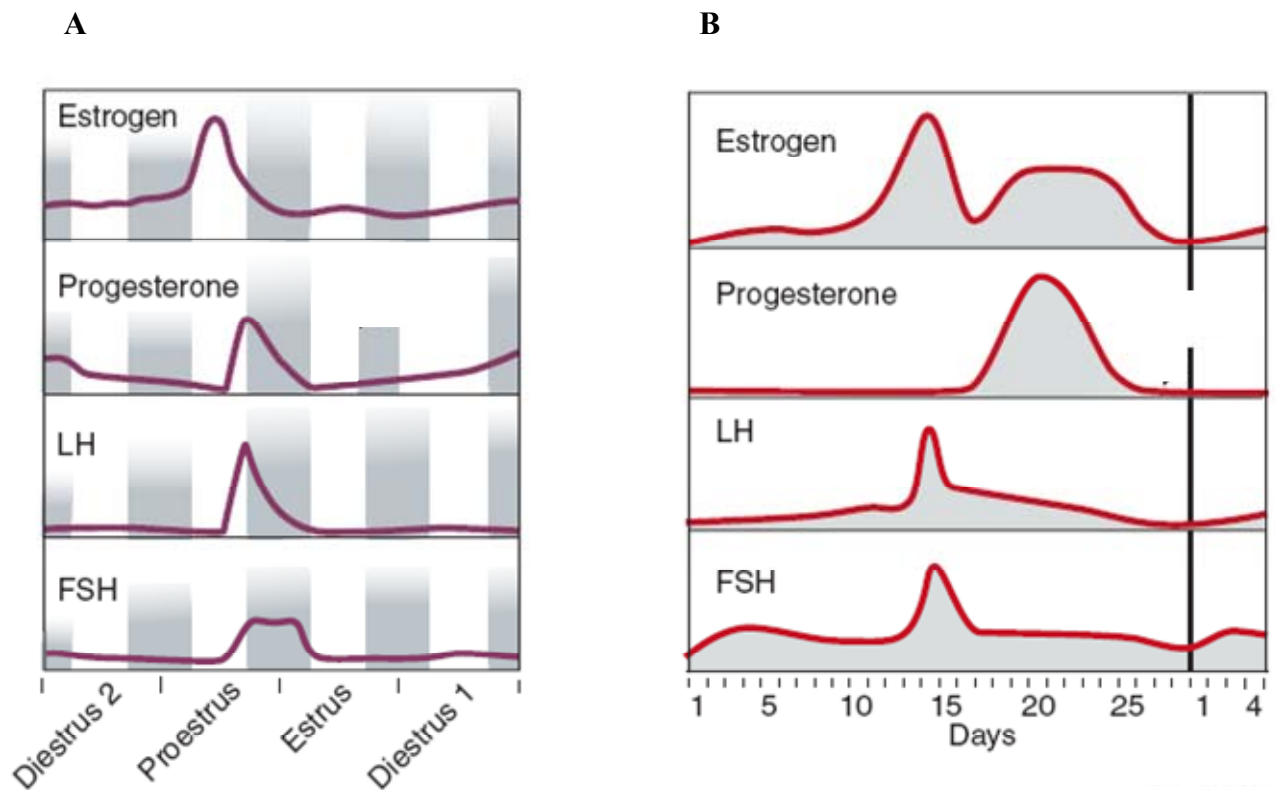


Figure 4.1: Fluctuations in circulating levels of oestrogen, progesterone, LH and FSH throughout the female (A) rat and (B) human reproductive cycle. Grey shaded areas in (A) represent the dark phase (approximately 7pm-7am). Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone. Adapted from (Staley and Scharfman, 2005).

4.1.3. Kisspeptin

The kisspeptins are coded for by the KiSS-1 gene and belong to the RF-amide family of peptides (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001). The RF-amide peptide family are so named due to the arginine-phenylalanine-amide residues present at the carboxyl terminus. In humans, the initial translation product of KiSS-1 is a 145 amino acid peptide which is cleaved to produce a 54-amino acid protein named kisspeptin-54 (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001). Shorter peptides of 14, 13 and 10 amino acids have also been identified, which may be degradation products of kisspeptin-54 (Kotani et al., 2001; Bilban et al., 2004). All four peptides (kisspeptin-54, -14, -13 and -10) share a common carboxyl terminal, which is essential for receptor binding and activation (Kotani et al., 2001) (Figure 4.2).

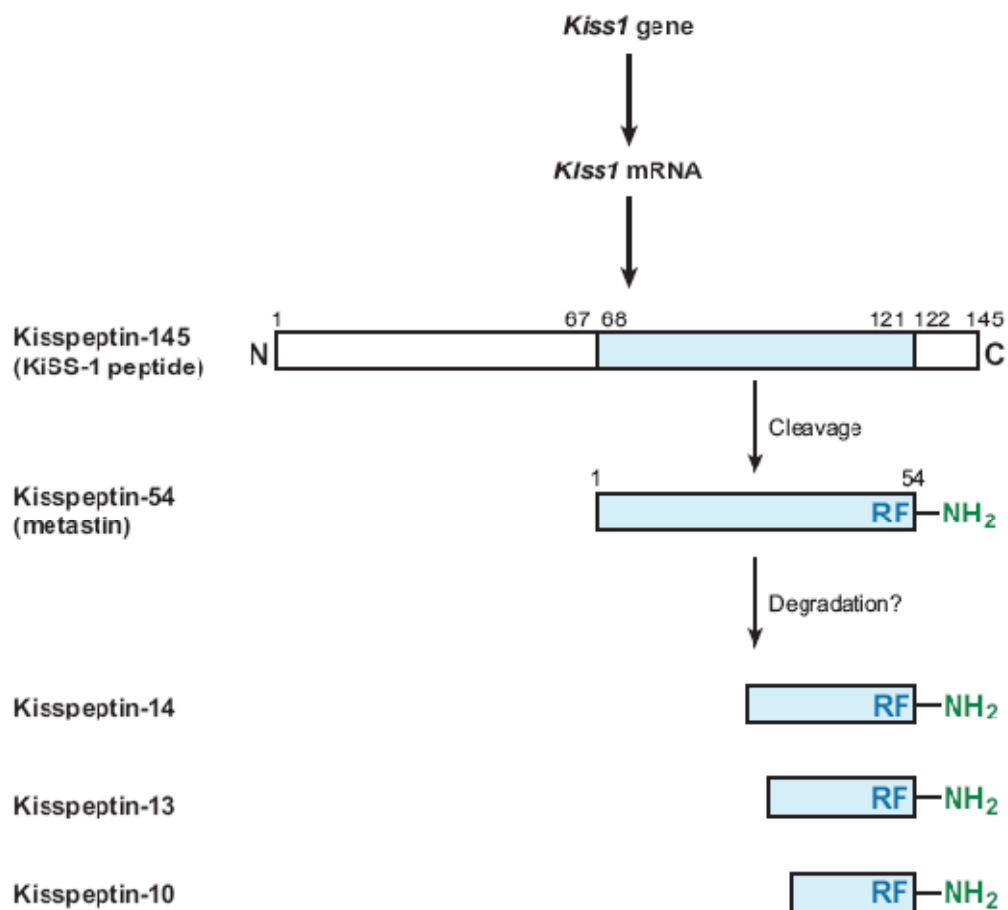


Figure 4.2: Products of the human KiSS-1 gene. Translation of the KiSS-1 gene produces a 145-amino acid propeptide, which is cleaved to generate kisspeptin-54. Shorter peptides (kisspeptin-14, -13 and -10) have also been identified, which all share a C terminus with kisspeptin-54. From (Popa et al., 2008).

In rats and mice, kisspeptin is a 52-amino acid peptide which shares approximately 46% sequence homology with human kisspeptin-54 (Terao et al., 2004). This diversity is mainly due to differences in the amino terminal of the peptide; the carboxyl terminal decapeptide is highly conserved between species (Stafford et al., 2002; Terao et al., 2004) (Figure 4.3).

Human: 112 Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe 121

Mouse: 110 Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr 119

Rat: 110 Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr 119

Figure 4.3: Alignment of the human, mouse and rat amino acid sequences corresponding to human kisspeptin 45-54 (kisspeptin-10). Abbreviations: Arg, arginine; Asn, asparagine; Gly, glycine; Leu, leucine; Phe, phenylalanine; Ser, serine; Trp, tryptophan; Tyr, tyrosine. From (Terao et al., 2004).

Kisspeptin is the endogenous ligand of the G-protein coupled receptor GPR54 (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001). GPR54 shares weak sequence homology with the galanin receptor, but does not bind either galanin or galanin-like peptide (Lee et al., 1999; Ohtaki et al., 2001). Kisspeptin-54, -14, -13 and -10 all bind to GPR54 with equal affinity and efficacy *in vitro* (Kotani et al., 2001).

4.1.4. Kisspeptin and GPR54 localisation

In humans, KiSS-1 mRNA is abundantly expressed in the placenta and brain, and is also expressed in the ovary, testis, pancreas, and small intestine (Muir et al., 2001; Ohtaki et al., 2001; Kotani et al., 2001). Within the human CNS, kisspeptin immunoreactive cell bodies are predominantly located in the infundibular nucleus of the hypothalamus, the equivalent of the rodent Arc (Hrabovszky et al., 2010). GPR54 mRNA is expressed at high levels within the placenta, pituitary gland and brain, specifically in the amygdala, nucleus accumbens, hypothalamus and hippocampus (Muir et al., 2001).

Distribution of KiSS-1 and GPR54 in rodents is similar to that observed in humans (Lee et al., 1999; Brailoiu et al., 2005). Within the rodent CNS, two major populations of kisspeptin immunoreactive cell bodies have been identified in the hypothalamus: in the Arc, and in the rostral periventricular region of the third ventricle, which includes the anteroventral periventricular nucleus (AVPV) (Clarkson and Herbison, 2006; Clarkson et al., 2009). The expression of KiSS-1 within the AVPV, but not the Arc, is sexually differentiated; females have up to 25 times more kisspeptin-expressing neuronal cell bodies in this area than males (Kauffman et al., 2007). Kisspeptin immunoreactive fibres are located within the medial preoptic area (MPOA), PVN, Arc, and in extra-hypothalamic areas including the amygdala, brainstem and thalamus (Brailoiu et al., 2005; Clarkson et al., 2009).

4.1.5. Kisspeptin and GPR54 are vital regulators of reproduction

A major role for the kisspeptin/GPR54 system in the control of reproduction was identified when it was noted that patients with homozygous mutations in the gene encoding GPR54 suffered from hypogonadotropic hypogonadism, defined as impaired pubertal development and reproductive function due to defective pituitary LH and FSH secretion (Seminara et al., 2003; de Roux N. et al., 2003). These patients have abnormally low levels of circulating gonadotrophins and sex steroids, and do not demonstrate normal sexual maturation. Mice lacking GPR54 show a similar phenotype (Seminara et al., 2003). Both humans and mice with defective GPR54 signalling respond to treatment with GnRH by increasing circulating gonadotrophin levels (Seminara et al., 2003). KiSS-1 knockout mice are also infertile, but are not as severely affected as GPR54 knockouts (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007).

Central and peripheral administration of kisspeptin markedly increases circulating levels of LH and FSH in numerous species, including humans (Irwig et al., 2004; Matsui et al., 2004; Thompson et al., 2004; Messenger et al., 2005; Dhillon et al., 2005). The mechanism by which kisspeptin stimulates the HPG axis is GnRH-dependent. ICV kisspeptin administration increases cerebrospinal fluid levels of GnRH in female sheep (Messenger et al., 2005), and the rise in circulating LH induced by kisspeptin administration is attenuated by pre-treatment with a GnRH antagonist in rats (Irwig et al., 2004). Approximately 77% of GnRH neurones express GPR54 mRNA, and kisspeptin is able to directly depolarize and increase firing rates of GnRH neurones (Pielecka-Fortuna et al., 2008). Additionally, ICV injection of kisspeptin induces Fos expression, a marker of neuronal activation, in GnRH neurones (Irwig et al., 2004). Kisspeptin neuronal

fibres terminate in close proximity to GnRH neuronal cell bodies and dendrites, particularly those located in the pre-optic area (Clarkson and Herbison, 2006).

4.1.6. Kisspeptin neurones relay gonadal steroid feedback to GnRH neurones

Feedback from gonadal steroids to hypothalamic GnRH neurones is crucial in maintaining HPG axis activity. In males, testosterone reduces GnRH and LH pulse frequency (Jackson et al., 1991). In females, oestrogen and progesterone negatively regulate the HPG axis at most stages of the oestrous cycle, except immediately prior to the LH surge and ovulation, when they exert positive feedback (Laycock J and Wise P, 1996). As GnRH neurones do not express receptors for gonadal steroids, an intermediary factor must relay these feedback signals (Shivers et al., 1983). The majority of hypothalamic kisspeptin neurones express receptors for oestrogen and testosterone, (Smith et al., 2005b), and project to areas where GnRH neurones reside (Brailoiu et al., 2005; Clarkson et al., 2009). This suggests that kisspeptin neurones may relay sex steroid signals from the gonads to GnRH neurones.

4.1.6.1. The arcuate nucleus

Kisspeptin neurones in the Arc may relay negative feedback signals to GnRH neurones. KiSS-1 mRNA expression within the Arc increases following castration or ovariectomy, and decreases following gender-appropriate sex steroid replacement (Smith et al., 2005b; Smith et al., 2005a). In female rats, KiSS-1 mRNA expression in the Arc reaches a peak during diestrus, when circulating oestrogen levels are low, and falls dramatically during the oestrogen surge (Smith et al., 2006b). This suggests that high circulating levels of gonadal steroids may negatively regulate Arc kisspeptin expression, and thus indirectly reduce GnRH, LH and FSH release.

Additionally, female mice lacking GPR54 do not exhibit a compensatory rise in circulating LH following ovariectomy (Dungan et al., 2007). Similarly, castrated male mice do not exhibit a rise in circulating LH if administered a kisspeptin antagonist following castration (Roseweir et al., 2009). This suggests that kisspeptin/GPR54 signalling is required for negative feedback regulation of GnRH and gonadotrophins (Figure 4.4).

In several species, kisspeptin neurones in the Arc co-express the neuropeptides neurokinin B (NKB) and dynorphin (Goodman et al., 2007; Navarro et al., 2009; Hrabovszky et al., 2010).

These neurones form autosynaptic contacts with one another, in addition to projecting to GnRH neurones, and it has been suggested that the autosynaptic action of kisspeptin, NKB and dynorphin drives the pulsatile secretion of kisspeptin and hence of GnRH release (Navarro et al., 2009).

4.1.6.2. The AVPV

It has been suggested that in female rodents, AVPV kisspeptin neurones are involved in the generation of the pre-ovulatory GnRH/LH surge. Administration of an anti-kisspeptin antibody into the pre-optic area of rats blocks the pre-ovulatory LH surge (Kinoshita et al., 2005), as does ICV infusion of a kisspeptin receptor antagonist (Pineda et al., 2010). These findings suggest that signalling from AVPV kisspeptin neurones to GnRH neurones mediates the positive feedback effects of oestrogen, and hence generates the pre-ovulatory GnRH/LH surge (Figure 4.4).

In contrast to the Arc, kisspeptin expression in the AVPV is positively regulated by gonadal steroids. In female mice, kisspeptin expression in the AVPV decreases following ovariectomy, and rises after oestradiol replacement (Smith et al., 2005a). Similarly, testosterone positively regulates KiSS-1 expression in the AVPV of male mice (Smith et al., 2005b). The number of KiSS-1-expressing cells in the AVPV peaks on the afternoon of proestrous in rats, and reaches a nadir at diestrous (Smith et al., 2006b). The majority of AVPV kisspeptin neurones express Fos on the afternoon of proestrous, suggesting that transcriptional activation occurs (Smith et al., 2006b). These data suggest that AVPV kisspeptin neurones mediate the positive feedback effects of sex steroids on the HPG axis.

It should be noted that these observations regarding the role of AVPV kisspeptin neurones are limited to rodents, as female sheep and primates (including humans) do not have a population of kisspeptin neurones equivalent to the AVPV. In these species, kisspeptin neurones in the Arc are hypothesised to relay both the negative and positive feedback effects of gonadal steroids to GnRH neurones, perhaps via discrete neuronal populations (Estrada et al., 2006).

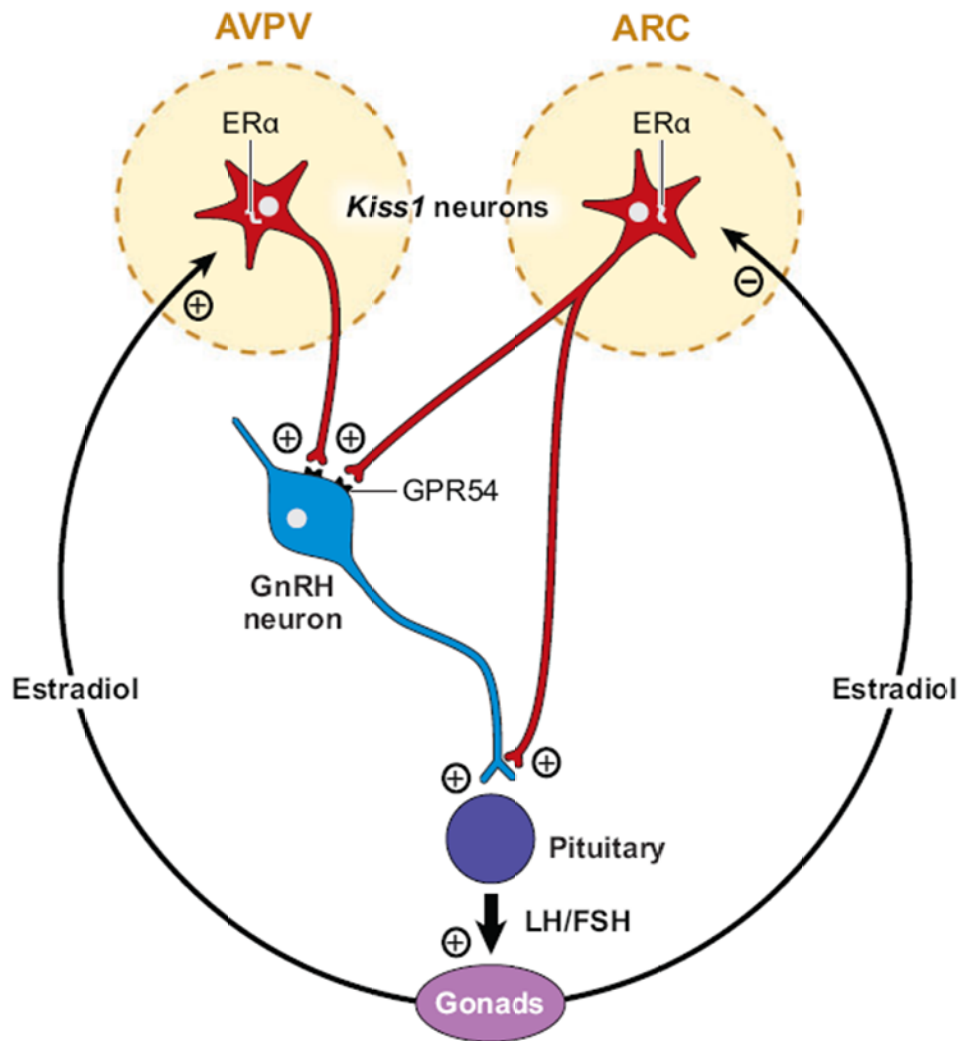


Figure 4.4: Schematic diagram demonstrating how kisspeptin neuronal populations in the female rodent Arc and AVPV may mediate the negative and positive feedback effects of gonadal steroids to GnRH neurones. Kisspeptin stimulates GnRH release by acting on GPR54. GnRH stimulates the release of gonadotrophins from the anterior pituitary gland, which in turn increase sex steroid release from the gonads. Oestradiol increases kisspeptin expression in the AVPV, and decreases expression in the Arc. Oestradiol is thought to exert its effects on kisspeptin neurones via the estrogen receptor α (ER α). Abbreviations: Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; GPR54, G protein-coupled receptor 54; GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. From (Popa et al., 2008).

In summary, it is assumed that kisspeptin neurones in the Arc provide a tonic, stimulatory drive to GnRH neurones. When circulating levels of sex steroids decline, Arc KiSS-1 expression increases, leading to an increase in GnRH and gonadotrophin secretion. This in turn facilitates a rise in circulating gonadal steroids. In contrast, in rodents it is hypothesised that kisspeptin neurones in the AVPV mediate the ‘surge’ mode of GnRH and LH release. However, conclusive evidence to support these assumptions is lacking. Although studies have investigated the acute effects of administration of anti-kisspeptin antibodies (Kinoshita et al., 2005) or a kisspeptin receptor antagonist (Li et al., 2009) into specific areas of the brain, the long-term effects of reducing kisspeptin expression in either the Arc or AVPV have not been investigated. One possible method to investigate the role of kisspeptin signalling within these discrete nuclei over long time periods is to use gene knockdown.

4.1.7. Gene transfer methods

Gene transfer allows modulation of gene expression either *in vitro* or *in vivo*. When administered *in vivo*, spatial and temporal control of gene expression can be achieved, and problems associated with transgenic models or chronic injection of a receptor agonist or antagonist can be avoided.

Gene vectors may be non-viral or viral. Injection of naked plasmid DNA is the simplest form of non-viral transfection. Although simple to produce and non-immunogenic, naked plasmid DNA is only expressed transiently and at a relatively low level *in vivo* (Yovandich et al., 1995). Plasmid DNA can be combined with cationic lipids or polymers to enhance cellular uptake and improve stability (Davis, 2002; Gardlik et al., 2005).

Viruses have developed specialised mechanisms to permit effective transport of DNA into cells, and hence are useful gene vectors. Viral vectors have been developed from numerous viruses including retrovirus, adenovirus, lentivirus and adeno-associated virus (AAV). The viral vector used in the work described in this chapter was AAV.

4.1.8. Adeno-associated virus

Adeno-associated viruses (AAVs) are single-stranded, non-enveloped DNA viruses which belong to the *Parvoviridae* family. The AAV genome consists of two inverted terminal repeats (ITRs) located at the 5' and 3' ends, and two large open reading frames which contain *rep* (replication) and *cap* (capsid) genes. The *rep* gene encodes four Rep proteins, which are essential for viral DNA replication and transcriptional control, and the *cap* gene encodes three capsid proteins. AAV is a dependovirus, as it requires a helper virus such as adenovirus to permit replication and infection. Although a number of AAV serotypes have been identified, AAV serotype 2 (AAV2) is the most widely used as a gene vector (Lu, 2004). AAV2 enters target cells by binding to the heparin sulphate proteoglycan cell surface receptor (Summerford and Samulski, 1998).

Recombinant AAV (rAAV) is a modified form of AAV. It is a good choice of gene vector because it is non-pathogenic and does not induce an inflammatory response when administered to animals (Chamberlin et al., 1998). Recombinant AAV2 is an ideal vector for gene transfer to neurones within the CNS because it is selectively taken up by neurones, and not by astrocytes or microglia (Bartlett et al., 1998). Additionally, transgene expression following rAAV administration is long-lasting, and has been reported to persist for at least 15 months (Xu et al., 2001).

In rAAV vectors, all viral DNA except the ITRs is removed and replaced with the gene of interest. Host cells are then co-transfected *in vitro* with this packaging plasmid and a helper plasmid (containing AAV *rep* and *cap* genes and several adenovirus helper genes), in order to produce rAAV particles (Grimm et al., 1998). The rAAV vector also contains the early cytomegalovirus (CMV) promoter, a constitutively active promoter in mammalian cells, and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which increases transgene expression (Loeb et al., 1999).

