VENTROMEDIAL HYPOTHALAMIC NEURONES INVOLVED IN ENERGY HOMEOSTASIS

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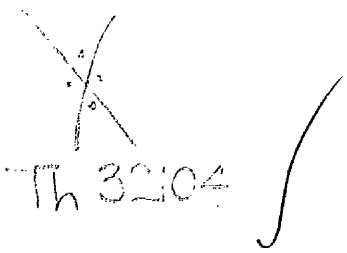

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Abstract

The processes that control energy homeostasis are extremely complex and involve interactions between peripheral organs, the hypothalamus and the brainstem, as well as inputs from sensory and reward centres of the brain. Researchers have come a long way in recent years in elucidating the networks that control energy balance, but recent transgenic models have shown that over-emphasis may have been put on the arcuate nucleus. Research is now shifting to extra-arcuate sites including the ventromedial nucleus (VMN) which, unlike other hypothalamic nuclei, remains poorly characterised in terms of its cellular phenotypes.

The aim of this thesis was to initially identify energy balance-related neuronal markers for the VMN by examining expressional changes of four VMN-enriched genes in response to metabolic stimuli. Particular emphasis was given to the regulatory responses to leptin. Semi-quantitative *in situ* hybridisation revealed that expression of all four genes tested is altered according to energy status, but the most robust changes were seen in expression of pituitary adenylate cyclase-activating polypeptide (PACAP). PACAP mRNA was markedly reduced by fasting or leptin deficiency and increased following leptin or high-energy diet.

The regulation of VMN PACAP expression is directionally appropriate for that of a catabolic factor, and it was subsequently shown that central PACAP administration decreased food intake and metabolic efficiency, and increased energy expenditure. We then went on to show that the feeding effects of PACAP are associated with those of the CRH, but not melanocortin pathway. The effects of PACAP are almost certainly mediated by the PACAP-specific PAC_1R, since equimolar VIP, a co-ligand at the VPACRs has no effect on energy balance. Furthermore genetic knockout of $VPAC_2R$ has no effect on the ability of PACAP to induce hypophagia and increase energy expenditure.

The *in vivo* effects of PACAP are similar to those of leptin, and we next sought to determine whether a functional relationship exists between the two peptides. The revelation that VMN targeted knockout