There are disadvantages to using rAAV as a viral vector. The genome of AAV is small, and hence it is not possible to efficiently incorporate transgenes larger than 4.9kb (Dong et al., 1996). Additionally, following administration of the vector, transgene expression is delayed because single stranded viral DNA must be converted to double stranded DNA (Ferrari et al., 1996).

4.1.9. Antisense RNA

Antisense RNA is a transcript which is complementary to an endogenous protein-coding mRNA sequence. Binding of antisense and sense RNA strands by Watson and Crick base-pairing prevents translation of the mRNA sequence, and hence reduces protein expression. Antisense RNA is a naturally occurring mechanism present in prokaryotic and eukaryotic cells to regulate gene expression (Werner and Sayer, 2009).

The precise mechanism by which antisense RNA reduces gene expression is not known, although the most widely accepted theory is that formation of double-stranded RNA induces RNA editing. Double-stranded RNA duplexes are targets for adenosine deaminases, or ADARs. These enzymes convert adenosine residues (A) to inosines (I). In long RNA duplexes, numerous A to I conversions cause the double-stranded RNA duplex to become unstable and partially unwind (Bass and Weintraub, 1988). The modified RNA then becomes trapped in the nucleus, and cannot associate with translation machinery in the cytoplasm (Kumar and Carmichael, 1997). If a smaller number of A to I modifications take place on a shorter RNA duplex, point mutations are introduced into the transcript which results in translation of a non-functioning protein (Kumar and Carmichael, 1998).

In this work I used a rAAV2 vector expressing kisspeptin antisense cDNA (rAAV-Kisspeptin-AS) as a tool to investigate the effects of kisspeptin down-regulation in the rat Arc. A schematic diagram of the vector used in this work is shown in Figure 4.5. The same vector containing enhanced green fluorescent protein (EGFP) instead of kisspeptin antisense was used as a control in this study. Green fluorescent protein was originally isolated from the jellyfish *Aequorea Victoria*, and is commonly used as a reporter gene. The enhanced variant has been modified to improve expression in mammalian cells (Zhang et al., 1996).

Although kisspeptin signalling is important in both males and females, I used female rats in this work, because vaginal smearing is a relatively non-disruptive method of assessing reproductive function.

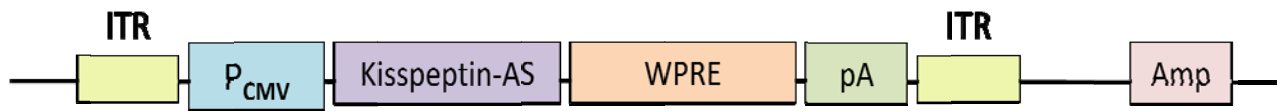


Figure 4.5: Structure of the recombinant adeno-associated virus kisspeptin-antisense vector. Abbreviations: Amp, ampicillin resistance gene; ITR, inverted terminal repeat; pA, polyadenylation signal; P_{CMV}, early cytomegalovirus promoter; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

4.1.10. Hypothesis

Reducing kisspeptin expression using rAAV-Kisspeptin-AS within the Arc will disrupt reproductive function in adult female rats.

4.1.11. Aims

In order to investigate the effect of reducing kisspeptin expression within the Arc on reproductive function I will:

- Verify that the rAAV-Kisspeptin-AS transgene is expressed *in vivo*.
- Determine the effect of intra-Arc rAAV-Kisspeptin-AS administration on oestrous cyclicity, reproductive hormones, food intake and body weight in female Wistar rats.

4.2. Methods

4.2.1. Production of rAAV-EGFP and rAAV-Kisspeptin-AS

The production of rAAV-Kisspeptin-AS and rAAV-EGFP was carried out by Dr James Gardiner (Division of Diabetes, Endocrinology and Metabolism, Imperial College London). The two plasmid system was used (Grimm et al., 1998). Briefly, host cells were co-transfected with the rAAV plasmid and a helper plasmid. As there was no homology between the two plasmids, they were unable to recombine to form wild-type virus. This resulted in the production of rAAV particles, which were released from the host cells by freeze-thawing, and subsequently purified.

Prior to the work described in this thesis, the kisspeptin-AS plasmid was tested *in vitro* to ensure that the construct was able to reduce kisspeptin release. Kisspeptin secretion from RIN 1056a cells was significantly reduced following transfection with rAAV-Kisspeptin-AS.

4.2.2. Animals

Female Wistar rats (specific pathogen free; Charles River) weighing 220-250g were maintained as described in section 2.2.2.

All animal procedures undertaken were approved by the British Home Office Animals (Scientific Procedures) Act 1986 (Project License 70/6377).

4.2.3. Confirmation of Arc coordinates with ink injections

Prior to administration of rAAV-Kisspeptin-AS, suitable stereotactic coordinates were identified using 'The Rat Brain in Stereotactic Co-ordinates' (Paxinos and Watson, 2007). Stereotactic coordinates calculated were as follows: 3.3mm posterior to the bregma, 0.5mm lateral from the midline and 10.5mm below the surface of the skull. These coordinates were verified by injection of 1µl 10% dilution of India ink and water.

Female Wistar rats (n=5) were terminally anaesthetised with intraperitoneal administration of 200mg/kg Pentobarbitone (Animalcare Ltd.). Ink injections were carried out as described in section 3.2.3.1.

Following dissection and slicing of the brains, slices were mounted onto polylysine coated slides (VWR) then counter-stained using cresyl violet.

4.2.4. Cresyl violet staining of ink injected rat brains

Cresyl violet staining was carried out as described in section 3.2.3.2.

4.2.5. Recombinant adeno-associated virus intra-nuclear administration to verify transgene expression

Prior to carrying out the main experiment I first verified that the rAAV encoding Kisspeptin-AS would result in transgene expression *in vivo*. Female Wistar rats (n=5) were anaesthetised with 2L/minute oxygen and 4% inhaled isoflurane (Abbott Laboratories Ltd). Animals were placed in the induction chamber until they lost the pedal reflex. Once anaesthetised, animals were administered intra-peritoneal prophylactic antibiotics (amoxycillin (37.5mg/kg) and flucloxacillin (37.5mg/kg)) to prevent post-operative infection. Rats were positioned on a stereotactic frame (David Kopf Instruments, supplied by Clark Electromedical Instruments) with the incisor bar 3mm below the interaural line. The head was cleaned with 10% w/v povidone/iodine solution (Ecolabs Ltd) and a 1.5cm longitudinal incision made in the scalp. Two holes were drilled using a stereotactically mounted electric drill (Department of Medical Engineering, Imperial College London, UK), using stereotactic coordinates described in section 4.2.3. Each animal was injected bilaterally into the Arc with 1µl rAAV-Kisspeptin-AS at a titre of 1.33×10^{15} genome particles per microlitre. The cannula and injector were maintained *in situ* for five minutes after injection to minimize backflow. The incision was sutured using 6.0 proline thread. Animals were administered subcutaneous buprenorphine (45mg/kg, Schering-Plough Corp.) for post-operative analgesia and rehydrated with 0.9% sodium chloride (5ml/rat).

Three weeks post injection, animals were culled and *in situ* hybridization performed to confirm expression of rAAV-Kisspeptin-AS.

4.2.6. Confirmation of transgene expression by *in situ* hybridisation

In situ hybridisation (ISH) was performed to confirm expression of the rAAV-Kisspeptin-AS construct using a ^{35}S radiolabelled probe specific for WPRE. WPRE is part of the rAAV construct and aids transgene expression (Loeb et al., 1999). As WPRE is not naturally expressed in mammalian tissues, a probe complementary to WPRE will only bind to tissue where the transgene is expressed.

Preparation of slides, production of a radiolabelled RNA probe and hybridisation of the probe to slides was carried out as described in section 2.2.11.2 (n=5). The ³⁵S antisense and sense WPRE riboprobes were labelled using pBluescript-WPRE as a template, which contains a 308bp fragment of WPRE. The template plasmid was linearised by digestion with ApaI for the anti-sense probe and SpeI for the sense probe.

4.2.7. Oestrous cycle stage determination

The rat oestrous cycle lasts on average for four days, and is characterised by four stages: proestrous, oestrous, metestrous and diestrous (Mandl 1951). Vaginal smear cytology can be used to determine each phase of the oestrous cycle. Proestrous is characterised by a predominance of round, nucleated epithelial cells. A smear taken at oestrous consists of irregular, anucleated cornified cells. Metestrous is characterised by an equal proportion of epithelial cells, cornified cells and leukocytes, and at diestrous the smear predominantly contains leukocytes (Mandl, 1951; Marcondes et al., 2002). Figure 4.6 shows representative vaginal smears taken at different stages of the rat oestrous cycle.

As the aim of my study was to determine the effect of knockdown of Arc kisspeptin expression on the oestrous cycle, it was important to determine that the animals used in my study exhibited normal cycles prior to rAAV administration. It has previously been reported that up to 25% of female rats do not exhibit regular oestrous cycles (Mandl, 1951). Prior to injection of rAAV-Kisspeptin-AS or rAAV-EGFP as described in section 4.2.8, oestrous cyclicity was monitored for 10 days. Only rats exhibiting regular oestrous cycles were included in the study (29 out of 40 rats).

Vaginal cytology of each of the rats was monitored by once-daily vaginal lavage, which was performed between 9am and 11am. Vaginal secretions were collected using a 1ml syringe filled with 100µl sterile water, by gently inserting the tip of the syringe into the rat vagina. The procedure was performed as gently as possible to eliminate the risk of the rats becoming pseudopregnant (Steuer et al., 1987). One drop of vaginal fluid from each rat was placed on a glass slide. Unstained fluid was observed under a light microscope (Jencons-PLS, Forest Row, East Sussex, UK) at 10x and 20x magnification. Cells were identified as described above, and the proportion of the cells observed was used to determine the stage of the oestrous cycle (Mandl, 1951).

Cycles were analysed using previously described methods. Cycles were classified as regular, extended or abnormal. A regular cycle lasted between 3 and 5 days, with either 1 or 2 days of oestrous, or 2 or 3 days of diestrous. An extended cycle contained either 3 or 4 days of oestrous, or 4 or 5 days of diestrous. An abnormal cycle contained more than 4 consecutive days of oestrous, or more than 6 days of diestrous (Goldman et al., 2007).

A second, more stringent analysis was also performed to detect subtler changes between groups. A 'complete' cycle was classified as one in which three stages of the oestrous cycle were observed in the correct order.

Finally, cycle length was determined by the number of days between each occurrence of oestrous (Goldman et al., 2007).

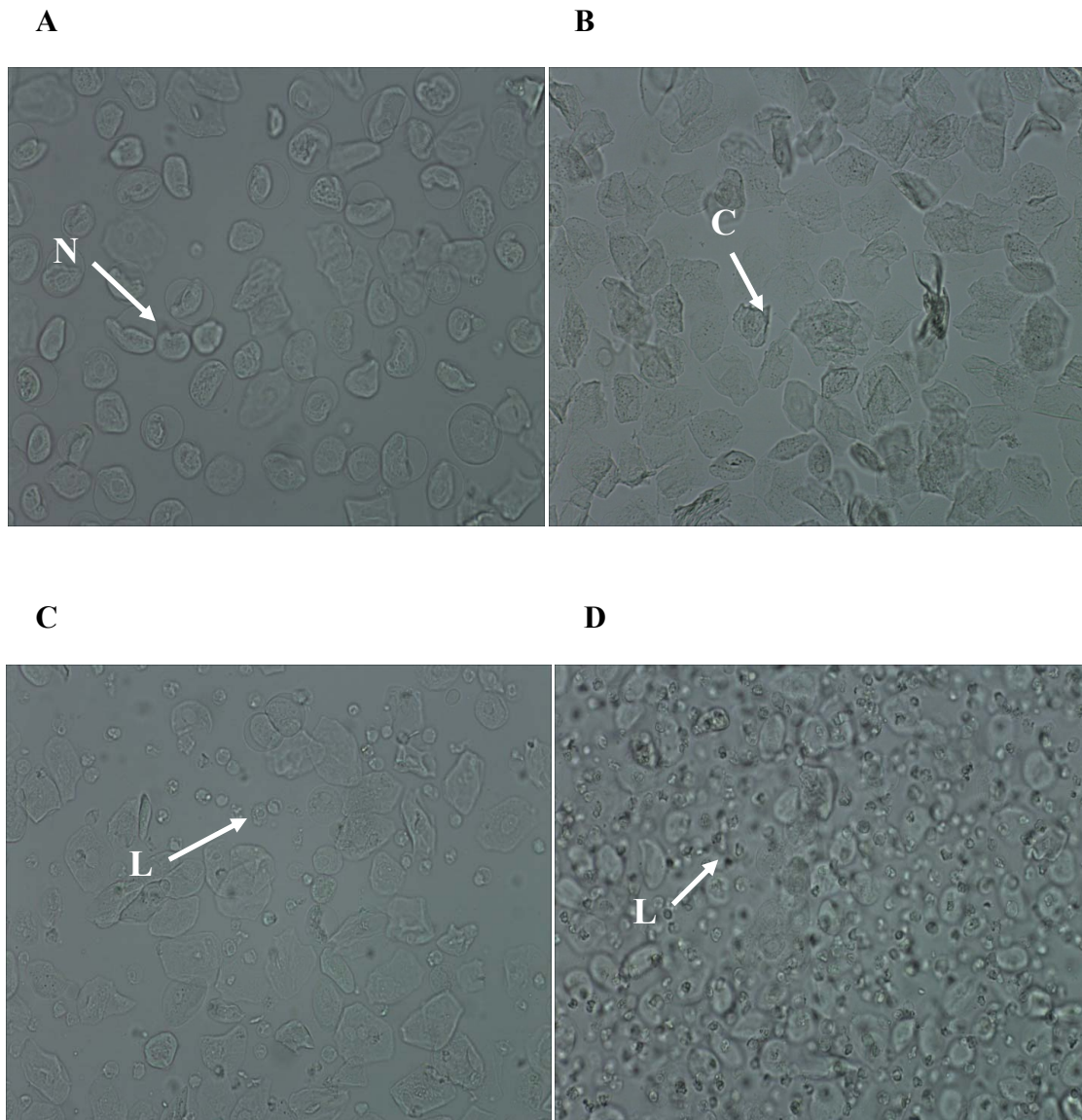


Figure 4.6: Photomicrographs of unstained vaginal smears from female rats in (A) proestrous, (B) oestrous, (C) metestrous, and (D) diestrous. Smears were viewed under a light microscope at 20x magnification. Examples of nucleated cells (N), cornified cells (C) and leukocytes (L) are labelled. The proportion of the three types of cells was used to determine oestrous cycle stage.

4.2.8. Recombinant adeno-associated virus intra-nuclear administration

Female Wistar rats (200-240g) were randomised into groups by both body weight and pre-surgery cycle length, and administered either rAAV-EGFP (titre 5×10^{12} genome particles per microlitre) (n=14) or rAAV-Kisspeptin-AS (n=15) bilaterally into the Arc using methods described in section 4.2.5.

After a four day recovery period, vaginal cytology was monitored daily for 100 days to determine the length and regularity of each individual animal's oestrous cycle. Smears were taken between 9am and 11am daily. Body weight and food intake were measured every other day for 100 days.

4.2.9. Tissue and plasma collection

The study ended on day 100 post-injection. Rats were culled in the diestrous phase of the oestrous cycle, as this is when basal LH levels are less variable and hence differences between groups may have been easier to detect.

Rats were killed by decapitation. Plasma was collected in plastic lithium heparin tubes containing 1000 kallikrein inactivator units of aprotinin (Nordic Pharma Ltd.). Plasma was separated by centrifugation at 1600g and snap frozen in liquid nitrogen. Samples were stored at -20°C until analysis for LH and oestradiol. LH was measured by RIA (see section 2.2.13). Oestradiol was measured using a mouse/rat oestradiol enzyme-linked immunosorbent assay (ELISA) kit (Calbiotech, Spring Valley, California, USA).

Brains were rapidly removed, cut to the level of the hypothalamus, snap frozen in isopentane and stored at -80°C until quantification of kisspeptin levels in the Arc.

4.2.10. Statistics

All data are presented as mean $\pm$ standard error of the mean (SEM). Oestrous cyclicity data and plasma hormone data were analysed using an unpaired *t*-test (GraphPad Prism version 5 for Windows; GraphPad Software, San Diego, CA). Percentage time spent in each stage of the oestrous cycle was analysed using the Mann-Whitney U test (Systat, Evanston, IL) as data were non-parametric. Body weight and food intake data were analysed using the generalized estimating equation (Stata 9, StataCorp LP, College Station, Texas). Three outliers were removed from data analysis due to being more than two standard deviations away from mean body weight and/or food intake measurements. In all cases *p* values <0.05 were taken as significant.

4.3. Results

4.3.1. Confirmation of Arc coordinates with ink injections

Prior to rAAV administration, correct coordinates were confirmed with ink injections. Ink was observed unilaterally in the Arc in 80% of animals (n=5). A representative photograph is shown in Figure 4.7A, and the corresponding diagram of the rat brain (Paxinos and Watson, 2007) is shown in Figure 4.7B.

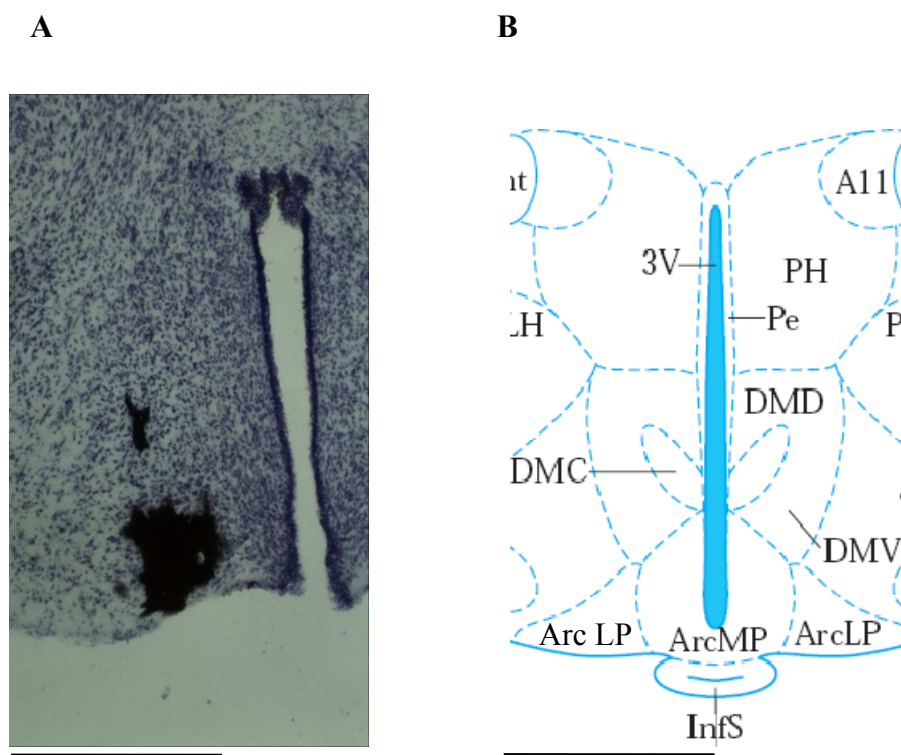


Figure 4.7: Confirmation of arcuate nucleus coordinates with ink injections. Female Wistar rats were injected unilaterally with India ink (coordinates 3.3mm posterior to the bregma, 0.5mm lateral from the midline and 10.5mm below the surface of the skull). Brain sections were stained with cresyl violet (A), and compared to ‘The Rat Brain in Stereotaxic Coordinates’ (Paxinos and Watson, 2007) (B). A coronal section -3.4mm from bregma is shown. Scale bars are equivalent to 1mm. Abbreviations: 3V, third ventricle; A11, dopamine cells; ArcLP, arcuate nucleus, lateroposterior part; ArcMP, arcuate nucleus, medial posterior part; DMC, dorsomedial nucleus, compact part; DMD, dorsomedial nucleus, dorsal part; DMV, dorsomedial nucleus, ventral part; InfS, infundibular stem; Pe, periventricular hypothalamic nucleus; PH, posterior hypothalamic nucleus.

4.3.2. Verification of transgene expression

Rats were killed three weeks post-injection (n=5) and ISH was performed to confirm transgene expression, using a radiolabelled probe complementary to WPRE.

All brains demonstrated transgene expression in the Arc. An image from a representative brain is shown in Figure 4.8A. No non-specific binding was observed when ISH was performed using a WPRE sense radiolabelled probe (Figure 4.8B).

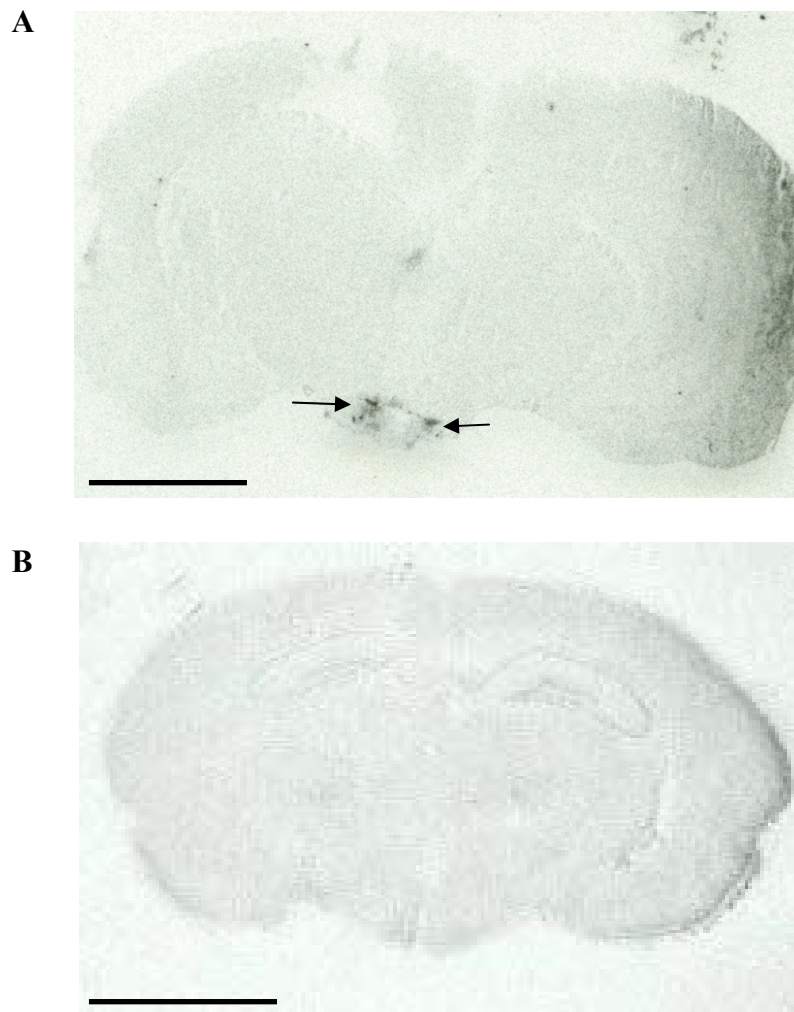


Figure 4.8: Representative microphotographs showing *in situ* hybridisation using (A) a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) anti-sense riboprobe and (B) a WPRE sense riboprobe to confirm signal specificity in an animal injected bilaterally intra-Arc with rAAV-Kisspeptin-AS. Arrows show riboprobe binding. No non-specific binding was observed. Sections of 12 μ m thickness were taken approximately 3.3mm caudal to bregma. Scale bars are equivalent to 5mm.

4.3.3. The effect of intra-Arc rAAV-Kisspeptin-AS administration on oestrous cyclicity

Animals administered intra-Arc rAAV-Kisspeptin-AS had significantly fewer ‘regular’ cycles between day 4 and day 100 post-injection compared to EGFP controls (number of ‘regular’ cycles: 17.12 ± 0.46 [rAAV-EGFP] vs 15.00 ± 0.87 [rAAV-Kisspeptin-AS] $p < 0.05$, $n = 12-14$ per group). There was also a trend for animals administered rAAV-Kisspeptin-AS to have more ‘extended’ and ‘abnormal’ cycles than controls, although this did not reach significance (Table 4.1).

A more stringent cycle analysis was performed to detect subtler changes in oestrous cyclicity between groups. Animals administered intra-Arc rAAV-Kisspeptin-AS had significantly fewer ‘complete’ cycles than animals administered rAAV-EGFP between days 4 and 100 post-injection (number of complete cycles: 10.00 ± 0.63 [rAAV-EGFP] vs 6.50 ± 0.82 [rAAV-Kisspeptin-AS] $p < 0.01$, $n = 12-14$ per group) (Table 4.1 and Figure 4.9A).

Animals injected with rAAV-Kisspeptin-AS had significantly longer cycles than controls, as demonstrated by an increased number of days between each occurrence of oestrous (average number of days per cycle: 3.84 ± 0.10 [rAAV-EGFP] vs 4.30 ± 0.11 [rAAV-Kisspeptin-AS] $p < 0.01$, $n = 12-14$ per group) (Table 4.1 and Figure 4.9B).

No significant differences between groups were observed in the percentage time spent in proestrous, oestrous, metestrous or diestrous (Figure 4.10).

Intra-Arc treatment	Regular cycles	Extended cycles	Abnormal cycles	Complete cycles	Average number of days per cycle
rAAV-EGFP	17.12 ± 0.46	0.58 ± 0.29	0.00 ± 0.00	10.00 ± 0.63	3.84 ± 0.10
rAAV-Kisspeptin-AS	15.00 ± 0.87*	1.38 ± 0.33	0.23 ± 0.17	6.50 ± 0.82**	4.30 ± 0.11**

Table 4.1: The effect of intra-Arc rAAV-EGFP or rAAV-Kisspeptin-AS administration on oestrous cyclicity in adult female Wistar rats. Vaginal cytology was analysed daily from day 4 to day 100 post-injection to determine oestrous cycle stage. The number of regular, extended and abnormal cycles was calculated. A regular cycle was one lasting between 3 and 5 days, with either 1 or 2 days of oestrous or 2 or 3 days of diestrous. An extended cycle contained either 3 or 4 days of oestrous, or 4 or 5 days of diestrous. An abnormal cycle contained more than 4 consecutive days of oestrous, or more than 6 days of diestrous (Goldman et al., 2007). Additionally, a complete cycle was counted as one in which 3 out of 4 cycle stages were observed in the correct order. Cycle length was calculated as the average number of days between each occurrence of oestrous (Goldman et al., 2007). Data is presented as mean ± SEM. *p<0.05, **p<0.01 vs. rAAV-EGFP group, n= 12-14 per group.

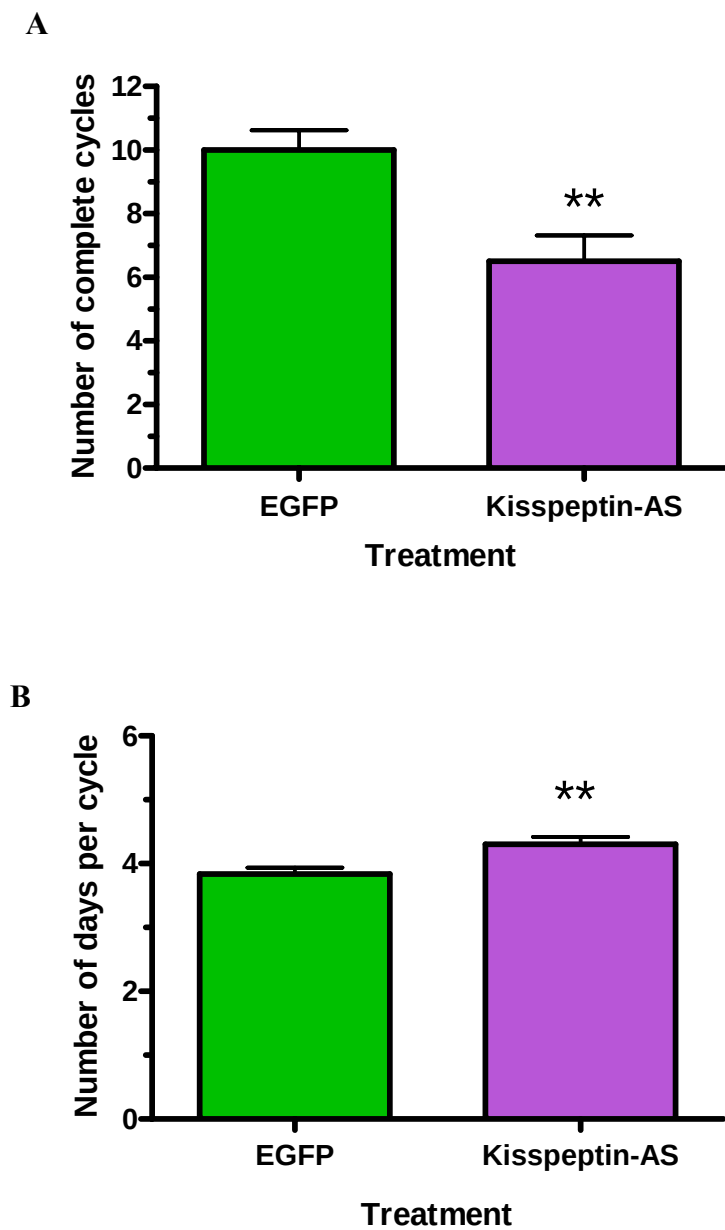
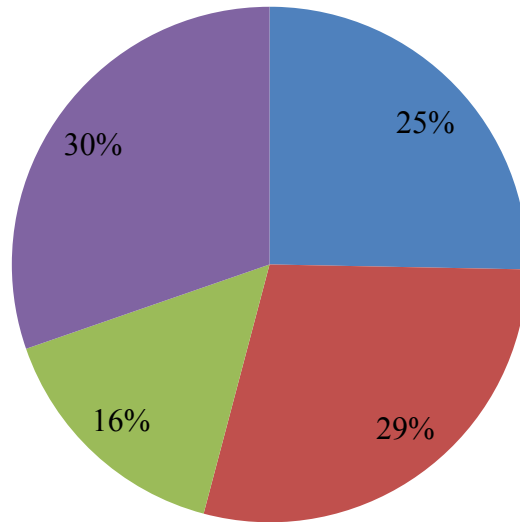
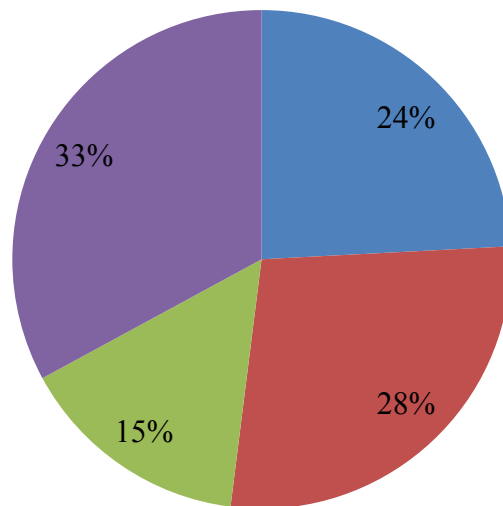


Figure 4.9: The effect of intra-Arc rAAV-Kisspeptin-AS administration on oestrous cyclicity in adult female Wistar rats. Vaginal cytology was analysed daily from day 4 to day 100 post-injection to determine oestrous cycle stage. Number of complete cycles (**A**) and average number of days per cycle (**B**) were calculated. Data is presented as mean $\pm$ SEM. ** $p < 0.01$ vs. rAAV-EGFP group, $n = 12-14$ per group.

A



B



■ Proestrous ■ Oestrous ■ Metestrous ■ Diestrous

Figure 4.10: The effect of intra-Arc rAAV-Kisspeptin-AS administration on percentage time spent in each stage of the oestrous cycle in adult female Wistar rats. Vaginal cytology was analysed daily from day 4 to day 100 post-injection to determine oestrous cycle stage. Percentage time spent in proestrous, oestrous, metestrous and diestrous in animals administered **(A)** rAAV-EGFP or **(B)** rAAV-Kisspeptin-AS was calculated. N= 12-14 per group.

4.3.4. The effect of intra-Arc rAAV-Kisspeptin-AS administration on plasma LH and oestradiol

No statistically significant differences in plasma LH (Figure 4.11A) or oestradiol (Figure 4.11B) were observed between animals administered rAAV-EGFP or rAAV-Kisspeptin-AS.

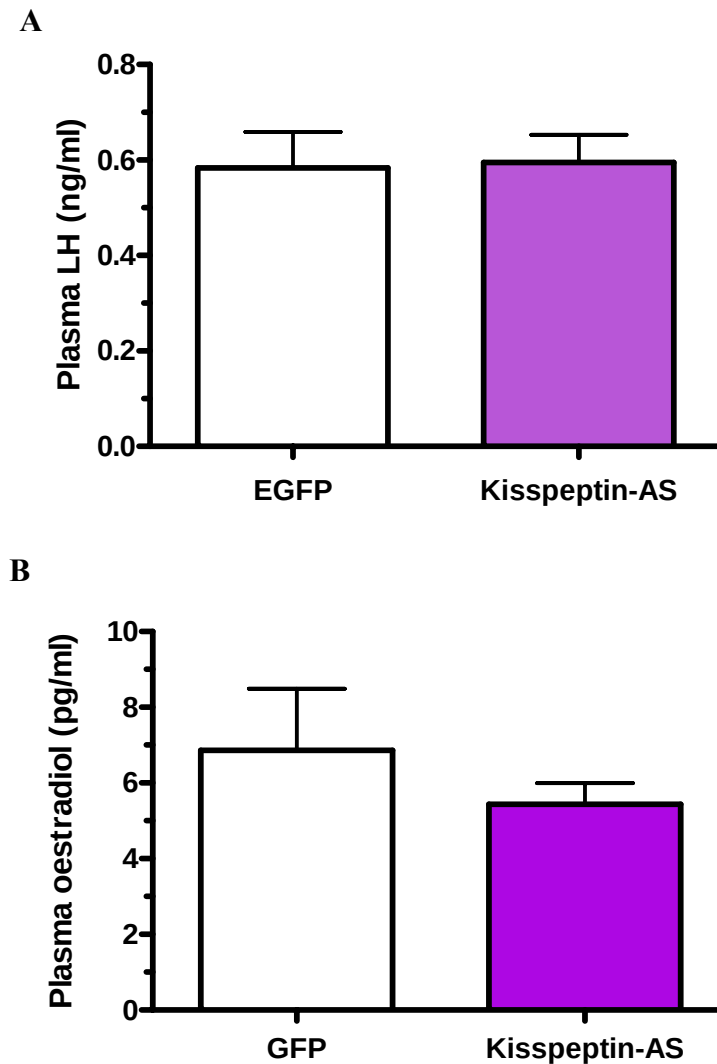


Figure 4.11: Plasma (A) luteinizing hormone (LH) and (B) oestradiol levels in female Wistar rats administered intra-Arc rAAV-EGFP or rAAV-Kisspeptin-AS. Animals were killed in diestrous between day 100 and day 105 post-injection. Plasma LH was measured by radioimmunoassay, and oestradiol by enzyme-linked immunosorbent assay. Data is presented as mean $\pm$ SEM, n=12-14 per group.

4.3.5. The effect of intra-Arc rAAV-Kisspeptin-AS administration on body weight and food intake

There was no difference in body weight between the rAAV-EGFP and rAAV-Kisspeptin-AS groups at the onset of the study. The body weights of both groups remained similar until the termination of the study on day 100 post-injection (Figure 4.12A).

Similarly, there was no difference in food intake between groups at any time point throughout the experiment (Figure 4.12B).

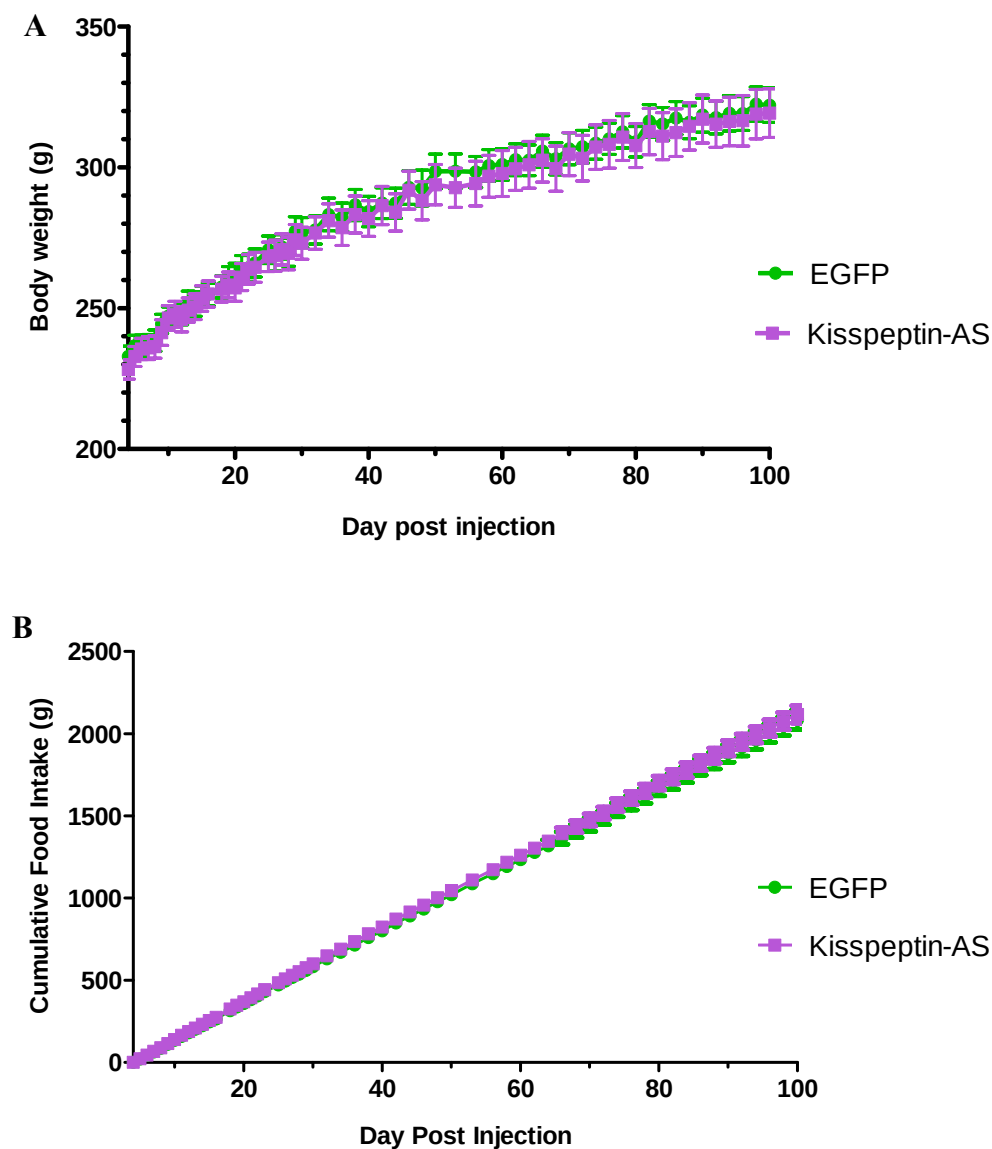


Figure 4.12: (A) Body weight and (B) cumulative food intake following bilateral intra-Arc injection of rAAV-EGFP or rAAV-Kisspeptin-AS to adult female Wistar rats. Body weight and food intake were measured every other day from day 4 to day 100 post-injection of rAAV-EGFP (green line) or rAAV-Kisspeptin-AS (pink line). Data is presented as mean $\pm$ SEM, n= 12-14 per group.

4.4. Discussion

In the work described in this chapter I have investigated the effects of rAAV-mediated expression of kisspeptin antisense RNA in the Arc of adult female rats on oestrous cyclicity, plasma levels of gonadotrophins and oestradiol, food intake and body weight.

In the rodent hypothalamus, kisspeptin neuronal cell bodies are predominantly located in the Arc and the AVPV (Clarkson and Herbison, 2006). Kisspeptin neurones in the Arc are hypothesised to regulate the tonic, pulsatile release of GnRH, and mediate the negative feedback effects of gonadal steroids on GnRH release (Smith et al., 2005a; Li et al., 2009). Conversely, kisspeptin neurones within the AVPV are thought to have a role in generating the preovulatory LH surge in females, and relay the positive feedback effect of oestrogen to GnRH neurones (Smith et al., 2005a; Kinoshita et al., 2005).

No studies have investigated the long-term effects of kisspeptin down-regulation in adult animals on the reproductive axis. Knockout models are extremely useful, but can be complicated by developmental compensation. Repeated injections of a kisspeptin antagonist or anti-kisspeptin antibodies avoid this problem, but are stressful for the animal, which may confound results. Recombinant AAV circumvents these problems and is a useful tool for investigating the long-term effects of transgene expression. The rAAV construct can be administered to adult animals, and rAAV-mediated transgene expression has been reported to be stable for up to 15 months in the rat CNS (Xu et al., 2001). Although the exact mechanism by which antisense RNA reduces protein expression is unknown, it is thought that antisense RNA binds to endogenous sense RNA, and hence forms a double-stranded RNA duplex. This duplex is then subjected to modification by ADAR enzymes, which convert approximately 50% of adenosine residues to inosines (Bass and Weintraub, 1988). The RNA duplex then becomes unstable and is retained in the nucleus, unable to be translated (Kumar and Carmichael, 1997). The newly formed mismatched RNA is also extremely susceptible to degradation by ribonucleases (Nishikura, 1992).

Vaginal lavage was performed daily to determine the cycle stage of each animal. Lavage was performed at a similar time each day to minimise variability and ensure consistency. Cycles were classified as regular, extended, or abnormal, as previously described (Goldman et al., 2007). A more stringent classification was also used to detect smaller changes between treatment groups; a 'complete' oestrous cycle was defined as one in which 3 out of 4 stages were observed in the correct order. Cycle length was calculated as the number of days between one oestrous and the

next. These methods have been previously used to evaluate the oestrous cycle in rodents (Goldman et al., 2007; Risvanli et al., 2010).

Intra-Arc administration of rAAV-Kisspeptin-AS to adult female rats significantly reduced the number of both regular and complete oestrous cycles over a 100-day period, and significantly increased the length of each cycle. This suggests that rAAV-Kisspeptin-AS administration lengthened each cycle sufficiently to result in a reduced number of total overall cycles. There was also a tendency for animals administered rAAV-Kisspeptin-AS to have more extended and abnormal cycles than controls, although this effect did not reach statistical significance. The percentage time spent in each stage of the cycle was calculated to determine whether rAAV-Kisspeptin-AS induced a tendency to remain in one stage of the cycle. However, no statistically significant differences were observed between groups.

Pulsatile GnRH release is crucial for normal functioning of the HPG axis. It has long been thought that the GnRH pulse generator resides in the Arc, as surgically isolating this nucleus in female rats induces persistent oestrous, but maintains pulsatile LH release (Blake and Sawyer, 1974). The exact mechanisms underlying the GnRH pulse generator are unknown. Numerous neurotransmitters, including NPY and glutamate, are known to regulate GnRH release (Crowley and Kalra, 1987; Bourguignon et al., 1989). Additionally, GnRH neurones possess intrinsic pulsatile firing properties (Wetsel et al., 1992). It has recently been suggested that Arc kisspeptin neurones may also regulate GnRH, and hence LH, pulsatility. Kisspeptin is released in a pulsatile fashion, and kisspeptin pulses closely match release of GnRH into the median eminence (Keen et al., 2008). In the goat, LH pulses closely follow multi-unit electrical activity volleys in the posterior Arc, where kisspeptin neurones reside (Ohkura et al., 2009). Additionally, intra-Arc administration of a kisspeptin antagonist to female rats prolongs LH pulse interval, resulting in fewer overall LH pulses measured over 8 hours (Li et al., 2009). This is presumed to be due to reduced stimulatory input to GnRH neurones. Administration of the same antagonist to the MPOA had no effect on LH pulse amplitude or frequency (Li et al., 2009).

It is therefore possible that intra-Arc administration of kisspeptin antisense reduced GnRH, and hence LH, pulse frequency, resulting in lengthened oestrous cycles. I was not able to measure the effect of intra-Arc rAAV-Kisspeptin-AS administration on LH pulsatility in this study; repeated blood sampling is stressful for rats and thus would likely disrupt oestrous cyclicity. However, it would be interesting to investigate this in the future. It is interesting to note that my results are in accord with previous work in which destruction of the Arc in female mice resulted in lengthening of oestrous cycles (May et al., 1989). At the time, the authors did not know which

neuronal population was responsible for this phenotype, but their findings may have reflected a reduction in Arc kisspeptin signalling.

Arcuate kisspeptin neurones are also thought to relay the negative feedback effects of gonadal steroids onto GnRH neurones (Smith et al., 2005a; Smith et al., 2005b). It would be interesting to investigate whether kisspeptin knockdown disrupts this process by, for example, determining whether oestradiol replacement is able to suppress high circulating LH levels induced by ovariectomy in female rats administered intra-Arc rAAV-Kisspeptin-AS.

My results suggest that intra-Arc administration of rAAV-Kisspeptin-AS increases the length of the oestrous cycle in adult female rats, but does not severely disrupt reproductive function. There are several reasons why this may not be. Firstly, the antisense construct may not have reduced kisspeptin expression sufficiently to have a marked phenotypic effect. Antisense RNA expression is unable to reduce protein levels by 100%, and so residual kisspeptin signalling may have been adequate to ensure that reproductive cycles continued. Previous published work from our laboratory has demonstrated that rAAV-NPY-AS administration into the Arc reduces hypothalamic NPY peptide content by over 50% at day fifteen post-injection (Gardiner et al., 2005). Although I have demonstrated transgene expression *in vivo* using *in situ* hybridisation, at the time of writing this thesis I have not yet been able to confirm that the rAAV-Kisspeptin-AS construct reduced arcuate levels of kisspeptin protein *in vivo*. There is currently no radioimmunoassay to quantify rat kisspeptin available, and I am currently in the process of establishing such an assay. Once this assay has been optimised, I plan to take punch biopsies of the arcuate nuclei of animals used in this study, and to compare levels of kisspeptin protein in rAAV-Kisspeptin-AS and rAAV-EGFP animals. At present it is therefore not possible to firmly conclude that the results obtained are due to a reduction in kisspeptin expression.

Secondly, it is possible that signalling by neuropeptides other than kisspeptin to GnRH neurones were sufficient to compensate for the reduction in kisspeptin, to ensure reproductive capacity was maintained. GnRH neurones receive stimulatory afferent inputs from numerous neurotransmitters including glutamate, NPY, galanin and vasoactive intestinal peptide (Bourguignon et al., 1989; Crowley and Kalra, 1987; Lopez and Negro-Vilar, 1990; Samson et al., 1981). Additionally, Arc kisspeptin neurones co-express dynorphin and NKB in several species (Goodman et al., 2007; Navarro et al., 2009). Administration of an NKB agonist increases plasma LH in female rats, and an autoregulatory circuit comprising kisspeptin, NKB and dynorphin signalling has been proposed to play an important role in generating pulsatile GnRH secretion (Navarro et al., 2011). The hypothesis that NKB signalling is an important

regulator of the HPG axis is supported by the finding that in humans, mutations of the genes encoding NKB or its receptor result in hypogonadotrophic hypogonadism (Topaloglu et al., 2009; Young et al., 2010). If Arc kisspeptin expression is reduced, NKB signalling may compensate to ensure reproductive capacity is maintained. However, such compensatory signalling is obviously insufficient to maintain fertility when either kisspeptin or GPR54 are completely absent, as KiSS-1 (d'Anglemont de Tassigny et al., 2007) and GPR54 (Seminara et al., 2003) knockout mice do not progress through puberty and are infertile.

Rats were culled in diestrous as this is when basal levels of LH and FSH are less variable, and hence small differences between groups may have been easier to detect. However, there were no differences in plasma LH between groups. This is perhaps not surprising, as LH secretion follows a pulsatile pattern, with peaks occurring every 20 to 40 minutes in the rat (Dierschke et al., 1970; Blake and Sawyer, 1974). It is therefore difficult to obtain true baseline measurements. Additionally, it has recently been demonstrated that ICV administration of a kisspeptin antagonist does not affect basal LH levels in male and female rats; only the pre-ovulatory LH surge was attenuated by antagonist administration in females (Roseweir et al., 2009; Pineda et al., 2010). It is likely that this represents blockade of kisspeptin neurones originating in the AVPV, not the Arc, as AVPV kisspeptin neurones become activated immediately prior to the LH surge (Smith et al., 2006b). Although plasma oestradiol levels were lower in animals administered rAAV-Kisspeptin-AS compared to EGFP controls, this effect did not reach statistical significance. It is perhaps unlikely that we would detect a significant change, as mice lacking a functional KiSS-1 gene do not have significantly lower plasma oestradiol levels compared to wild-type controls (d'Anglemont de Tassigny et al., 2007).

Expression of kisspeptin antisense RNA had no effect on food intake or body weight over a 100-day period. This confirms that the differences in oestrous cyclicity observed were not due to differences in energy status. Fertility is dependent on adequate body fat reserves. In rats, food deprivation and body weight loss suppress circulating LH and sex steroid levels and prevent normal reproductive function (Badger et al., 1985; Castellano et al., 2005; Terry et al., 2005; Glass et al., 1976). Kisspeptin has been hypothesised to mediate the permissive effects of leptin on the HPG axis, as Arc kisspeptin neurones co-express *ObRb* mRNA, and leptin-deficient *ob/ob* mice have significantly lower hypothalamic KiSS-1 mRNA levels than wild type controls (Smith et al., 2006a). It is therefore reassuring that there were no differences in body weight or food intake between groups, which may have confounded my results. Conversely, kisspeptin neurones have recently been shown to directly stimulate anorexigenic POMC neurones, and so

administration of kisspeptin antisense RNA might have been postulated to modulate energy homeostasis (Fu and van den Pol, 2010). However, no differences between groups were observed.

To continue with this work I would like to administer rAAV-Kisspeptin-AS to the AVPV of adult female rats, and monitor oestrous cyclicity to determine whether reproductive function is impaired. It would be particularly interesting to determine the effect of AVPV kisspeptin knockdown on the pre-ovulatory LH surge.

Kisspeptin signalling has a crucial role in the initiation of puberty. Humans and mice lacking GPR54 do not progress through puberty (de Roux N. et al., 2003; Seminara et al., 2003), and chronic ICV administration of kisspeptin to immature female rats advances pubertal onset (Navarro et al., 2004). Kisspeptin expression increases at the onset of puberty in both sexes, particularly in the AVPV, as does the number of appositions between kisspeptin immunoreactive fibres and GnRH neurones (Clarkson and Herbison, 2006). It would therefore be interesting to determine whether kisspeptin antisense administration to pre-pubertal male and female rats delays puberty.

In conclusion, my work has demonstrated that intra-Arc administration of kisspeptin antisense to adult female rats resulted in significantly fewer oestrous cycles over a 100 day period, and increased the average length of each cycle, compared to controls. No significant differences in plasma LH or oestradiol were observed. Although this work has begun to investigate the long-term effects of Arc kisspeptin knockdown on reproduction, more work is required to further characterise the importance of kisspeptin signalling in both the Arc and AVPV.

Chapter 5: General discussion

5.1. Introduction

The hypothalamus has a crucial role in maintaining energy homeostasis and reproduction. Nutrients, gut hormones and metabolic factors such as leptin signal to the hypothalamus, where appropriate downstream circuits are activated to modulate food intake and energy expenditure. Energy homeostasis and fertility are intimately linked, as fat reserves must be adequate to permit pubertal onset and normal functioning of the HPG axis, which is regulated by the hypothalamus.

The CNS reward system also has an important role in the control of feeding. It is hypothesised that dominance of hedonic signals over homeostatic signals may be a major contributing factor to the current obesity epidemic, particularly in an environment where palatable, high-fat food is readily available. However, the reward pathways involved in the regulation of feeding are not well characterised.

This thesis has described work investigating the role of the hypothalamus in food intake and reproduction, and the role of the reward system in food intake.

5.2. Investigating the role of Cerebellin 1 in the regulation of energy homeostasis and neuroendocrine function

Cerebellin 1 (Cbln1) is a 16-amino acid peptide derived from the precursor Pre-Cbln1 (Yiangou et al., 1989; Urade et al., 1991). Cbln1 was originally isolated from the cerebellum, and it is thought to have a role in cerebellar development and motor coordination (Slemmon et al., 1984; Hirai et al., 2005). Cbln1 is also highly expressed in the hypothalamus, particularly in the VMN (Burnet et al., 1988; Miura et al., 2006). My studies focused on the effect of Cbln1 on food intake and neuroendocrine function.

My work suggests that Cbln1 is a novel orexigenic peptide. ICV administration of Cbln1 significantly increased food intake in both fasted and *ad libitum* fed rats, although the effect was more pronounced in fed animals. Hypothalamic explants treated with Cbln1 had significantly increased NPY release, suggesting that Cbln1 may mediate its orexigenic effect via NPY. However, pre-treatment with an NPY Y1 receptor antagonist did not significantly attenuate the effect of Cbln1 on feeding *in vivo*, suggesting that signalling by this receptor is not necessary for the orexigenic effect of Cbln1, and that additional pathways are likely to be involved. It would be interesting to block other orexigenic hypothalamic neuropeptide pathways using, for example, antagonists or antibodies to the orexins and MCH, and determine the effect on Cbln1-induced

feeding. Alternatively, Cbln1 may reduce expression of the anorectic neuropeptide BDNF, which is, like Cbln1, highly expressed in the VMN (Xu et al., 2003).

I demonstrated that Pre-Cbln1 mRNA expression within the VMN altered following changes in nutritional status. This implies that Cbln1 may have a physiological role in the control of food intake. It would be interesting to measure levels of hypothalamic Pre-Cbln1 mRNA in lean and obese rodents; differences in expression may indicate a possible role for Cbln1 in the development of obesity.

ICV Cbln1 administration significantly reduced circulating levels of TSH. This suggests that in addition to increasing food intake, Cbln1 may reduce energy expenditure. Other orexigenic neuropeptides, for example NPY and AgRP, have also been shown to reduce energy expenditure (Fekete et al., 2001; Kim et al., 2000b). NPY decreases energy expenditure by reducing activity of the thyroid axis, and by reducing sympathetic outflow to brown adipose tissue (BAT), a major site of adaptive thermogenesis in rodents (Fekete et al., 2001; Egawa et al., 1991). It would be interesting to determine whether central administration of Cbln1 also affects the activity of BAT by, for example, quantifying UCP-1 expression.

It would be interesting to determine which hypothalamic pathways are activated following Cbln1 administration. I would like to localise and quantify expression of Fos, a marker of neuronal activation, in specific hypothalamic nuclei following central injection of Cbln1. It would also be interesting to perform dual staining for Fos and NPY following Cbln1 administration. If co-localisation was observed, it would confirm that NPY neurones are stimulated by Cbln1. Investigating whether Cbln1 is able to increase food intake in NPY knockout animals would determine whether Cbln1 is dependent on NPY for its orexigenic effect.

The receptor through which Cbln1 exerts its orexigenic effect is unknown, and therefore cannot be targeted by an antagonist. To determine whether a reduction in hypothalamic Cbln1 signalling affects food intake, it would be possible to administer an anti-Cbln1 antibody ICV. If anti-Cbln1 administration reduced food intake, it would provide strong evidence that Cbln1 has a physiological role in the control of energy intake. Administering Pre-Cbln1 anti-sense DNA via a suitable vector, such as rAAV, into the VMN of rats and monitoring their food intake and body weight would also help to determine the physiological role of Cbln1. Knockdown of Cbln1 expression would be expected to cause a reduction in food intake and body weight gain if Cbln1 has a physiological orexigenic role.

5.3. Investigating the role of VTA melanocortin signalling in the regulation of food intake

Cortico-limbic structures involved in cognition, emotion and reward play an important role in the control of feeding. The VTA, which predominantly contains dopamine neurones and GABAergic inter-neurones, is an important component of the CNS motivational circuitry and has a major role in mediating the rewarding and reinforcing properties of palatable food (Kenny, 2011).

Recently, it has been suggested that hypothalamic neuropeptides may have a role in the hedonic control of feeding, in addition to their role in maintaining energy homeostasis (Davis et al., 2010; Zheng et al., 2007). My studies focused on the effect of melanocortin administration into the VTA on food intake. I found that intra-VTA administration of NDP-MSH potently reduced food intake when administered to *ad libitum* fed rats, even at extremely low doses. However, NDP-MSH had no significant early effect on food intake when administered to fasted rats. This suggests that the effect of VTA melanocortin signalling on food intake may vary with nutritional status; in times of caloric deficit, homeostatic signals may override hedonic signals to promote food intake and protect body fat reserves. The anorectic effect of intra-VTA NDP-MSH administration was attenuated by pre-administration of a GABA_A receptor antagonist, suggesting that NDP-MSH may act via GABAergic interneurones in the VTA to reduce food intake.

Intra-VTA administration of AgRP increased food intake and body weight in *ad libitum* fed rats. This effect was attenuated by pre-treatment with a dopamine receptor antagonist. Similarly, injection of an MC4R antagonist stimulated feeding, and this effect was completely abolished by pre-treatment with a dopamine receptor antagonist. From these results it is possible to construct a hypothetical model of how melanocortin signalling in the VTA may influence feeding. Activation of melanocortin receptors within the VTA may stimulate GABAergic neurones, causing suppression of dopamine neuronal firing. Antagonism of melanocortin receptor signalling by AgRP may disinhibit dopamine neurones, leading to increased neuronal firing, increased dopamine release and increased food intake.

However, further work is required to support this hypothesis. Although the MC3R and MC4R have been found in the VTA (Mountjoy et al., 1994; Roselli-Reh fuss et al., 1993), co-localisation studies are necessary to determine whether melanocortin receptors are located on GABAergic and/or dopamine neurones. I would also like to determine whether intra-VTA administration of AgRP increases dopamine turnover in the NAc and pre-frontal cortex.

The finding that blocking MC4R signalling in the VTA increases food intake suggests that there is an endogenous melanocortin tone acting in the VTA. Mice lacking a functional MC4R gene

are obese and hyperphagic (Huszar et al., 1997). It is possible to purchase mice with a *loxP*-flanked transcriptional blocking sequence that prevents normal transcription and translation of the endogenous MC4R gene. The blocking sequence can be removed by injecting the enzyme Cre-recombinase. It would be interesting to investigate whether re-expression of the MC4R specifically within the VTA of these mice could partially reverse their obese, hyperphagic phenotype. This would help determine the importance of endogenous MC4R signalling in the VTA in the modulation of energy homeostasis.

In contrast to hypothalamic pathways, relatively little is known about the role of the reward system in feeding. There has been much interest recently in the role of the reward system in the development of obesity. It has been suggested that both hypo- and hyper-sensitivity to reward may predispose to obesity. An increase in the responsiveness of reward circuitries may lead to overeating and weight gain (Gautier et al., 2000). Conversely, ‘reward deficiency’ has been suggested to result in overconsumption of palatable food, to compensate for hypo-functioning of the reward circuitry. Reward deficiency may be caused by genetic factors; patients with a polymorphism downstream of the D2R gene have reduced D2R expression in the striatum, and are more likely to become obese (Stice et al., 2008). Additionally, studies in humans and rodents have suggested that over-consumption of palatable food and weight gain reduce D2R availability in the striatum and hence reduce responsiveness to reward (Colantuoni et al., 2001; Johnson and Kenny, 2010; Stice et al., 2010). Interestingly, the functional changes observed in the mesolimbic dopamine pathway in obese patients are similar to those observed in drug addicts, suggesting that there is overlap in the pathways controlling hedonic food intake and drug abuse (Wang et al., 1997a).

Although the reward system may be an attractive therapeutic target for treating obesity, care must be taken when manipulating this pathway. Rimonabant, a cannabinoid receptor antagonist, was previously prescribed as an effective weight loss agent. However, it was withdrawn from clinical practice in 2008. Its side-effects, which included severe depression and increased suicidal intention, may have been due to the actions of endogenous cannabinoids in the reward system. Once a better understanding of the reward system is achieved, more specific drugs targeting this pathway can be developed for the treatment of obesity.

5.4. Investigating the role of arcuate kisspeptin signalling in the control of reproduction

Kisspeptin signals upstream of GnRH to stimulate the HPG axis, and has a vital role in the control of reproduction (Irwig et al., 2004; Matsui et al., 2004; Pielecka-Fortuna et al., 2008). Kisspeptin neurones are hypothesised to relay metabolic signals, for example leptin, to GnRH neurones, and hence to couple energy reserves with fertility (Castellano et al., 2005; Smith et al., 2006a). However, recent studies suggest that leptin can influence fertility in the absence of kisspeptin signalling, suggesting that kisspeptin is not critical for this effect (Donato et al., 2010).

Kisspeptin neurones may also relay gonadal steroid signals to GnRH neurones. Within the rodent hypothalamus, kisspeptin neurones in the Arc are hypothesised to relay negative feedback signals to GnRH neurones, and to provide tonic stimulatory input to GnRH neurones. In contrast, kisspeptin neurones in the AVPV may relay positive feedback signals to GnRH neurones, and mediate the pre-ovulatory GnRH and LH surge (Smith et al., 2005a; Smith et al., 2005b; Dungan et al., 2007; Pineda et al., 2010).

I used recombinant AAV expressing kisspeptin antisense cDNA to investigate the effects of kisspeptin down-regulation in the rat Arc on reproductive function. I chose to use a viral vector because it permits spatial and temporal control of gene expression, and allows the phenotype resulting from transgene expression to be monitored for long periods of time.

Intra-Arc administration of rAAV-Kisspeptin-AS to adult female rats resulted in a significant reduction in the number of both regular and complete oestrous cycles between days 4 and 100 post-injection. Cycle length was also significantly increased in Kisspeptin-AS animals. Plasma analysis revealed no statistically significant differences in levels of LH or oestradiol between groups.

Kisspeptin neurones in the Arc are hypothesised to regulate the pulsatile release of GnRH into the pituitary portal circulation, and hence LH release from the anterior pituitary gland (Keen et al., 2008; Ohkura et al., 2009). It is therefore possible that the alterations in oestrous cyclicity observed in animals administered rAAV-Kisspeptin-AS were due to disruption of the GnRH pulse generator. Administration of a kisspeptin receptor antagonist into the Arc has been shown to suppress LH pulse frequency over 5 hours (Li et al., 2009). However, it is unclear whether this experiment specifically measured the effects of inhibiting Arc kisspeptin neuronal signalling, or whether the antagonist may, for example, have reached the median eminence and inhibited

kisspeptin signalling at this location. It would be interesting to monitor LH pulsatility in animals administered intra-Arc rAAV-Kisspeptin-AS in future studies.

This work has begun to elucidate the physiological role of kisspeptin signalling within the Arc. However, further studies are required to determine the role of hypothalamic kisspeptin. I plan to administer rAAV-Kisspeptin-AS into the AVPV of female rats and monitor oestrous cyclicity. It will be interesting to determine whether the pre-ovulatory LH surge and hence ovulation is impaired in these animals, as might be predicted from acute studies using antagonists or kisspeptin antibodies (Pineda et al., 2010; Kinoshita et al., 2005).

I am also establishing a radioimmunoassay for kisspeptin to quantify kisspeptin peptide levels in the Arc and AVPV of rAAV-Kisspeptin-AS-treated animals. It would be interesting to use this assay in conjunction with a hypothalamic explant system to determine the responsiveness of kisspeptin neurones to factors for which they express receptors, for example testosterone, oestrogen and leptin (Smith et al., 2005b; Smith et al., 2006a), at different stages of the oestrous cycle.

Kisspeptin is a promising agent for the treatment of reproductive disorders. Acute and chronic administration of kisspeptin-54 to women with hypothalamic amenorrhea has been shown to significantly increase plasma levels of LH and FSH (Jayasena et al., 2009; Jayasena et al., 2010). Further understanding of the kisspeptin system may therefore aid the development of novel agents for the treatment of infertility.

5.5. Final summary

In the work described in this thesis I have described a novel role for the neuropeptide Cbln1 in the control of energy homeostasis, and have begun to characterise the role of the melanocortins in reward pathway signalling. Further work is required to improve our understanding of both systems, and to determine how homeostatic and hedonic pathways interact to modulate feeding. I have also investigated the role of arcuate kisspeptin signalling in the control of reproduction in female rats, although more work is also required to further characterise this complex system.

Reference List

- Abbott,C.R., Rossi,M., Wren,A.M., Murphy,K.G., Kennedy,A.R., Stanley,S.A., Zollner,A.N., Morgan,D.G., Morgan,I., Ghatei,M.A., Small,C.J., and Bloom,S.R. (2001). Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. *Endocrinology* 142, 3457-3463.
- Abizaid,A., Liu,Z.W., Andrews,Z.B., Shanabrough,M., Borok,E., Elsworth,J.D., Roth,R.H., Sleeman,M.W., Picciotto,M.R., Tschop,M.H., Gao,X.B., and Horvath,T.L. (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J. Clin. Invest* 116, 3229-3239.
- Ahima,R.S., Prabakaran,D., Mantzoros,C., Qu,D., Lowell,B., Maratos-Flier,E., and Flier,J.S. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature* 382, 250-252.
- Albertin,G., Malendowicz,L.K., Macchi,C., Markowska,A., and Nussdorfer,G.G. (2000). Cerebellin stimulates the secretory activity of the rat adrenal gland: in vitro and in vivo studies. *Neuropeptides* 34, 7-11.
- Allen,Y.S., Adrian,T.E., Allen,J.M., Tatemoto,K., Crow,T.J., Bloom,S.R., and Polak,J.M. (1983). Neuropeptide Y distribution in the rat brain. *Science* 221, 877-879.
- Anand,B.K. and Brobeck,J.S. (1951). Localization of a "feeding center" in the hypothalamus of the rat. *Proc. Soc. Exp. Biol. Med.* 77, 323-324.
- Aponte,Y., Atasoy,D., and Sternson,S.M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat. Neurosci.*
- Arase,K., York,D.A., Shimizu,H., Shargill,N., and Bray,G.A. (1988). Effects of Corticotropin-Releasing Factor on Food-Intake and Brown Adipose-Tissue Thermogenesis in Rats. *American Journal of Physiology* 255, E255-E259.
- Aravich,P.F. and Scalfani,A. (1983). Paraventricular hypothalamic lesions and medial hypothalamic knife cuts produce similar hyperphagia syndromes. *Behav. Neurosci.* 97, 970-983.
- Asarian,L. and Geary,N. (2006). Modulation of appetite by gonadal steroid hormones. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 361, 1251-1263.
- Badger,T.M., Lynch,E.A., and Fox,P.H. (1985). Effects of fasting on luteinizing hormone dynamics in the male rat. *J. Nutr.* 115, 788-797.
- Bagnol,D., Lu,X.Y., Kaelin,C.B., Day,H.E., Ollmann,M., Gantz,I., Akil,H., Barsh,G.S., and Watson,S.J. (1999). Anatomy of an endogenous antagonist: relationship between Agouti-related protein and proopiomelanocortin in brain. *J. Neurosci.* 19, RC26.
- Bai,F.L., Yamano,M., Shiotani,Y., Emson,P.C., Smith,A.D., Powell,J.F., and Tohyama,M. (1985). An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. *Brain Res.* 331, 172-175.
- Banks,W.A., Kastin,A.J., Huang,W., Jaspan,J.B., and Maness,L.M. (1996). Leptin enters the brain by a saturable system independent of insulin. *Peptides* 17, 305-311.

- Bao,D., Pang,Z., and Morgan,J.I. (2005). The structure and proteolytic processing of Cbln1 complexes. *J. Neurochem.* 95, 618-629.
- Bartlett,J.S., Samulski,R.J., and McCown,T.J. (1998). Selective and rapid uptake of adeno-associated virus type 2 in brain. *Hum. Gene Ther.* 9, 1181-1186.
- Bass,B.L. and Weintraub,H. (1988). An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* 55, 1089-1098.
- Bauer-Dantoin,A.C., McDonald,J.K., and Levine,J.E. (1991). Neuropeptide Y potentiates luteinizing hormone (LH)-releasing hormone-stimulated LH surges in pentobarbital-blocked proestrous rats. *Endocrinology* 129, 402-408.
- Bayer,V.E. and Pickel,V.M. (1991). GABA-labeled terminals form proportionally more synapses with dopaminergic neurons containing low densities of tyrosine hydroxylase-immunoreactivity in rat ventral tegmental area. *Brain Res.* 559, 44-55.
- Belchetz,P.E., Plant,T.M., Nakai,Y., Keogh,E.J., and Knobil,E. (1978). Hypophysial responses to continuous and intermittent delivery of hypophthalamic gonadotropin-releasing hormone. *Science* 202, 631-633.
- Bell,S.M., Stewart,R.B., Thompson,S.C., and Meisch,R.A. (1997). Food-deprivation increases cocaine-induced conditioned place preference and locomotor activity in rats. *Psychopharmacology (Berl)* 131, 1-8.
- Benoit,S.C., Schwartz,M.W., Lachey,J.L., Hagan,M.M., Rushing,P.A., Blake,K.A., Yagaloff,K.A., Kurylko,G., Franco,L., Danhoo,W., and Seeley,R.J. (2000). A novel selective melanocortin-4 receptor agonist reduces food intake in rats and mice without producing aversive consequences. *J. Neurosci.* 20, 3442-3448.
- Bergevin,A., Girardot,D., Bourque,M.J., and Trudeau,L.E. (2002). Presynaptic mu-opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons. *Neuropharmacology* 42, 1065-1078.
- Berridge,K.C. (1996). Food reward: brain substrates of wanting and liking. *Neurosci. Biobehav. Rev.* 20, 1-25.
- Berthoud,H.R. (2006). Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance. *Obesity. (Silver. Spring)* 14 Suppl 5, 197S-200S.
- Berthoud,H.R. (2002). Multiple neural systems controlling food intake and body weight. *Neurosci. Biobehav. Rev.* 26, 393-428.
- Bilban,M., Ghaffari-Tabrizi,N., Hintermann,E., Bauer,S., Molzer,S., Zoratti,C., Malli,R., Sharabi,A., Hiden,U., Graier,W., Knofler,M., Andreae,F., Wagner,O., Quaranta,V., and Desoye,G. (2004). Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. *J. Cell Sci.* 117, 1319-1328.
- Billington,C.J., Briggs,J.E., Grace,M., and Levine,A.S. (1991). Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. *Am. J. Physiol* 260, R321-R327.
- Blake,C.A. and Sawyer,C.H. (1974). Effects of hypothalamic deafferentation on the pulsatile rhythm in plasma concentrations of luteinizing hormone in ovariectomized rats. *Endocrinology* 94, 730-736.
- Blake,N.G., Eckland,D.J., Foster,O.J., and Lightman,S.L. (1991). Inhibition of hypothalamic thyrotropin-releasing hormone messenger ribonucleic acid during food deprivation. *Endocrinology* 129, 2714-2718.

- Bourguignon,J.P., Gerard,A., Mathieu,J., Simons,J., and Franchimont,P. (1989). Pulsatile release of gonadotropin-releasing hormone from hypothalamic explants is restrained by blockade of N-methyl-D,L-aspartate receptors. *Endocrinology* 125, 1090-1096.
- Bradberry,C.W. and Roth,R.H. (1989). Cocaine increases extracellular dopamine in rat nucleus accumbens and ventral tegmental area as shown by in vivo microdialysis. *Neurosci. Lett.* 103, 97-102.
- Brady,L.S., Smith,M.A., Gold,P.W., and Herkenham,M. (1990). Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology* 52, 441-447.
- Brailoiu,G.C., Dun,S.L., Ohsawa,M., Yin,D., Yang,J., Chang,J.K., Brailoiu,E., and Dun,N.J. (2005). KiSS-1 expression and metastin-like immunoreactivity in the rat brain. *J. Comp Neurol.* 481, 314-329.
- Branco,M., Ribeiro,M., Negrao,N., and Bianco,A.C. (1999). 3,5,3'-Triiodothyronine actively stimulates UCP in brown fat under minimal sympathetic activity. *Am. J. Physiol* 276, E179-E187.
- Broadwell,R.D. and Brightman,M.W. (1976). Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood. *J. Comp Neurol.* 166, 257-283.
- Broberger,C., Johansen,J., Johansson,C., Schalling,M., and Hokfelt,T. (1998). The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. *Proc. Natl. Acad. Sci. U. S. A* 95, 15043-15048.
- Bryant,M.G. (1982). Radioactive Labelling. In *Radioimmunoassay of Gut Regulatory Peptides*, S.Bloom and R.G.Long, eds. W.B. Saunders Company Ltd.), pp. 21-27.
- Bull,L.S., Hurley,W.L., Kennett,W.S., Tamplin,C.B., and Williams,W.F. (1974). Effect of sex hormones on feed intake in rats. *J. Nutr.* 104, 968-975.
- Burnet,P.W., Bretherton-Watt,D., Ghatei,M.A., and Bloom,S.R. (1988). Cerebellin-like peptide: tissue distribution in rat and guinea-pig and its release from rat cerebellum, hypothalamus and cerebellar synaptosomes in vitro. *Neuroscience* 25, 605-612.
- Caine,S.B., Negus,S.S., Mello,N.K., and Bergman,J. (2000). Effects of dopamine D1-like and D2-like agonists in rats trained to discriminate cocaine from saline: influence of experimental history. *Exp. Clin. Psychopharmacol.* 8, 404-414.
- Campbell,B.A. and Fibiger,H.C. (1971). Potentiation of amphetamine-induced arousal by starvation. *Nature* 233, 424-425.
- Campfield,L.A., Smith,F.J., Guisez,Y., Devos,R., and Burn,P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269, 546-549.
- Canteras,N.S., Simerly,R.B., and Swanson,L.W. (1994). Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. *J. Comp Neurol.* 348, 41-79.
- Carr,D.B. and Sesack,S.R. (2000). GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. *Synapse* 38, 114-123.
- Carr,K.D., Kim,G., and Cabeza,d., V (2000). Hypoinsulinemia may mediate the lowering of self-stimulation thresholds by food restriction and streptozotocin-induced diabetes. *Brain Res.* 863, 160-168.
- Carr,K.D., Tsimberg,Y., Berman,Y., and Yamamoto,N. (2003). Evidence of increased dopamine receptor signaling in food-restricted rats. *Neuroscience* 119, 1157-1167.

- Carroll, M.E., France, C.P., and Meisch, R.A. (1979). Food deprivation increases oral and intravenous drug intake in rats. *Science* 205, 319-321.
- Carroll, M.E. and Meisch, R.A. (1981). Determinants of increased drug self-administration due to food deprivation. *Psychopharmacology (Berl)* 74, 197-200.
- Carter, C.J. (1982). Topographical distribution of possible glutamatergic pathways from the frontal cortex to the striatum and substantia nigra in rats. *Neuropharmacology* 21, 379-383.
- Castellano, J.M., Navarro, V.M., Fernandez-Fernandez, R., Nogueiras, R., Tovar, S., Roa, J., Vazquez, M.J., Vigo, E., Casanueva, F.F., Aguilar, E., Pinilla, L., Dieguez, C., and Tena-Sempere, M. (2005). Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology* 146, 3917-3925.
- Chamberlin, N.L., Du, B., de, L.S., and Saper, C.B. (1998). Recombinant adeno-associated virus vector: use for transgene expression and anterograde tract tracing in the CNS. *Brain Res.* 793, 169-175.
- Chehab, F.F., Lim, M.E., and Lu, R. (1996). Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat. Genet.* 12, 318-320.
- Chemelli, R.M., Willie, J.T., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., Fitch, T.E., Nakazato, M., Hammer, R.E., Saper, C.B., and Yanagisawa, M. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98, 437-451.
- Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L., Metzger, J.M., Strack, A.M., Camacho, R.E., Mellin, T.N., Nunes, C.N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D.E., Chen, H.Y., and Van Der Ploeg, L.H. (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat. Genet.* 26, 97-102.
- Cheung, C.C., Clifton, D.K., and Steiner, R.A. (1997). Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* 138, 4489-4492.
- Clarkson, J., d'Anglemont de Tassigny, X., Colledge, W.H., Caraty, A., and Herbison, A.E. (2009). Distribution of kisspeptin neurones in the adult female mouse brain. *J. Neuroendocrinol.* 21, 673-682.
- Clarkson, J., d'Anglemont de Tassigny, X., Moreno, A.S., Colledge, W.H., and Herbison, A.E. (2008). Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J. Neurosci.* 28, 8691-8697.
- Clarkson, J. and Herbison, A.E. (2006). Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* 147, 5817-5825.
- Clayton, R.N., Channabasavaiah, K., Stewart, J.M., and Catt, K.J. (1982). Hypothalamic regulation of pituitary gonadotropin-releasing hormone receptors: effects of hypothalamic lesions and a gonadotropin-releasing hormone antagonist. *Endocrinology* 110, 1108-1115.
- Clegg, D.J., Air, E.L., Benoit, S.C., Sakai, R.S., Seeley, R.J., and Woods, S.C. (2003). Intraventricular melanin-concentrating hormone stimulates water intake independent of food intake. *Am. J. Physiol Regul. Integr. Comp Physiol* 284, R494-R499.
- Colantuoni, C., Schwenker, J., McCarthy, J., Rada, P., Ladenheim, B., Cadet, J.L., Schwartz, G.J., Moran, T.H., and Hoebel, B.G. (2001). Excessive sugar intake alters binding to dopamine and mu-opioid receptors in the brain. *Neuroreport* 12, 3549-3552.

- Connors,J.M., DeVito,W.J., and Hedge,G.A. (1985). Effects of food deprivation on the feedback regulation of the hypothalamic-pituitary-thyroid axis of the rat. *Endocrinology* 117, 900-906.
- Considine,R.V., Sinha,M.K., Heiman,M.L., Kriauciunas,A., Stephens,T.W., Nyce,M.R., Ohannesian,J.P., Marco,C.C., McKee,L.J., Bauer,T.L., and . (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* 334, 292-295.
- Contreras,P.C. and Takemori,A.E. (1984). Antagonism of morphine-induced analgesia, tolerance and dependence by alpha-melanocyte-stimulating hormone. *J. Pharmacol. Exp. Ther.* 229, 21-26.
- Cousins,M.S., Wei,W., and Salamone,J.D. (1994). Pharmacological characterization of performance on a concurrent lever pressing/feeding choice procedure: effects of dopamine antagonist, cholinomimetic, sedative and stimulant drugs. *Psychopharmacology (Berl)* 116, 529-537.
- Cowley,M.A., Smart,J.L., Rubinstein,M., Cerdan,M.G., Diano,S., Horvath,T.L., Cone,R.D., and Low,M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411, 480-484.
- Crowley,W.R. and Kalra,S.P. (1987). Neuropeptide Y stimulates the release of luteinizing hormone-releasing hormone from medial basal hypothalamus in vitro: modulation by ovarian hormones. *Neuroendocrinology* 46, 97-103.
- d'Anglemont de Tassigny,X. and Colledge,W.H. (2010). The role of kisspeptin signaling in reproduction. *Physiology. (Bethesda.)* 25, 207-217.
- d'Anglemont de Tassigny,X., Fagg,L.A., Dixon,J.P., Day,K., Leitch,H.G., Hendrick,A.G., Zahn,D., Franceschini,I., Caraty,A., Carlton,M.B., Aparicio,S.A., and Colledge,W.H. (2007). Hypogonadotropic hypogonadism in mice lacking a functional *Kiss1* gene. *Proc. Natl. Acad. Sci. U. S. A* 104, 10714-10719.
- Dalton,L.D., Carpenter,R.G., and Grossman,S.P. (1981). Ingestive behavior in adult rats with dorsomedial hypothalamic lesions. *Physiol Behav.* 26, 117-123.
- Daniels,A.J., Grizzle,M.K., Wiard,R.P., Matthews,J.E., and Heyer,D. (2002). Food intake inhibition and reduction in body weight gain in lean and obese rodents treated with GW438014A, a potent and selective NPY-Y5 receptor antagonist. *Regul. Pept.* 106, 47-54.
- Date,Y., Murakami,N., Toshinai,K., Matsukura,S., Nijima,A., Matsuo,H., Kangawa,K., and Nakazato,M. (2002). The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 123, 1120-1128.
- David,V., Durkin,T.P., and Cazala,P. (1997). Self-administration of the GABAA antagonist bicuculline into the ventral tegmental area in mice: dependence on D2 dopaminergic mechanisms. *Psychopharmacology (Berl)* 130, 85-90.
- Davidowa,H., Heidel,E., and Plagemann,A. (2002). Differential involvement of dopamine D1 and D2 receptors and inhibition by dopamine of hypothalamic VMN neurons in early postnatally overfed juvenile rats. *Nutr. Neurosci.* 5, 27-36.
- Davis,J.F., Choi,D.L., Shurdak,J.D., Krause,E.G., Fitzgerald,M.F., Lipton,J.W., Sakai,R.R., and Benoit,S.C. (2010). Central melanocortins modulate mesocorticolimbic activity and food seeking behavior in the rat. *Physiol Behav.*
- Davis,M.E. (2002). Non-viral gene delivery systems. *Curr. Opin. Biotechnol.* 13, 128-131.

- de Quidt, M.E. and Emson, P.C. (1986). Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system--I. Radioimmunoassay and chromatographic characterisation. *Neuroscience* 18, 527-543.
- de Roux N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., and Milgrom, E. (2003). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. U. S. A* 100, 10972-10976.
- De Souza, M.J., Miller, B.E., Loucks, A.B., Luciano, A.A., Pescatello, L.S., Campbell, C.G., and Lasley, B.L. (1998). High frequency of luteal phase deficiency and anovulation in recreational women runners: blunted elevation in follicle-stimulating hormone observed during luteal-follicular transition. *J. Clin. Endocrinol. Metab* 83, 4220-4232.
- Dellovade, T.L., Young, M., Ross, E.P., Henderson, R., Caron, K., Parker, K., and Tobet, S.A. (2000). Disruption of the gene encoding SF-1 alters the distribution of hypothalamic neuronal phenotypes. *J. Comp Neurol.* 423, 579-589.
- Dhillon, W.S., Chaudhri, O.B., Patterson, M., Thompson, E.L., Murphy, K.G., Badman, M.K., McGowan, B.M., Amber, V., Patel, S., Ghatei, M.A., and Bloom, S.R. (2005). Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J. Clin. Endocrinol. Metab* 90, 6609-6615.
- Dhillon, H., Zigman, J.M., Ye, C., Lee, C.E., McGovern, R.A., Tang, V., Kenny, C.D., Christiansen, L.M., White, R.D., Edelstein, E.A., Coppari, R., Balthasar, N., Cowley, M.A., Chua S Jr, Elmquist, J.K., and Lowell, B.B. (2006). Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* 49, 191-203.
- Di Chiara, G. (2002). Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behav. Brain Res.* 137, 75-114.
- Di Chiara, G. and Imperato, A. (1988). Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *J. Pharmacol. Exp. Ther.* 244, 1067-1080.
- Dierschke, D.J., Bhattacharya, A.N., Atkinson, L.E., and Knobil, E. (1970). Circoral oscillations of plasma LH levels in the ovariectomized rhesus monkey. *Endocrinology* 87, 850-853.
- Donato, J., Cravo, R.M., Frazao, R., Gautron, L., Scott, M.M., Lachey, J., Castro, I.A., Margatho, L.O., Lee, S., Lee, C., Richardson, J.A., Friedman, J., Chua S Jr, Coppari, R., Zigman, J.M., Elmquist, J.K., and Elias, C.F. (2010). Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. *J. Clin. Invest.*
- Dong, J.Y., Fan, P.D., and Frizzell, R.A. (1996). Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum. Gene Ther.* 7, 2101-2112.
- Douglass, J., McKinzie, A.A., and Couceyro, P. (1995). PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. *J. Neurosci.* 15, 2471-2481.
- Dungan, H.M., Gottsch, M.L., Zeng, H., Gragerov, A., Bergmann, J.E., Vassilatis, D.K., Clifton, D.K., and Steiner, R.A. (2007). The role of kisspeptin-GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. *J. Neurosci.* 27, 12088-12095.
- Dunn, A.J., Berridge, C.W., Lai, Y.I., and Yachabach, T.L. (1987). CRF-induced excessive grooming behavior in rats and mice. *Peptides* 8, 841-844.
- Egawa, M., Yoshimatsu, H., and Bray, G.A. (1991). Neuropeptide Y suppresses sympathetic activity to interscapular brown adipose tissue in rats. *Am. J. Physiol* 260, R328-R334.

- Elias,C.F., Aschkenasi,C., Lee,C., Kelly,J., Ahima,R.S., Bjorbaek,C., Flier,J.S., Saper,C.B., and Elmquist,J.K. (1999). Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 23, 775-786.
- Elias,C.F., Lee,C., Kelly,J., Aschkenasi,C., Ahima,R.S., Couceyro,P.R., Kuhar,M.J., Saper,C.B., and Elmquist,J.K. (1998a). Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 21, 1375-1385.
- Elias,C.F., Saper,C.B., Maratos-Flier,E., Tritos,N.A., Lee,C., Kelly,J., Tatro,J.B., Hoffman,G.E., Ollmann,M.M., Barsh,G.S., Sakurai,T., Yanagisawa,M., and Elmquist,J.K. (1998b). Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *J. Comp Neurol.* 402, 442-459.
- Elmquist,J.K., Ahima,R.S., Elias,C.F., Flier,J.S., and Saper,C.B. (1998a). Leptin activates distinct projections from the dorsomedial and ventromedial hypothalamic nuclei. *Proc. Natl. Acad. Sci. U. S. A* 95, 741-746.
- Elmquist,J.K., Ahima,R.S., Maratos-Flier,E., Flier,J.S., and Saper,C.B. (1997). Leptin activates neurons in ventrobasal hypothalamus and brainstem. *Endocrinology* 138, 839-842.
- Elmquist,J.K., Bjorbaek,C., Ahima,R.S., Flier,J.S., and Saper,C.B. (1998b). Distributions of leptin receptor mRNA isoforms in the rat brain. *J. Comp Neurol.* 395, 535-547.
- Elsworth,J.D. and Roth,R.H. (1997). Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. *Exp. Neurol.* 144, 4-9.
- Erickson,J.C., Clegg,K.E., and Palmiter,R.D. (1996). Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* 381, 415-421.
- Estrada,K.M., Clay,C.M., Pompolo,S., Smith,J.T., and Clarke,I.J. (2006). Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. *J. Neuroendocrinol.* 18, 806-809.
- Fadel,J. and Deutch,A.Y. (2002). Anatomical substrates of orexin-dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. *Neuroscience* 111, 379-387.
- Fekete,C., Kelly,J., Mihaly,E., Sarkar,S., Rand,W.M., Legradi,G., Emerson,C.H., and Lechan,R.M. (2001). Neuropeptide Y has a central inhibitory action on the hypothalamic-pituitary-thyroid axis. *Endocrinology* 142, 2606-2613.
- Fekete,C., Legradi,G., Mihaly,E., Huang,Q.H., Tatro,J.B., Rand,W.M., Emerson,C.H., and Lechan,R.M. (2000). alpha-Melanocyte-stimulating hormone is contained in nerve terminals innervating thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and prevents fasting-induced suppression of prothyrotropin-releasing hormone gene expression. *J. Neurosci.* 20, 1550-1558.
- Ferrari,F.K., Samulski,T., Shenk,T., and Samulski,R.J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J. Virol.* 70, 3227-3234.
- Figlewicz,D.P., Bennett,J., Evans,S.B., Kaiyala,K., Sipols,A.J., and Benoit,S.C. (2004). Intraventricular insulin and leptin reverse place preference conditioned with high-fat diet in rats. *Behav. Neurosci.* 118, 479-487.
- Figlewicz,D.P., Bennett,J.L., Naleid,A.M., Davis,C., and Grimm,J.W. (2006). Intraventricular insulin and leptin decrease sucrose self-administration in rats. *Physiol Behav.* 89, 611-616.

- Figlewicz,D.P., Evans,S.B., Murphy,J., Hoen,M., and Baskin,D.G. (2003). Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat. *Brain Res.* 964, 107-115.
- Figlewicz,D.P., Higgins,M.S., Ng-Evans,S.B., and Havel,P.J. (2001). Leptin reverses sucrose-conditioned place preference in food-restricted rats. *Physiol Behav.* 73, 229-234.
- Figlewicz,D.P., Szot,P., Chavez,M., Woods,S.C., and Veith,R.C. (1994). Intraventricular insulin increases dopamine transporter mRNA in rat VTA/substantia nigra. *Brain Res.* 644, 331-334.
- Finn,P.D., Cunningham,M.J., Pau,K.Y., Spies,H.G., Clifton,D.K., and Steiner,R.A. (1998). The stimulatory effect of leptin on the neuroendocrine reproductive axis of the monkey. *Endocrinology* 139, 4652-4662.
- Fray,P.J., Sahakian,B.J., Robbins,T.W., Koob,G.F., and Iversen,S.D. (1980). An observational method for quantifying the behavioural effects of dopamine agonists: contrasting effects of d-amphetamine and apomorphine. *Psychopharmacology (Berl)* 69, 253-259.
- Frederich,R.C., Lollmann,B., Hamann,A., Napolitano-Rosen,A., Kahn,B.B., Lowell,B.B., and Flier,J.S. (1995). Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. *J. Clin. Invest* 96, 1658-1663.
- Fu,L.Y. and van den Pol,A.N. (2010). Kisspeptin directly excites anorexigenic proopiomelanocortin neurons but inhibits orexigenic neuropeptide Y cells by an indirect synaptic mechanism. *J. Neurosci.* 30, 10205-10219.
- Fukushima,M., Tokunaga,K., Lupien,J., Kemnitz,J.W., and Bray,G.A. (1987). Dynamic and static phases of obesity following lesions in PVN and VMH. *Am. J. Physiol* 253, R523-R529.
- Fulton,S., Pissios,P., Manchon,R.P., Stiles,L., Frank,L., Pothos,E.N., Maratos-Flier,E., and Flier,J.S. (2006). Leptin regulation of the mesoaccumbens dopamine pathway. *Neuron* 51, 811-822.
- Fulton,S., Woodside,B., and Shizgal,P. (2000). Modulation of brain reward circuitry by leptin. *Science* 287, 125-128.
- Funes,S., Hedrick,J.A., Vassileva,G., Markowitz,L., Abbondanzo,S., Golovko,A., Yang,S., Monsma,F.J., and Gustafson,E.L. (2003). The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem. Biophys. Res. Commun.* 312, 1357-1363.
- Gardiner,J.V., Kong,W.M., Ward,H., Murphy,K.G., Dhillo,W.S., and Bloom,S.R. (2005). AAV mediated expression of anti-sense neuropeptide Y cRNA in the arcuate nucleus of rats results in decreased weight gain and food intake. *Biochem. Biophys. Res. Commun.* 327, 1088-1093.
- Gardlik,R., Palffy,R., Hodosy,J., Lukacs,J., Turna,J., and Celec,P. (2005). Vectors and delivery systems in gene therapy. *Med. Sci. Monit.* 11, RA110-RA121.
- Gautier,J.F., Chen,K., Salbe,A.D., Bandy,D., Pratley,R.E., Heiman,M., Ravussin,E., Reiman,E.M., and Tataranni,P.A. (2000). Differential brain responses to satiation in obese and lean men. *Diabetes* 49, 838-846.
- Giraud,S.Q., Billington,C.J., and Levine,A.S. (1998). Feeding effects of hypothalamic injection of melanocortin 4 receptor ligands. *Brain Res.* 809, 302-306.
- Glass,A.R., Harrison,R., and Swerdloff,R.S. (1976). Effect of undernutrition and amino acid deficiency on the timing of puberty in rats. *Pediatr. Res.* 10, 951-955.

- Glaum, S.R., Hara, M., Bindokas, V.P., Lee, C.C., Polonsky, K.S., Bell, G.I., and Miller, R.J. (1996). Leptin, the obese gene product, rapidly modulates synaptic transmission in the hypothalamus. *Mol. Pharmacol.* 50, 230-235.
- Goldman, J.M., Murr, A.S., and Cooper, R.L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res. B Dev. Reprod. Toxicol.* 80, 84-97.
- Gonon, F.G. (1988). Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. *Neuroscience* 24, 19-28.
- Goodman, R.L., Lehman, M.N., Smith, J.T., Coolen, L.M., de Oliveira, C.V., Jafarzadehshirazi, M.R., Pereira, A., Iqbal, J., Caraty, A., Ciofi, P., and Clarke, I.J. (2007). Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* 148, 5752-5760.
- Gottsch, M.L., Cunningham, M.J., Smith, J.T., Popa, S.M., Acohido, B.V., Crowley, W.F., Seminara, S., Clifton, D.K., and Steiner, R.A. (2004). A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* 145, 4073-4077.
- Grill, H.J. and Smith, G.P. (1988). Cholecystokinin decreases sucrose intake in chronic decerebrate rats. *Am. J. Physiol* 254, R853-R856.
- Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J.A. (1998). Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum. Gene Ther.* 9, 2745-2760.
- Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol. Cell Biol.* 19, 1720-1730.
- Hagan, J.J., Leslie, R.A., Patel, S., Evans, M.L., Wattam, T.A., Holmes, S., Benham, C.D., Taylor, S.G., Routledge, C., Hemmati, P., Munton, R.P., Ashmeade, T.E., Shah, A.S., Hatcher, J.P., Hatcher, P.D., Jones, D.N., Smith, M.I., Piper, D.C., Hunter, A.J., Porter, R.A., and Upton, N. (1999). Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc. Natl. Acad. Sci. U. S. A* 96, 10911-10916.
- Hagan, M.M., Benoit, S.C., Rushing, P.A., Pritchard, L.M., Woods, S.C., and Seeley, R.J. (2001). Immediate and prolonged patterns of Agouti-related peptide-(83--132)-induced c-Fos activation in hypothalamic and extrahypothalamic sites. *Endocrinology* 142, 1050-1056.
- Hahn, T.M., Breininger, J.F., Baskin, D.G., and Schwartz, M.W. (1998). Coexpression of AgRP and NPY in fasting-activated hypothalamic neurons. *Nat. Neurosci.* 1, 271-272.
- Hakan, R.L. and Henriksen, S.J. (1989). Opiate influences on nucleus accumbens neuronal electrophysiology: dopamine and non-dopamine mechanisms. *J. Neurosci.* 9, 3538-3546.
- Hakansson, M.L., Brown, H., Ghilardi, N., Skoda, R.C., and Meister, B. (1998). Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *J. Neurosci.* 18, 559-572.
- Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269, 543-546.
- Halliday, G.M. and Tork, I. (1986). Comparative anatomy of the ventromedial mesencephalic tegmentum in the rat, cat, monkey and human. *J. Comp Neurol.* 252, 423-445.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557-580.

- Harris,A.R., Christianson,D., Smith,M.S., Fang,S.L., Braverman,L.E., and Vagenakis,A.G. (1978). The physiological role of thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone and prolactin secretion in the rat. *J. Clin. Invest* 61, 441-448.
- Heimer,L., Zahm,D.S., Churchill,L., Kalivas,P.W., and Wohltmann,C. (1991). Specificity in the projection patterns of accumbal core and shell in the rat. *Neuroscience* 41, 89-125.
- Hernandez,L. and Hoebel,B.G. (1988). Feeding and hypothalamic stimulation increase dopamine turnover in the accumbens. *Physiol Behav.* 44, 599-606.
- Hetherington, A. W and Ranson, S. W. Hypothalamic lesions and adiposity in the rat. *The Anatomical Record* 78[2], 149-172. 1940.
Ref Type: Generic
- Hewson,A.K. and Dickson,S.L. (2000). Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J. Neuroendocrinol.* 12, 1047-1049.
- Heymsfield,S.B., Greenberg,A.S., Fujioka,K., Dixon,R.M., Kushner,R., Hunt,T., Lubina,J.A., Patane,J., Self,B., Hunt,P., and McCamish,M. (1999). Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* 282, 1568-1575.
- Hikiji,K., Inoue,K., Iwasaki,S., Ichihara,K., and Kirriike,N. (2004). Local perfusion of mCPP into ventromedial hypothalamic nucleus, but not into lateral hypothalamic area and frontal cortex, inhibits food intake in rats. *Psychopharmacology (Berl)* 174, 190-196.
- Hirai,H., Pang,Z., Bao,D., Miyazaki,T., Li,L., Miura,E., Parris,J., Rong,Y., Watanabe,M., Yuzaki,M., and Morgan,J.I. (2005). Cbln1 is essential for synaptic integrity and plasticity in the cerebellum. *Nat. Neurosci.* 8, 1534-1541.
- Hochol,A., Neri,G., Majchrzak,M., Ziolkowska,A., Nussdorfer,G.G., and Malendowicz,L.K. (2001). Prolonged cerebellin administration inhibits the growth, but enhances steroidogenic capacity of rat adrenal cortex. *Endocr. Res.* 27, 11-17.
- Hoebel,B.G., Monaco,A.P., Hernandez,L., Aulisi,E.F., Stanley,B.G., and Lenard,L. (1983). Self-injection of amphetamine directly into the brain. *Psychopharmacology (Berl)* 81, 158-163.
- Holmes,A.M., Rudd,J.A., Tattersall,F.D., Aziz,Q., and Andrews,P.L. (2009). Opportunities for the replacement of animals in the study of nausea and vomiting. *Br. J. Pharmacol.* 157, 865-880.
- Hommel,J.D., Trinko,R., Sears,R.M., Georgescu,D., Liu,Z.W., Gao,X.B., Thurmon,J.J., Marinelli,M., and DiLeone,R.J. (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. *Neuron* 51, 801-810.
- Hou,J., Zheng,D.Z., Zhou,J.Y., and Zhou,S.W. (2010). Orexigenic effect of cocaine- and amphetamine-regulated transcript (CART) after injection into hypothalamic nuclei in streptozotocin-diabetic rats. *Clin. Exp. Pharmacol. Physiol* 37, 989-995.
- Hrabovszky,E., Ciofi,P., Vida,B., Horvath,M.C., Keller,E., Caraty,A., Bloom,S.R., Ghatei,M.A., Dhillo,W.S., Liposits,Z., and Kallo,I. (2010). The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. *Eur. J. Neurosci.* 31, 1984-1998.
- Hsu,R., Taylor,J.R., Newton,S.S., Alvaro,J.D., Haile,C., Han,G., Hruby,V.J., Nestler,E.J., and Duman,R.S. (2005). Blockade of melanocortin transmission inhibits cocaine reward. *Eur. J. Neurosci.* 21, 2233-2242.

- Hurd, Y.L., Lindefors, N., Brodin, E., Brene, S., Persson, H., Ungerstedt, U., and Hokfelt, T. (1992). Amphetamine regulation of mesolimbic dopamine/cholecystokinin neurotransmission. *Brain Res.* 578, 317-326.
- Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P., and Lee, F. (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88, 131-141.
- Ida, T., Nakahara, K., Katayama, T., Murakami, N., and Nakazato, M. (1999). Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioral activities of rats. *Brain Res.* 821, 526-529.
- Iijima, T., Miura, E., Matsuda, K., Kamekawa, Y., Watanabe, M., and Yuzaki, M. (2007). Characterization of a transneuronal cytokine family Cbln--regulation of secretion by heteromeric assembly. *Eur. J. Neurosci.* 25, 1049-1057.
- Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res. Rev.* 56, 27-78.
- Irwig, M.S., Fraley, G.S., Smith, J.T., Acohido, B.V., Popa, S.M., Cunningham, M.J., Gottsch, M.L., Clifton, D.K., and Steiner, R.A. (2004). Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology* 80, 264-272.
- Ito-Ishida, A., Miura, E., Emi, K., Matsuda, K., Iijima, T., Kondo, T., Kohda, K., Watanabe, M., and Yuzaki, M. (2008). Cbln1 regulates rapid formation and maintenance of excitatory synapses in mature cerebellar Purkinje cells in vitro and in vivo. *J. Neurosci.* 28, 5920-5930.
- Jackson, G.L., Kuehl, D., and Rhim, T.J. (1991). Testosterone inhibits gonadotropin-releasing hormone pulse frequency in the male sheep. *Biol. Reprod.* 45, 188-194.
- Jansen, A.S., Hoffman, J.L., and Loewy, A.D. (1997). CNS sites involved in sympathetic and parasympathetic control of the pancreas: a viral tracing study. *Brain Res.* 766, 29-38.
- Jayasena, C.N., Nijher, G.M., Abbara, A., Murphy, K.G., Lim, A., Patel, D., Mehta, A., Todd, C., Donaldson, M., Trew, G.H., Ghatei, M.A., Bloom, S.R., and Dhillo, W.S. (2010). Twice-weekly administration of kisspeptin-54 for 8 weeks stimulates release of reproductive hormones in women with hypothalamic amenorrhea. *Clin. Pharmacol. Ther.* 88, 840-847.
- Jayasena, C.N., Nijher, G.M., Chaudhri, O.B., Murphy, K.G., Ranger, A., Lim, A., Patel, D., Mehta, A., Todd, C., Ramachandran, R., Salem, V., Stamp, G.W., Donaldson, M., Ghatei, M.A., Bloom, S.R., and Dhillo, W.S. (2009). Subcutaneous injection of kisspeptin-54 acutely stimulates gonadotropin secretion in women with hypothalamic amenorrhea, but chronic administration causes tachyphylaxis. *J. Clin. Endocrinol. Metab* 94, 4315-4323.
- Johnson, P.M. and Kenny, P.J. (2010). Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats. *Nat. Neurosci.* 13, 635-641.
- Joseph, S.A., Pilcher, W.H., and Knigge, K.M. (1985). Anatomy of the corticotropin-releasing factor and opiomelanocortin systems of the brain. *Fed. Proc.* 44, 100-107.
- Josselyn, S.A. and Beninger, R.J. (1993). Neuropeptide Y: intraaccumbens injections produce a place preference that is blocked by cis-flupenthixol. *Pharmacol. Biochem. Behav.* 46, 543-552.
- Kalra, S.P. and Crowley, W.R. (1984). Norepinephrine-like effects of neuropeptide Y on LH release in the rat. *Life Sci.* 35, 1173-1176.

Kalra,S.P., Dube,M.G., Sahu,A., Phelps,C.P., and Kalra,P.S. (1991). Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. *Proc. Natl. Acad. Sci. U. S. A* 88, 10931-10935.

Kastin,A.J., Akerstrom,V., and Hackler,L. (2000). Agouti-related protein(83-132) aggregates and crosses the blood-brain barrier slowly. *Metabolism* 49, 1444-1448.

Kauffman,A.S., Gottsch,M.L., Roa,J., Byquist,A.C., Crown,A., Clifton,D.K., Hoffman,G.E., Steiner,R.A., and Tena-Sempere,M. (2007). Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* 148, 1774-1783.

Kebabian,J.W. (1978). Multiple classes of dopamine receptors in mammalian central nervous system: the involvement of dopamine-sensitive adenylyl cyclase. *Life Sci.* 23, 479-483.

Keen,K.L., Wegner,F.H., Bloom,S.R., Ghatei,M.A., and Terasawa,E. (2008). An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. *Endocrinology* 149, 4151-4157.

Kelsey,J.E., Carlezon,W.A., Jr., and Falls,W.A. (1989). Lesions of the nucleus accumbens in rats reduce opiate reward but do not alter context-specific opiate tolerance. *Behav. Neurosci.* 103, 1327-1334.

Kennedy,A.R., Todd,J.F., Stanley,S.A., Abbott,C.R., Small,C.J., Ghatei,M.A., and Bloom,S.R. (2001). Melanin-concentrating hormone (MCH) suppresses thyroid stimulating hormone (TSH) release, in vivo and in vitro, via the hypothalamus and the pituitary. *Endocrinology* 142, 3265-3268.

Kenny,P.J. (2011). Reward mechanisms in obesity: new insights and future directions. *Neuron* 69, 664-679.

Kernie,S.G., Liebl,D.J., and Parada,L.F. (2000). BDNF regulates eating behavior and locomotor activity in mice. *EMBO J.* 19, 1290-1300.

Kim,M.S., Rossi,M., Abbott,C.R., AlAhmed,S.H., Smith,D.M., and Bloom,S.R. (2002). Sustained orexigenic effect of Agouti related protein may be not mediated by the melanocortin 4 receptor. *Peptides* 23, 1069-1076.

Kim,M.S., Rossi,M., Abusnana,S., Sunter,D., Morgan,D.G., Small,C.J., Edwards,C.M., Heath,M.M., Stanley,S.A., Seal,L.J., Bhatti,J.R., Smith,D.M., Ghatei,M.A., and Bloom,S.R. (2000a). Hypothalamic localization of the feeding effect of agouti-related peptide and alpha-melanocyte-stimulating hormone. *Diabetes* 49, 177-182.

Kim,M.S., Small,C.J., Stanley,S.A., Morgan,D.G., Seal,L.J., Kong,W.M., Edwards,C.M., Abusnana,S., Sunter,D., Ghatei,M.A., and Bloom,S.R. (2000b). The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. *J. Clin. Invest* 105, 1005-1011.

Kinoshita,M., Tsukamura,H., Adachi,S., Matsui,H., Uenoyama,Y., Iwata,K., Yamada,S., Inoue,K., Ohtaki,T., Matsumoto,H., and Maeda,K. (2005). Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. *Endocrinology* 146, 4431-4436.

Kishore,U., Gaboriaud,C., Waters,P., Shrive,A.K., Greenhough,T.J., Reid,K.B., Sim,R.B., and Arlaud,G.J. (2004). C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends Immunol.* 25, 551-561.

Klentrou,P. and Plyley,M. (2003). Onset of puberty, menstrual frequency, and body fat in elite rhythmic gymnasts compared with normal controls. *Br. J. Sports Med.* 37, 490-494.

- Klusa, V., Svirskis, S., Opmane, B., Muceniece, R., Skujins, A., Mutulis, F., Wikberg, J.E., and Schioth, H.B. (1998). Evaluation of behavioural effects of neural melanocortin receptor antagonists injected ICV and in VTA in rats. *Neuropeptides* 32, 573-580.
- Kohno, D., Gao, H.Z., Muroya, S., Kikuyama, S., and Yada, T. (2003). Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca²⁺ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* 52, 948-956.
- Korotkova, T.M., Sergeeva, O.A., Eriksson, K.S., Haas, H.L., and Brown, R.E. (2003). Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. *J. Neurosci.* 23, 7-11.
- Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.M., Le, P.E., Brezillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C., Schiffmann, S.N., Vassart, G., and Parmentier, M. (2001). The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J. Biol. Chem.* 276, 34631-34636.
- Kreek, M.J., LaForge, K.S., and Butelman, E. (2002). Pharmacotherapy of addictions. *Nat. Rev. Drug Discov.* 1, 710-726.
- Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J., and Hastrup, S. (1998). Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393, 72-76.
- Kumar, M. and Carmichael, G.G. (1998). Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol. Mol. Biol. Rev.* 62, 1415-1434.
- Kumar, M. and Carmichael, G.G. (1997). Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts. *Proc. Natl. Acad. Sci. U. S. A* 94, 3542-3547.
- Kyrkouli, S.E., Stanley, B.G., and Leibowitz, S.F. (1986). Galanin: stimulation of feeding induced by medial hypothalamic injection of this novel peptide. *Eur. J. Pharmacol.* 122, 159-160.
- Lambert, P.D., Couceyro, P.R., McGirr, K.M., Il Vechia, S.E., Smith, Y., and Kuhar, M.J. (1998). CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* 29, 293-298.
- Lapatto, R., Pallais, J.C., Zhang, D., Chan, Y.M., Mahan, A., Cerrato, F., Le, W.W., Hoffman, G.E., and Seminara, S.B. (2007). Kiss1^{-/-} mice exhibit more variable hypogonadism than Gpr54^{-/-} mice. *Endocrinology* 148, 4927-4936.
- Lavin, A., Nogueira, L., Lapish, C.C., Wightman, R.M., Phillips, P.E., and Seamans, J.K. (2005). Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. *J. Neurosci.* 25, 5013-5023.
- Laviolette, S.R. and van der Kooy, D. (2004). The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat. Rev. Neurosci.* 5, 55-65.
- Laycock J and Wise P (1996). Female Reproductive Endocrinology. In *Essential Endocrinology*, Oxford University Press), pp. 163-202.
- Lebrethon, M.C., Vandersmissen, E., Gerard, A., Parent, A.S., Junien, J.L., and Bourguignon, J.P. (2000). In vitro stimulation of the prepubertal rat gonadotropin-releasing hormone pulse generator by leptin and neuropeptide Y through distinct mechanisms. *Endocrinology* 141, 1464-1469.
- Lee, D.K., Nguyen, T., O'Neill, G.P., Cheng, R., Liu, Y., Howard, A.D., Coulombe, N., Tan, C.P., Tang-Nguyen, A.T., George, S.R., and O'Dowd, B.F. (1999). Discovery of a receptor related to the galanin receptors. *FEBS Lett.* 446, 103-107.

- Legradi, G. and Lechan, R.M. (1999). Agouti-related protein containing nerve terminals innervate thyrotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. *Endocrinology* 140, 3643-3652.
- Leonard, C.M. (1969). The prefrontal cortex of the rat. I. Cortical projection of the mediodorsal nucleus. II. Efferent connections. *Brain Res.* 12, 321-343.
- Levine, A.S. and Morley, J.E. (1984). Neuropeptide Y: a potent inducer of consummatory behavior in rats. *Peptides* 5, 1025-1029.
- Lewis, D.E., Shellard, L., Koeslag, D.G., Boer, D.E., McCarthy, H.D., McKibbin, P.E., Russell, J.C., and Williams, G. (1993). Intense exercise and food restriction cause similar hypothalamic neuropeptide Y increases in rats. *Am. J. Physiol* 264, E279-E284.
- Li, X.F., Kinsey-Jones, J.S., Cheng, Y., Knox, A.M., Lin, Y., Petrou, N.A., Roseweir, A., Lightman, S.L., Milligan, S.R., Millar, R.P., and O'Byrne, K.T. (2009). Kisspeptin signalling in the hypothalamic arcuate nucleus regulates GnRH pulse generator frequency in the rat. *PLoS. One.* 4, e8334.
- Lindblom, J., Johansson, A., Holmgren, A., Grandin, E., Nedergard, C., Fredriksson, R., and Schioth, H.B. (2006). Increased mRNA levels of tyrosine hydroxylase and dopamine transporter in the VTA of male rats after chronic food restriction. *Eur. J. Neurosci.* 23, 180-186.
- Ljungberg, T., Apicella, P., and Schultz, W. (1992). Responses of monkey dopamine neurons during learning of behavioral reactions. *J. Neurophysiol.* 67, 145-163.
- Loeb, J.E., Cordier, W.S., Harris, M.E., Weitzman, M.D., and Hope, T.J. (1999). Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. *Hum. Gene Ther.* 10, 2295-2305.
- Lopez, F.J. and Negro-Vilar, A. (1990). Galanin stimulates luteinizing hormone-releasing hormone secretion from arcuate nucleus-median eminence fragments in vitro: involvement of an alpha-adrenergic mechanism. *Endocrinology* 127, 2431-2436.
- Lu, X.Y., Nicholson, J.R., Akil, H., and Watson, S.J. (2001). Time course of short-term and long-term orexigenic effects of Agouti-related protein (86-132). *Neuroreport* 12, 1281-1284.
- Lu, Y. (2004). Recombinant adeno-associated virus as delivery vector for gene therapy--a review. *Stem Cells Dev.* 13, 133-145.
- Lynch, R.M., Tompkins, L.S., Brooks, H.L., Dunn-Meynell, A.A., and Levin, B.E. (2000). Localization of glucokinase gene expression in the rat brain. *Diabetes* 49, 693-700.
- Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., and . (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat. Med.* 1, 1155-1161.
- Mahler, S.V., Smith, K.S., and Berridge, K.C. (2007). Endocannabinoid hedonic hotspot for sensory pleasure: anandamide in nucleus accumbens shell enhances 'liking' of a sweet reward. *Neuropsychopharmacology* 32, 2267-2278.
- Majdic, G., Young, M., Gomez-Sanchez, E., Anderson, P., Szczepaniak, L.S., Dobbins, R.L., McGarry, J.D., and Parker, K.L. (2002). Knockout mice lacking steroidogenic factor 1 are a novel genetic model of hypothalamic obesity. *Endocrinology* 143, 607-614.
- Maldonado, R., Saiardi, A., Valverde, O., Samad, T.A., Roques, B.P., and Borrelli, E. (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* 388, 586-589.

- Mandl,A.M. (1951). The phases of the oestrous cycle in the adult white rat. *J. Exp. Biol* 28, 576-584.
- Marcondes,F.K., Bianchi,F.J., and Tanno,A.P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. *Braz. J. Biol* 62, 609-614.
- Marcus,J.N., Aschkenasi,C.J., Lee,C.E., Chemelli,R.M., Saper,C.B., Yanagisawa,M., and Elmquist,J.K. (2001). Differential expression of orexin receptors 1 and 2 in the rat brain. *J. Comp Neurol.* 435, 6-25.
- Margules,D.L. and Olds,J. (1962). Identical "feeding" and "rewarding" systems in the lateral hypothalamus of rats. *Science* 135, 374-375.
- Maric,T., Tobin,S., Quinn,T., and Shalev,U. (2008). Food deprivation-like effects of neuropeptide Y on heroin self-administration and reinstatement of heroin seeking in rats. *Behav. Brain Res.* 194, 39-43.
- Matsuda,K., Miura,E., Miyazaki,T., Kakegawa,W., Emi,K., Narumi,S., Fukazawa,Y., Ito-Ishida,A., Kondo,T., Shigemoto,R., Watanabe,M., and Yuzaki,M. (2010). Cbln1 is a ligand for an orphan glutamate receptor delta2, a bidirectional synapse organizer. *Science* 328, 363-368.
- Matsui,H., Takatsu,Y., Kumano,S., Matsumoto,H., and Ohtaki,T. (2004). Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. *Biochem. Biophys. Res. Commun.* 320, 383-388.
- Matthews,R.T. and German,D.C. (1984). Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. *Neuroscience* 11, 617-625.
- May,P.C., Kohama,S.G., and Finch,C.E. (1989). N-methyl-aspartic acid lesions of the arcuate nucleus in adult C57BL/6J mice: a new model for age-related lengthening of the estrous cycle. *Neuroendocrinology* 50, 605-612.
- Mazzocchi,G., Andreis,P.G., De,C.R., Aragona,F., Gottardo,L., and Nussdorfer,G.G. (1999). Cerebellin enhances in vitro secretory activity of human adrenal gland. *J. Clin. Endocrinol. Metab* 84, 632-635.
- Mercer,J.G., Hoggard,N., Williams,L.M., Lawrence,C.B., Hannah,L.T., Morgan,P.J., and Trayhurn,P. (1996a). Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. *J. Neuroendocrinol.* 8, 733-735.
- Mercer,J.G., Hoggard,N., Williams,L.M., Lawrence,C.B., Hannah,L.T., and Trayhurn,P. (1996b). Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett.* 387, 113-116.
- Meredith,G.E. (1999). The synaptic framework for chemical signaling in nucleus accumbens. *Ann. N. Y. Acad. Sci.* 877, 140-156.
- Messenger,S., Chatzidaki,E.E., Ma,D., Hendrick,A.G., Zahn,D., Dixon,J., Thresher,R.R., Malinge,I., Lomet,D., Carlton,M.B., Colledge,W.H., Caraty,A., and Aparicio,S.A. (2005). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc. Natl. Acad. Sci. U. S. A* 102, 1761-1766.
- Miura,E., Iijima,T., Yuzaki,M., and Watanabe,M. (2006). Distinct expression of Cbln family mRNAs in developing and adult mouse brains. *Eur. J. Neurosci.* 24, 750-760.
- Mizuno,Y., Takahashi,K., Totsune,K., Ohneda,M., Konno,H., Murakami,O., Satoh,F., Sone,M., Takase,S., Itoyama,Y., and . (1995). Decrease in cerebellin and corticotropin-releasing hormone in the cerebellum of olivopontocerebellar atrophy and Shy-Drager syndrome. *Brain Res.* 686, 115-118.

- Montague, C.T., Farooqi, I.S., Whitehead, J.P., Soos, M.A., Rau, H., Wareham, N.J., Sewter, C.P., Digby, J.E., Mohammed, S.N., Hurst, J.A., Cheetham, C.H., Earley, A.R., Barnett, A.H., Prins, J.B., and O'Rahilly, S. (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 387, 903-908.
- Morgan, J.I., Slemmon, J.R., Danho, W., Hempstead, J., Berrebi, A.S., and Mugnaini, E. (1988). Cerebellin and related postsynaptic peptides in the brain of normal and neurodevelopmentally mutant vertebrates. *Synapse* 2, 117-124.
- Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.* 8, 1298-1308.
- Mugnaini, E. and Morgan, J.I. (1987). The neuropeptide cerebellin is a marker for two similar neuronal circuits in rat brain. *Proc. Natl. Acad. Sci. U. S. A* 84, 8692-8696.
- Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekeres, P.G., Sarau, H.M., Chambers, J.K., Murdock, P., Steplewski, K., Shabon, U., Miller, J.E., Middleton, S.E., Darker, J.G., Larminie, C.G., Wilson, S., Bergsma, D.J., Emson, P., Faull, R., Philpott, K.L., and Harrison, D.C. (2001). AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J. Biol. Chem.* 276, 28969-28975.
- Munzberg, H. (2008). Differential leptin access into the brain--a hierarchical organization of hypothalamic leptin target sites? *Physiol Behav.* 94, 664-669.
- Naleid, A.M., Grace, M.K., Cummings, D.E., and Levine, A.S. (2005). Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. *Peptides* 26, 2274-2279.
- Narita, M., Nagumo, Y., Hashimoto, S., Narita, M., Khotib, J., Miyatake, M., Sakurai, T., Yanagisawa, M., Nakamachi, T., Shioda, S., and Suzuki, T. (2006). Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. *J. Neurosci.* 26, 398-405.
- Nauta, W.J., Smith, G.P., Faull, R.L., and Domesick, V.B. (1978). Efferent connections and nigral afferents of the nucleus accumbens septi in the rat. *Neuroscience* 3, 385-401.
- Navarro, V.M., Castellano, J.M., McConkey, S.M., Pineda, R., Ruiz-Pino, F., Pinilla, L., Clifton, D.K., Tena-Sempere, M., and Steiner, R.A. (2011). Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am. J. Physiol Endocrinol. Metab* 300, E202-E210.
- Navarro, V.M., Fernandez-Fernandez, R., Castellano, J.M., Roa, J., Mayen, A., Barreiro, M.L., Gaytan, F., Aguilar, E., Pinilla, L., Dieguez, C., and Tena-Sempere, M. (2004). Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *J. Physiol* 561, 379-386.
- Navarro, V.M., Gottsch, M.L., Chavkin, C., Okamura, H., Clifton, D.K., and Steiner, R.A. (2009). Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J. Neurosci.* 29, 11859-11866.
- Nequin, L.G. and Schwartz, N.B. (1971). Adrenal participation in the timing of mating and LH release in the cyclic rat. *Endocrinology* 88, 325-331.
- Nestler, E.J. (2005). Is there a common molecular pathway for addiction? *Nat. Neurosci.* 8, 1445-1449.

- Nishikura, K. (1992). Modulation of double-stranded RNAs in vivo by RNA duplex unwindase. *Ann. N. Y. Acad. Sci.* 660, 240-250.
- O'Donnell, P. and Grace, A.A. (1995). Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input. *J. Neurosci.* 15, 3622-3639.
- O'Shea, D., Morgan, D.G., Meeran, K., Edwards, C.M., Turton, M.D., Choi, S.J., Heath, M.M., Gunn, I., Taylor, G.M., Howard, J.K., Bloom, C.I., Small, C.J., Haddo, O., Ma, J.J., Callinan, W., Smith, D.M., Ghatei, M.A., and Bloom, S.R. (1997). Neuropeptide Y induced feeding in the rat is mediated by a novel receptor. *Endocrinology* 138, 196-202.
- Oakman, S.A., Faris, P.L., Kerr, P.E., Cozzari, C., and Hartman, B.K. (1995). Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area. *J. Neurosci.* 15, 5859-5869.
- Ohkura, S., Takase, K., Matsuyama, S., Mogi, K., Ichimaru, T., Wakabayashi, Y., Uenoyama, Y., Mori, Y., Steiner, R.A., Tsukamura, H., Maeda, K.I., and Okamura, H. (2009). Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J. Neuroendocrinol.* 21, 813-821.
- Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., and Fujino, M. (2001). Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 411, 613-617.
- Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., and Barsh, G.S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 278, 135-138.
- Olson, V.G. and Nestler, E.J. (2007). Topographical organization of GABAergic neurons within the ventral tegmental area of the rat. *Synapse* 61, 87-95.
- Ono, M., Ichihara, J., Nonomura, T., Itakura, Y., Taiji, M., Nakayama, C., and Noguchi, H. (1997). Brain-derived neurotrophic factor reduces blood glucose level in obese diabetic mice but not in normal mice. *Biochem. Biophys. Res. Commun.* 238, 633-637.
- Overton, P.G. and Clark, D. (1997). Burst firing in midbrain dopaminergic neurons. *Brain Res. Brain Res. Rev.* 25, 312-334.
- Pang, Z., Zuo, J., and Morgan, J.I. (2000). Cbln3, a novel member of the precerebellin family that binds specifically to Cbln1. *J. Neurosci.* 20, 6333-6339.
- Patterson, T.A., Brot, M.D., Zavosh, A., Schenk, J.O., Szot, P., and Figlewicz, D.P. (1998). Food deprivation decreases mRNA and activity of the rat dopamine transporter. *Neuroendocrinology* 68, 11-20.
- Paxinos, G. and Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates*. Elsevier).
- Pecina, S. and Berridge, K.C. (2000). Opioid site in nucleus accumbens shell mediates eating and hedonic 'liking' for food: map based on microinjection Fos plumes. *Brain Res.* 863, 71-86.
- Pecina, S., Cagniard, B., Berridge, K.C., Aldridge, J.W., and Zhuang, X. (2003). Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. *J. Neurosci.* 23, 9395-9402.
- Pelley, M.A., Cullen, M.J., and Wellman, C.L. (1995). Characteristics of BDNF-induced weight loss. *Exp. Neurol.* 131, 229-238.

- Peoples,L.L. and West,M.O. (1996). Phasic firing of single neurons in the rat nucleus accumbens correlated with the timing of intravenous cocaine self-administration. *J. Neurosci.* *16*, 3459-3473.
- Pettit,H.O. and Justice,J.B., Jr. (1989). Dopamine in the nucleus accumbens during cocaine self-administration as studied by in vivo microdialysis. *Pharmacol. Biochem. Behav.* *34*, 899-904.
- Phillips,A.G. and Nikaido,R.S. (1975). Disruption of brain stimulation-induced feeding by dopamine receptor blockade. *Nature* *258*, 750-751.
- Pielecka-Fortuna,J., Chu,Z., and Moenter,S.M. (2008). Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. *Endocrinology* *149*, 1979-1986.
- Pineda,R., Garcia-Galiano,D., Roseweir,A., Romero,M., Sanchez-Garrido,M.A., Ruiz-Pino,F., Morgan,K., Pinilla,L., Millar,R.P., and Tena-Sempere,M. (2010). Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology* *151*, 722-730.
- Popa,S.M., Clifton,D.K., and Steiner,R.A. (2008). The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. *Annu. Rev. Physiol* *70*, 213-238.
- Pothos,E.N., Creese,I., and Hoebel,B.G. (1995). Restricted eating with weight loss selectively decreases extracellular dopamine in the nucleus accumbens and alters dopamine response to amphetamine, morphine, and food intake. *J. Neurosci.* *15*, 6640-6650.
- Pothos,E.N., Sulzer,D., and Hoebel,B.G. (1998). Plasticity of quantal size in ventral midbrain dopamine neurons: possible implications for the neurochemistry of feeding and reward. *Appetite* *31*, 405.
- Qu,D., Ludwig,D.S., Gammeltoft,S., Piper,M., Pelleymounter,M.A., Cullen,M.J., Mathes,W.F., Przypek,R., Kanarek,R., and Maratos-Flier,E. (1996). A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature* *380*, 243-247.
- Rachon,D. and Teede,H. (2010). Ovarian function and obesity--interrelationship, impact on women's reproductive lifespan and treatment options. *Mol. Cell Endocrinol.* *316*, 172-179.
- Risvanli,A., Apaydin,A.M., Bulut,H., Timurkaan,N., and Saat,N. (2010). The effects of Kisspeptin antibodies on delayed estrus in rats. *Gynecol. Endocrinol.* *26*, 297-301.
- Ritz,M.C., Lamb,R.J., Goldberg,S.R., and Kuhar,M.J. (1987). Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* *237*, 1219-1223.
- Roberts,D.C., Koob,G.F., Klonoff,P., and Fibiger,H.C. (1980). Extinction and recovery of cocaine self-administration following 6-hydroxydopamine lesions of the nucleus accumbens. *Pharmacol. Biochem. Behav.* *12*, 781-787.
- Roselli-Reh fuss,L., Mountjoy,K.G., Robbins,L.S., Mortrud,M.T., Low,M.J., Tatro,J.B., Entwistle,M.L., Simerly,R.B., and Cone,R.D. (1993). Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc. Natl. Acad. Sci. U. S. A* *90*, 8856-8860.
- Roseweir,A.K., Kauffman,A.S., Smith,J.T., Guerriero,K.A., Morgan,K., Pielecka-Fortuna,J., Pineda,R., Gottsch,M.L., Tena-Sempere,M., Moenter,S.M., Terasawa,E., Clarke,I.J., Steiner,R.A., and Millar,R.P. (2009). Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J. Neurosci.* *29*, 3920-3929.

- Rossi,M., Choi,S.J., O'Shea,D., Miyoshi,T., Ghatei,M.A., and Bloom,S.R. (1997). Melanin-concentrating hormone acutely stimulates feeding, but chronic administration has no effect on body weight. *Endocrinology* 138, 351-355.
- Rossi,M., Kim,M.S., Morgan,D.G., Small,C.J., Edwards,C.M., Sunter,D., Abusnana,S., Goldstone,A.P., Russell,S.H., Stanley,S.A., Smith,D.M., Yagaloff,K., Ghatei,M.A., and Bloom,S.R. (1998). A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology* 139, 4428-4431.
- Rucinski,M. and Malendowicz,L.K. (2009). Precerebellin-related genes and precerebellin 1 peptide in endocrine glands of the rat - pattern of their expression. *Int. J. Mol. Med.* 23, 113-119.
- Sahu,A., Crowley,W.R., Tatemoto,K., Balasubramaniam,A., and Kalra,S.P. (1987). Effects of neuropeptide Y, NPY analog (norleucine4-NPY), galanin and neuropeptide K on LH release in ovariectomized (ovx) and ovx estrogen, progesterone-treated rats. *Peptides* 8, 921-926.
- Sakurai,T., Amemiya,A., Ishii,M., Matsuzaki,I., Chemelli,R.M., Tanaka,H., Williams,S.C., Richardson,J.A., Kozlowski,G.P., Wilson,S., Arch,J.R., Buckingham,R.E., Haynes,A.C., Carr,S.A., Annan,R.S., McNulty,D.E., Liu,W.S., Terrett,J.A., Elshourbagy,N.A., Bergsma,D.J., and Yanagisawa,M. (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 1.
- Samson,W.K., Burton,K.P., Reeves,J.P., and McCann,S.M. (1981). Vasoactive intestinal peptide stimulates luteinizing hormone-releasing hormone release from median eminence synaptosomes. *Regul. Pept.* 2, 253-264.
- Saper,C.B., Swanson,L.W., and Cowan,W.M. (1979). An autoradiographic study of the efferent connections of the lateral hypothalamic area in the rat. *J. Comp Neurol.* 183, 689-706.
- Sarkar,S. and Lechan,R.M. (2003). Central administration of neuropeptide Y reduces alpha-melanocyte-stimulating hormone-induced cyclic adenosine 5'-monophosphate response element binding protein (CREB) phosphorylation in pro-thyrotropin-releasing hormone neurons and increases CREB phosphorylation in corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. *Endocrinology* 144, 281-291.
- Satoh,F., Takahashi,K., Murakami,O., Totsune,K., Ohneda,M., Mizuno,Y., Sone,M., Miura,Y., Takase,S., Hayashi,Y., Sasano,H., and Mouri,T. (1997). Cerebellin and cerebellin mRNA in the human brain, adrenal glands and the tumour tissues of adrenal tumour, ganglioneuroblastoma and neuroblastoma. *J. Endocrinol.* 154, 27-34.
- Sawyer,T.K., Sanfilippo,P.J., Hruby,V.J., Engel,M.H., Heward,C.B., Burnett,J.B., and Hadley,M.E. (1980). 4-Norleucine, 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity. *Proc. Natl. Acad. Sci. U. S. A* 77, 5754-5758.
- Schwartz,M.W., Erickson,J.C., Baskin,D.G., and Palmiter,R.D. (1998). Effect of fasting and leptin deficiency on hypothalamic neuropeptide Y gene transcription in vivo revealed by expression of a lacZ reporter gene. *Endocrinology* 139, 2629-2635.
- Schwartz,M.W., Marks,J.L., Sipols,A.J., Baskin,D.G., Woods,S.C., Kahn,S.E., and Porte,D., Jr. (1991). Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. *Endocrinology* 128, 2645-2647.
- Schwartz,M.W., Seeley,R.J., Woods,S.C., Weigle,D.S., Campfield,L.A., Burn,P., and Baskin,D.G. (1997). Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 46, 2119-2123.

- Schwartz,M.W., Woods,S.C., Porte,D., Jr., Seeley,R.J., and Baskin,D.G. (2000). Central nervous system control of food intake. *Nature* 404, 661-671.
- Scott,V., Kimura,N., Stark,J.A., and Luckman,S.M. (2005). Intravenous peptide YY3-36 and Y2 receptor antagonism in the rat: effects on feeding behaviour. *J. Neuroendocrinol.* 17, 452-457.
- Seeman,P. and Van Tol,H.H. (1994). Dopamine receptor pharmacology. *Trends Pharmacol. Sci.* 15, 264-270.
- Segal,J.P., Stallings,N.R., Lee,C.E., Zhao,L., Socci,N., Viale,A., Harris,T.M., Soares,M.B., Childs,G., Elmquist,J.K., Parker,K.L., and Friedman,J.M. (2005). Use of laser-capture microdissection for the identification of marker genes for the ventromedial hypothalamic nucleus. *J. Neurosci.* 25, 4181-4188.
- Seminara,S.B., Messenger,S., Chatzidaki,E.E., Thresher,R.R., Acierno,J.S., Jr., Shagoury,J.K., Bo-Abbas,Y., Kuohung,W., Schwinof,K.M., Hendrick,A.G., Zahn,D., Dixon,J., Kaiser,U.B., Slaugenhaupt,S.A., Gusella,J.F., O'Rahilly,S., Carlton,M.B., Crowley,W.F., Jr., Aparicio,S.A., and Colledge,W.H. (2003). The GPR54 gene as a regulator of puberty. *N. Engl. J. Med.* 349, 1614-1627.
- Semple,R.K., Achermann,J.C., Ellery,J., Farooqi,I.S., Karet,F.E., Stanhope,R.G., O'Rahilly,S., and Aparicio,S.A. (2005). Two novel missense mutations in g protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. *J. Clin. Endocrinol. Metab* 90, 1849-1855.
- Seth,A., Stanley,S., Dhillo,W., Murphy,K., Ghatei,M., and Bloom,S. (2003). Effects of galanin-like peptide on food intake and the hypothalamo-pituitary-thyroid axis. *Neuroendocrinology* 77, 125-131.
- Shapiro,L. and Scherer,P.E. (1998). The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr. Biol.* 8, 335-338.
- Shimada,M., Tritos,N.A., Lowell,B.B., Flier,J.S., and Maratos-Flier,E. (1998). Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature* 396, 670-674.
- Shivers,B.D., Harlan,R.E., Morrell,J.I., and Pfaff,D.W. (1983). Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. *Nature* 304, 345-347.
- Shutter,J.R., Graham,M., Kinsey,A.C., Scully,S., Luthy,R., and Stark,K.L. (1997). Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. *Genes Dev.* 11, 593-602.
- Silverman,A.J., Jhamandas,J., and Renaud,L.P. (1987). Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. *J. Neurosci.* 7, 2312-2319.
- Simonian,S.X., Spratt,D.P., and Herbison,A.E. (1999). Identification and characterization of estrogen receptor alpha-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. *J. Comp Neurol.* 411, 346-358.
- Sipols,A.J., Bayer,J., Bennett,R., and Figlewicz,D.P. (2002). Intraventricular insulin decreases kappa opioid-mediated sucrose intake in rats. *Peptides* 23, 2181-2187.
- Sipols,A.J., Stuber,G.D., Klein,S.N., Higgins,M.S., and Figlewicz,D.P. (2000). Insulin and raclopride combine to decrease short-term intake of sucrose solutions. *Peptides* 21, 1361-1367.
- Skofitsch,G., Jacobowitz,D.M., and Zamir,N. (1985). Immunohistochemical localization of a melanin concentrating hormone-like peptide in the rat brain. *Brain Res. Bull.* 15, 635-649.
- Slemmon,J.R., Blacher,R., Danho,W., Hempstead,J.L., and Morgan,J.I. (1984). Isolation and sequencing of two cerebellum-specific peptides. *Proc. Natl. Acad. Sci. U. S. A* 81, 6866-6870.

- Slemmon, J.R., Danho, W., Hempstead, J.L., and Morgan, J.I. (1985). Cerebellin: a quantifiable marker for Purkinje cell maturation. *Proc. Natl. Acad. Sci. U. S. A* 82, 7145-7148.
- Slemmon, J.R., Goldowitz, D., Blacher, R., and Morgan, J.I. (1988). Evidence for the transneuronal regulation of cerebellin biosynthesis in developing Purkinje cells. *J. Neurosci.* 8, 4603-4611.
- Small, C.J., Liu, Y.L., Stanley, S.A., Connoley, I.P., Kennedy, A., Stock, M.J., and Bloom, S.R. (2003). Chronic CNS administration of Agouti-related protein (Agrp) reduces energy expenditure. *Int. J. Obes. Relat Metab Disord.* 27, 530-533.
- Smith, G.P., Jerome, C., Cushin, B.J., Eterno, R., and Simansky, K.J. (1981). Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. *Science* 213, 1036-1037.
- Smith, J.T., Acohido, B.V., Clifton, D.K., and Steiner, R.A. (2006a). KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *J. Neuroendocrinol.* 18, 298-303.
- Smith, J.T., Cunningham, M.J., Rissman, E.F., Clifton, D.K., and Steiner, R.A. (2005a). Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* 146, 3686-3692.
- Smith, J.T., Dungan, H.M., Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., Clifton, D.K., and Steiner, R.A. (2005b). Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* 146, 2976-2984.
- Smith, J.T., Popa, S.M., Clifton, D.K., Hoffman, G.E., and Steiner, R.A. (2006b). Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J. Neurosci.* 26, 6687-6694.
- Smith, K.L., Patterson, M., Dhillon, W.S., Patel, S.R., Semjonous, N.M., Gardiner, J.V., Ghatei, M.A., and Bloom, S.R. (2006c). Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. *Endocrinology* 147, 3510-3518.
- Smith, M.S. (1993). Lactation alters neuropeptide-Y and proopiomelanocortin gene expression in the arcuate nucleus of the rat. *Endocrinology* 133, 1258-1265.
- Spyraki, C., Fibiger, H.C., and Phillips, A.G. (1982). Attenuation by haloperidol of place preference conditioning using food reinforcement. *Psychopharmacology (Berl)* 77, 379-382.
- Stafford, L.J., Xia, C., Ma, W., Cai, Y., and Liu, M. (2002). Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. *Cancer Res.* 62, 5399-5404.
- Staley, K. and Scharfman, H. (2005). A woman's prerogative. *Nat. Neurosci.* 8, 697-699.
- Stanley, B.G., Daniel, D.R., Chin, A.S., and Leibowitz, S.F. (1985). Paraventricular nucleus injections of peptide YY and neuropeptide Y preferentially enhance carbohydrate ingestion. *Peptides* 6, 1205-1211.
- Stanley, B.G., Kyrkouli, S.E., Lampert, S., and Leibowitz, S.F. (1986). Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides* 7, 1189-1192.
- Stanley, B.G. and Leibowitz, S.F. (1984). Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sci.* 35, 2635-2642.
- Stanley, S.A., Small, C.J., Kim, M.S., Heath, M.M., Seal, L.J., Russell, S.H., Ghatei, M.A., and Bloom, S.R. (1999). Agouti related peptide (Agrp) stimulates the hypothalamo pituitary gonadal axis in vivo & in vitro in male rats. *Endocrinology* 140, 5459-5462.

- Steele, K.E., Prokopowicz, G.P., Schweitzer, M.A., Magunsuon, T.H., Lidor, A.O., Kuwabawa, H., Kumar, A., Brasic, J., and Wong, D.F. (2010). Alterations of central dopamine receptors before and after gastric bypass surgery. *Obes. Surg.* 20, 369-374.
- Sternson, S.M., Shepherd, G.M., and Friedman, J.M. (2005). Topographic mapping of VMH --> arcuate nucleus microcircuits and their reorganization by fasting. *Nat. Neurosci.* 8, 1356-1363.
- Steuer, M.A., Thompson, A.C., Doerr, J.C., Youakim, M., and Kristal, M.B. (1987). Induction of maternal behavior in rats: effects of pseudopregnancy termination and placenta-smeared pups. *Behav. Neurosci.* 101, 219-227.
- Stice, E., Spoor, S., Bohon, C., and Small, D.M. (2008). Relation between obesity and blunted striatal response to food is moderated by TaqIA A1 allele. *Science* 322, 449-452.
- Stice, E., Yokum, S., Blum, K., and Bohon, C. (2010). Weight gain is associated with reduced striatal response to palatable food. *J. Neurosci.* 30, 13105-13109.
- Stratford, T.R. and Kelley, A.E. (1999). Evidence of a functional relationship between the nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior. *J. Neurosci.* 19, 11040-11048.
- Strowski, M.Z., Kaczmarek, P., Mergler, S., Wiedenmann, B., Domin, D., Szwajkowski, P., Wojciechowicz, T., Skrzypski, M., Szczepankiewicz, D., Szkudelski, T., Rucinski, M., Malendowicz, L.K., and Nowak, K.W. (2009). Insulinostatic activity of cerebellum--evidence from in vivo and in vitro studies in rats. *Regul. Pept.* 157, 19-24.
- Sugita, S., Johnson, S.W., and North, R.A. (1992). Synaptic inputs to GABAA and GABAB receptors originate from discrete afferent neurons. *Neurosci. Lett.* 134, 207-211.
- Summerford, C. and Samulski, R.J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* 72, 1438-1445.
- Suzuki, T., Kohno, H., Sakurada, T., Tadano, T., and Kisara, K. (1982). Intracranial injection of thyrotropin releasing hormone (TRH) suppresses starvation-induced feeding and drinking in rats. *Pharmacol. Biochem. Behav.* 17, 249-253.
- Swanson, L.W. (1982). The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res. Bull.* 9, 321-353.
- Swanson, L.W. and Kuypers, H.G. (1980). The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J. Comp Neurol.* 194, 555-570.
- Swanson, L.W. and Sawchenko, P.E. (1983). Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. *Annu. Rev. Neurosci.* 6, 269-324.
- Swerdloff, R.S., Batt, R.A., and Bray, G.A. (1976). Reproductive hormonal function in the genetically obese (ob/ob) mouse. *Endocrinology* 98, 1359-1364.
- Swerdlow, N.R., van der, K.D., Koob, G.F., and Wenger, J.R. (1983). Cholecystokinin produces conditioned place-aversions, not place-preferences, in food-deprived rats: evidence against involvement in satiety. *Life Sci.* 32, 2087-2093.
- Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Wool, E.A.,

- Monroe, C.A., and Tepper, R.I. (1995). Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83, 1263-1271.
- Ter Horst, G.J., de, B.P., Luiten, P.G., and van Willigen, J.D. (1989). Ascending projections from the solitary tract nucleus to the hypothalamus. A Phaseolus vulgaris lectin tracing study in the rat. *Neuroscience* 31, 785-797.
- Terao, Y., Kumano, S., Takatsu, Y., Hattori, M., Nishimura, A., Ohtaki, T., and Shintani, Y. (2004). Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. *Biochim. Biophys. Acta* 1678, 102-110.
- Terry, K.K., Chatman, L.A., Foley, G.L., Kadyszewski, E., Fleeman, T.L., Hurtt, M.E., and Chapin, R.E. (2005). Effects of feed restriction on fertility in female rats. *Birth Defects Res. B Dev. Reprod. Toxicol.* 74, 431-441.
- Thompson, E.L., Patterson, M., Murphy, K.G., Smith, K.L., Dhillo, W.S., Todd, J.F., Ghatei, M.A., and Bloom, S.R. (2004). Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. *J. Neuroendocrinol.* 16, 850-858.
- Thompson, R.H., Canteras, N.S., and Swanson, L.W. (1996). Organization of projections from the dorsomedial nucleus of the hypothalamus: a PHA-L study in the rat. *J. Comp Neurol.* 376, 143-173.
- Thompson, R.H. and Swanson, L.W. (1998). Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. *Brain Res. Brain Res. Rev.* 27, 89-118.
- Tindell, A.J., Berridge, K.C., and Aldridge, J.W. (2004). Ventral pallidal representation of pavlovian cues and reward: population and rate codes. *J. Neurosci.* 24, 1058-1069.
- Toni, R., Jackson, I.M., and Lechan, R.M. (1990). Neuropeptide-Y-immunoreactive innervation of thyrotropin-releasing hormone-synthesizing neurons in the rat hypothalamic paraventricular nucleus. *Endocrinology* 126, 2444-2453.
- Topaloglu, A.K., Reimann, F., Guclu, M., Yalin, A.S., Kotan, L.D., Porter, K.M., Serin, A., Mungan, N.O., Cook, J.R., Ozbek, M.N., Imamoglu, S., Akalin, N.S., Yuksel, B., O'Rahilly, S., and Semple, R.K. (2009). TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat. Genet.* 41, 354-358.
- Tracy, A.L., Clegg, D.J., Johnson, J.D., Davidson, T.L., and Benoit, S.C. (2008). The melanocortin antagonist AgRP (83-132) increases appetitive responding for a fat, but not a carbohydrate, reinforcer. *Pharmacol. Biochem. Behav.* 89, 263-271.
- Tsujii, S. and Bray, G.A. (1989). Acetylation alters the feeding response to MSH and beta-endorphin. *Brain Res. Bull.* 23, 165-169.
- Ungerstedt, U. (1971). Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system. *Acta Physiol Scand. Suppl* 367, 95-122.
- Urade, Y., Oberdick, J., Molinar-Rode, R., and Morgan, J.I. (1991). Precerebellin is a cerebellum-specific protein with similarity to the globular domain of complement C1q B chain. *Proc. Natl. Acad. Sci. U. S. A* 88, 1069-1073.
- Uramura, K., Funahashi, H., Muroya, S., Shioda, S., Takigawa, M., and Yada, T. (2001). Orexin-a activates phospholipase C- and protein kinase C-mediated Ca²⁺ signaling in dopamine neurons of the ventral tegmental area. *Neuroreport* 12, 1885-1889.

- Usiello,A., Baik,J.H., Rouge-Pont,F., Picetti,R., Dierich,A., LeMeur,M., Piazza,P.V., and Borrelli,E. (2000). Distinct functions of the two isoforms of dopamine D2 receptors. *Nature* 408, 199-203.
- Usuda,I., Tanaka,K., and Chiba,T. (1998). Efferent projections of the nucleus accumbens in the rat with special reference to subdivision of the nucleus: biotinylated dextran amine study. *Brain Res.* 797, 73-93.
- Van Bockstaele,E.J. and Pickel,V.M. (1995). GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. *Brain Res.* 682, 215-221.
- Van Heek,M., Compton,D.S., France,C.F., Tedesco,R.P., Fawzi,A.B., Graziano,M.P., Sybertz,E.J., Strader,C.D., and Davis,H.R., Jr. (1997). Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *J. Clin. Invest* 99, 385-390.
- Vaughan,C., Moore,M., Haskell-Luevano,C., and Rowland,N.E. (2006). Food motivated behavior of melanocortin-4 receptor knockout mice under a progressive ratio schedule. *Peptides* 27, 2829-2835.
- Volkow,N.D., Fowler,J.S., Wang,G.J., Hitzemann,R., Logan,J., Schlyer,D.J., Dewey,S.L., and Wolf,A.P. (1993). Decreased dopamine D2 receptor availability is associated with reduced frontal metabolism in cocaine abusers. *Synapse* 14, 169-177.
- Volkow,N.D., Fowler,J.S., Wang,G.J., and Swanson,J.M. (2004). Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. *Mol. Psychiatry* 9, 557-569.
- Wang,G.J., Volkow,N.D., Fowler,J.S., Logan,J., Abumrad,N.N., Hitzemann,R.J., Pappas,N.S., and Pascani,K. (1997a). Dopamine D2 receptor availability in opiate-dependent subjects before and after naloxone-precipitated withdrawal. *Neuropsychopharmacology* 16, 174-182.
- Wang,G.J., Volkow,N.D., Logan,J., Pappas,N.R., Wong,C.T., Zhu,W., Netusil,N., and Fowler,J.S. (2001). Brain dopamine and obesity. *Lancet* 357, 354-357.
- Wang,Q., Bing,C., Al-Barazanji,K., Mossakowaska,D.E., Wang,X.M., McBay,D.L., Neville,W.A., Taddayon,M., Pickavance,L., Dryden,S., Thomas,M.E., McHale,M.T., Gloyer,I.S., Wilson,S., Buckingham,R., Arch,J.R., Trayhurn,P., and Williams,G. (1997b). Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes* 46, 335-341.
- Welter,M., Vallone,D., Samad,T.A., Meziane,H., Usiello,A., and Borrelli,E. (2007). Absence of dopamine D2 receptors unmasks an inhibitory control over the brain circuitries activated by cocaine. *Proc. Natl. Acad. Sci. U. S. A* 104, 6840-6845.
- Werner,A. and Sayer,J.A. (2009). Naturally occurring antisense RNA: function and mechanisms of action. *Curr. Opin. Nephrol. Hypertens.* 18, 343-349.
- Wetsel,W.C., Valenca,M.M., Merchenthaler,I., Liposits,Z., Lopez,F.J., Weiner,R.I., Mellon,P.L., and Negro-Vilar,A. (1992). Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. *Proc. Natl. Acad. Sci. U. S. A* 89, 4149-4153.
- White,J.D. and Kershaw,M. (1990). Increased hypothalamic neuropeptide Y expression following food deprivation. *Mol. Cell Neurosci.* 1, 41-48.
- White,R.G. (1963). Factors affecting the antibody response. *Br. Med. Bull.* 19, 207-213.
- Wilcox,B.J., Corp,E.S., Dorsa,D.M., Figlewicz,D.P., Greenwood,M.R., Woods,S.C., and Baskin,D.G. (1989). Insulin binding in the hypothalamus of lean and genetically obese Zucker rats. *Peptides* 10, 1159-1164.

- Wilson,C., Nomikos,G.G., Collu,M., and Fibiger,H.C. (1995). Dopaminergic correlates of motivated behavior: importance of drive. *J. Neurosci.* 15, 5169-5178.
- Winokur,A. and Utiger,R.D. (1974). Thyrotropin-releasing hormone: regional distribution in rat brain. *Science* 185, 265-267.
- Wise,R.A., Spindler,J., deWit,H., and Gerberg,G.J. (1978). Neuroleptic-induced "anhedonia" in rats: pimozide blocks reward quality of food. *Science* 201, 262-264.
- Xu,B., Goulding,E.H., Zang,K., Cepoi,D., Cone,R.D., Jones,K.R., Tecott,L.H., and Reichardt,L.F. (2003). Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nat. Neurosci.* 6, 736-742.
- Xu,R., Janson,C.G., Mastakov,M., Lawlor,P., Young,D., Mouravlev,A., Fitzsimons,H., Choi,K.L., Ma,H., Dragunow,M., Leone,P., Chen,Q., Dicker,B., and During,M.J. (2001). Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. *Gene Ther.* 8, 1323-1332.
- Yasuda,T., Masaki,T., Kakuma,T., and Yoshimatsu,H. (2004). Hypothalamic melanocortin system regulates sympathetic nerve activity in brown adipose tissue. *Exp. Biol. Med.* (Maywood.) 229, 235-239.
- Yen,P.M. (2001). Physiological and molecular basis of thyroid hormone action. *Physiol Rev.* 81, 1097-1142.
- Yeo,G.S., Farooqi,I.S., Aminian,S., Halsall,D.J., Stanhope,R.G., and O'Rahilly,S. (1998). A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat. Genet.* 20, 111-112.
- Yiangou,Y., Burnet,P., Nikou,G., Chrysanthou,B.J., and Bloom,S.R. (1989). Purification and characterisation of cerebellins from human and porcine cerebellum. *J. Neurochem.* 53, 886-889.
- Young,J., Bouligand,J., Francou,B., Raffin-Sanson,M.L., Gaillez,S., Jeanpierre,M., Grynberg,M., Kamenicky,P., Chanson,P., Brailly-Tabard,S., and Guiochon-Mantel,A. (2010). TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J. Clin. Endocrinol. Metab* 95, 2287-2295.
- Young,J.I., Otero,V., Cerdan,M.G., Falzone,T.L., Chan,E.C., Low,M.J., and Rubinstein,M. (1998). Authentic cell-specific and developmentally regulated expression of pro-opiomelanocortin genomic fragments in hypothalamic and hindbrain neurons of transgenic mice. *J. Neurosci.* 18, 6631-6640.
- Yovandich,J., O'Malley,B., Jr., Sikes,M., and Ledley,F.D. (1995). Gene transfer to synovial cells by intra-articular administration of plasmid DNA. *Hum. Gene Ther.* 6, 603-610.
- Zarjevski,N., Cusin,I., Vettor,R., Rohnerjeanrenaud,F., and Jeanrenaud,B. (1993). Chronic Intracerebroventricular Neuropeptide-y Administration to Normal Rats Mimics Hormonal and Metabolic Changes of Obesity. *Endocrinology* 133, 1753-1758.
- Zarrindast,M.R., Hosseini-Nia,T., and lah-Maddadi,S. (1989). Food intake suppressant effect of baclofen in rats. *Gen. Pharmacol.* 20, 701-703.
- Zhang,G., Gurtu,V., and Kain,S.R. (1996). An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem. Biophys. Res. Commun.* 227, 707-711.
- Zhang,M., Gosnell,B.A., and Kelley,A.E. (1998). Intake of high-fat food is selectively enhanced by mu opioid receptor stimulation within the nucleus accumbens. *J. Pharmacol. Exp. Ther.* 285, 908-914.
- Zhang,Y., Proenca,R., Maffei,M., Barone,M., Leopold,L., and Friedman,J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372, 425-432.

Zheng,H., Patterson,L.M., and Berthoud,H.R. (2007). Orexin signaling in the ventral tegmental area is required for high-fat appetite induced by opioid stimulation of the nucleus accumbens. *J. Neurosci.* 27, 11075-11082.

Zheng,H., Townsend,R.L., Shin,A.C., Patterson,L.M., Phifer,C.B., and Berthoud,H.R. (2010). High-fat intake induced by mu-opioid activation of the nucleus accumbens is inhibited by Y1R-blockade and MC3/4R- stimulation. *Brain Res.* 1350, 131-138.

Zhou,Q.Y. and Palmiter,R.D. (1995). Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. *Cell* 83, 1197-1209.

Appendix I: Solutions used in this thesis

5M ammonium acetate

Dissolve 385g $\text{CH}_3\text{COONH}_4$ in 600ml GDW and make up to 1l.

Artificial cerebrospinal fluid (aCSF)

To make 500ml aCSF, mix 100ml 0.1M NaHCO_3 , 100ml 0.63M NaCl , 5ml 0.09M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 5ml 0.59M KCl , 5ml 0.09M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 450mg glucose, 88mg ascorbic acid, 5ml aprotinin (Bayer Corp.) and 270ml GDW. Saturate with 95% O_2 and 5% CO_2 . Add 10ml 0.07M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ after gassing.

Potassium-enriched aCSF

To make 100ml 56mM KCl aCSF, mix 20ml 0.1M NaHCO_3 , 11.6ml 0.63M NaCl , 1ml 0.09M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 10ml 0.59M KCl , 1ml 0.09M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 90mg glucose, 17.6mg ascorbic acid, 1ml aprotinin (Bayer Corp.) and 53.4ml GDW. Saturate with 95% O_2 and 5% CO_2 . Add 2ml 0.07M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ after gassing.

Stock solutions for aCSF:

0.1M sodium bicarbonate: Dissolve 4.62g NaHCO_3 in 500ml GDW

0.63M sodium chloride: Dissolve 18.43g NaCl in 500ml GDW

0.09M disodium hydrogen orthophosphate dihydrate: Dissolve 1.56g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 100ml GDW

0.59M potassium chloride: Dissolve 4.42g KCl in 100ml GDW

0.09M magnesium sulphate heptahydrate: Dissolve 2.2g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 100ml GDW.

0.07M calcium chloride dihydrate: Dissolve 1.04g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100ml GDW.

Alkaline sodium dodecyl sulphate (SDS)

Add 2ml 10M NaOH and 1g SDS to 100ml GDW.

Antifreeze solution

Add 30% (v/v) ethylene glycol and 20% (v/v) glycerol to 0.01M PBS

Cresyl violet stain

Mix 2g cresyl violet, 300ml GDW, 30ml 1M NaAc and 170ml acetic acid, and pH to 3.7-3.9. Filter before use.

Caesium chloride saturated isopropanol

Mix 100g CsCl₂, 100ml GDW and 100ml isopropanol and leave to settle.

Dextran-coated charcoal

Add 2.4g charcoal and 0.24g dextran to 100ml phosphate buffer with gelatin and mix for 20 minutes.

Dextran sulphate

Dissolve 1g dextran sulphate in 1.5ml GDW. Warm at 60°C for 2-3 hours to dissolve and vortex before use.

DNase buffer

Add 0.8ml 1M Tris/HCl pH 7.4, 120µl 1M MgCl₂ and 40µl CsCl₂ to 99ml GDW.

dNTP mix for PCR and reverse transcription

Mix 20µl each of 100mM dGTP, dCTP, dATP and dTTP in 120µl filtered GDW.

0.5M ethylenediaminetetra-acetic acid (EDTA)

Dissolve 186.1g $\text{C}_{10}\text{H}_{14}\text{H}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (EDTA) in 800ml GDW and adjust pH to 8.0. Make up to 1l with GDW.

4% formaldehyde solution

Dissolve 100mls 40% formaldehyde in 1l GDW.

Gel loading buffer

Mix 3.125ml 80% glycerol, 50µl 0.5M EDTA and 6.075ml GDW, and add 10mg orange G.

Glucose-Tris-EDTA buffer

Mix 2.5ml 1M Tris HCl pH 8.0, 2ml 0.5M EDTA and 5ml 18% glucose and make up to 100ml with GDW. Sterilise by passing through a 0.2µm filter.

Kemteck buffer

Dissolve 22.4g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 9.96g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 14.8g $\text{C}_{10}\text{H}_{14}\text{H}_2\text{ONa}_2 \cdot 2\text{H}_2\text{O}$, 1g $\text{C}_9\text{H}_9\text{HgNaO}_2\text{S}$ and 26.6ml 30% BSA in 5l GDW that has been boiled and allowed to cool. Adjust pH to 7.4 and store at 4°C.

Lysogeny broth

Dissolve 10g NaCl, 10g tryptone and 5g yeast extract in 2l GDW. Sterilise by autoclaving.

20x MOPS

Dissolve 83.6g 3-(N-Morpholino)propanesulphonic acid (MOPS), 8.1g sodium acetate, 7.4g $\text{C}_{10}\text{H}_{14}\text{H}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$ and 2.5ml formaldehyde in 800ml water. Adjust to pH 7.0 with 10M sodium hydroxide and make up to 1l.

Phosphate buffer

Dissolve 48g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.13g KHPO_4 , 18.61g $\text{C}_{10}\text{H}_{14}\text{H}_2\text{ONa}_2 \cdot 2\text{H}_2\text{O}$ and 2.5g NaN_3 in 5l GDW that has been boiled and allowed to cool. Adjust pH to 7.4 and store at 4°C.

Phosphate buffer with gelatine

The buffer is produced as above, except that 12.5g gelatine is added to the boiled water prior to cooling, and before addition of the other reagents.

10x phosphate buffered saline (PBS) (0.1M)

Add 80g NaCl, 2g KCl, 14.4g Na_2HPO_4 and 2.4g KH_2PO_4 to 800ml GDW. Adjust volume to 1l and pH to 7.4.

5M potassium acetate

Dissolve 490.6g CH_3COOK in 500ml GDW and add 115ml acetic acid. Make up to 1l with GDW.

RNase A (10mg/ml)

Add 10mg RNase A, 50µl 2M Tris and 30µl 5M NaCl to 9.92ml GDW. Boil for 15 minutes. Allow to cool and then aliquot.

20x saline sodium citrate (SSC)

Add 1753g NaCl and 882g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ to 8l GDW. Adjust to pH 7.0 and make up to 10l.

2M sodium acetate

Dissolve 164.1g CH_3COONa in 800ml GDW. Adjust pH to 5.2 with acetic acid and make up to 1l with GDW.

5M sodium chloride

Dissolve 292.2g NaCl in 1l GDW.

100x TE

Dissolve 121.1g Trizma base and 3.7g $\text{C}_{10}\text{H}_{14}\text{H}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$ in 800ml GDW and adjust pH to 7.5. Make up to 1l with GDW.

TES

Mix 25ml 1M Tris-HCl pH 8.0, 5ml 5M NaCl, and 5ml $\text{C}_{10}\text{H}_{14}\text{H}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$. Make up to a final volume of 500ml with GDW.

Transformation buffer I

Dissolve 589mg CH_3COOK , 2.418g RbCl, 1.979g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 438mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ in autoclaved distilled water. Add 37.5 ml 80% glycerol and adjust the volume to 200ml with water. Sterilise by passing through a 0.2 μm filter.

Transformation buffer II

Dissolve 418mg MOPS, 3.28g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 242mg RbCl in autoclaved distilled water. Add 37.5ml 80% glycerol and adjust the volume to 200ml with water. Sterilise by passing through a 0.2 μm filter.

1M Triethanolamine (TEA) pH 8.0

Dissolve 7.42g $\text{C}_6\text{H}_{15}\text{NO}_3 \cdot \text{HCl}$ (triethanolamine-hydrochloride) in 30ml GDW. Add NaOH to pH to 8.0, then make up to 40mls with GDW.

50x Tris-acetate-EDTA buffer (TAE)

Dissolve 242g Trizma base in 843ml GDW and mix in 57ml acetic acid and 100ml 0.5M $\text{C}_{10}\text{H}_{14}\text{H}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$.

2M Tris-HCl (pH 8.0)

Dissolve 121.1g Trizma base in 450ml GDW and adjust pH to 8.0 with HCl. Make up to 500ml with GDW.

Appendix II: List of Suppliers

Abbott Laboratories Ltd., Queenborough, Kent, UK
Animalcare Ltd., Dunnington, Yorkshire, UK
Associated Dental Products Ltd., Swindon, Wiltshire, UK
ATCC, Teddington, Middlesex, UK
Bachem, St Helens, Merseyside, UK
Bright Instrument Company, Huntingdon, Cambridgeshire, UK
Calbiotech, Spring Valley, California, USA
Charles River, Margate, Kent, UK
Clark Electromedical Instruments, Reading, Berkshire, UK
Ecolabs Ltd., Leeds, Yorkshire, UK
Euro-Diagnostica, Huntingdon, Cambridgeshire, UK
Fisher Scientific, Loughborough, Leicestershire, UK
GE Healthcare, Amersham, Buckinghamshire, UK
Harvard Apparatus, Massachusetts, USA
Invitrogen, Paisley, UK
Jencons-PLS, Forest Row, East Sussex, UK
Jet X-Ray, London, UK
Kodak, Hemel Hempstead, Hertfordshire, UK
Microfield Scientific Ltd., Dartmouth, Devon, UK
MP Biomedicals Inc., Illkirch, France
New England Biolabs, Hitchin, Hertfordshire, UK
Nordic Pharma Ltd., Reading, UK
Perkin Elmer, Waltham, Massachusetts, USA
Pharmacia Diagnostics, Uppsala, Sweden
Phoenix Pharmaceuticals Inc, Burlingame, California, USA
Plastics One Inc., Roanoke, Virginia, USA
Promega, Madison, Wisconsin, USA
Schering-Plough Corp., Welwyn Garden City, Hertfordshire, UK
Shimadzu Corporation Analytical Instruments, Shimadzu, Kyoto, Japan
Sigma-Aldrich, Gillingham, Dorset, UK
Special Diet Service Ltd., Witham, Essex, UK
Stratagene Ltd., Amsterdam, The Netherlands

The Peptide Institute, Osaka, Japan

Thermo Electron Corporation, Marietta, Ohio, USA

Thermo Fisher Scientific, Epsom, Surrey, UK

Tocris Bioscience, Bristol, UK

VWR, Leicester, Leicestershire, UK

Waters, Milford, Connecticut, USA

Appendix III: Amino acid codes

Amino acid	Three letter code	Single letter code
Glycine	Gly	G
Proline	Pro	P
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Cysteine	Cys	C
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Glutamine	Gln	Q
Asparagine	Asn	N
Glutamic Acid	Glu	E
Aspartic Acid	Asp	D
Serine	Ser	S
Threonine	Thr	T

Publications

Accurate measurement of body weight and food intake in environmentally enriched male Wistar rats

Beale KE, Murphy KG, Harrison EK, Kerton AJ, Ghatei MA, Bloom SR, Smith KL. Obesity (Silver Spring). 2011 Jan 13.

Cerebellin1 is a novel orexigenic peptide

Gardiner JV, **Beale KE**, Roy D, Boughton CK, Bataveljic A, Campbell DC, Bewick GA, Patel NA, Patterson M, Leavy EM, Ghatei MA, Bloom SR, Dhillon WS. Diabetes, Obesity and Metabolism. 2010 Oct;12(10):883-90.

Alarin stimulates food intake and gonadotrophin release in male rats

Boughton CK, Patterson M, Bewick GA, Tadross JA, Gardiner JV, **Beale KE**, Chaudery F, Hunter G, Busbridge M, Leavy EM, Ghatei MA, Bloom SR, Murphy KG. British Journal of Pharmacology. 2010 Oct;161(3):601-13.

Augurin stimulates the hypothalamic-pituitary-adrenal axis via the release of corticotrophin-releasing factor in rats

Tadross JA, Patterson M, Suzuki K, **Beale KE**, Boughton CK, Smith KL, Moore S, Ghatei MA, Bloom SR. British Journal of Pharmacology. 2010 Apr;159(8):1663-71.

Investigation of structure-activity relationships of Oxyntomodulin (Oxm) using Oxm analogues

Druce MR, Minnion JS, Field BC, Patel SR, Shillito JC, Tilby M, **Beale KE**, Murphy KG, Ghatei MA, Bloom SR. Endocrinology. 2009 Apr;150(4):1712-22.

Poster presentations

Chronic peripheral administration of PK2 causes a potent reduction in food intake and body weight in diet-induced obese mice. **KEL Beale**, K Hostomska, JV Gardiner, GA Bewick, MA Ghatei, SR Bloom and WS Dhillo. The Endocrine Society's 93rd Annual Meeting. Boston, USA, June 2011.

Age of vaginal opening as a predictor of leptin sensitivity in rats. ML Sleeth, EL Thompson, **KEL Beale**, JE Baxter, SR Bloom and KG Murphy. The Endocrine Society's 93rd Annual Meeting. Boston, USA, June 2011.

The effect of environmental enrichment on food intake, body weight and the HPA axis in laboratory rats. **KEL Beale**, EK Harrison, KL Smith, A Kerton, M Ghatei, SR Bloom and KG Murphy. Society for Endocrinology British Endocrine Society Meeting. Birmingham, April 2011.

The use of vaginal opening age as an indicator of leptin sensitivity in female Sprague Dawley rats. ML Sleeth, **KEL Beale**, EL Thompson, JE Baxter, SR Bloom and KG Murphy. Society for Endocrinology British Endocrine Society Meeting. Birmingham, April 2011.

The effect of environmental enrichment on food intake, body weight and the hypothalamo-pituitary-adrenal axis in laboratory rats. **KEL Beale**, E Harrison, KL Smith, A Kerton, M Ghatei, SR Bloom and KG Murphy. Integrative Mammalian Biology Animal Welfare/3Rs Meeting. University of Manchester, April 2011.

Does environmental enrichment affect food intake and body weight in singly housed male Wistar rats? **KEL Beale**, A Kerton and KL Smith. 7th James Black Conference, British Pharmacology Society. London, September 2009.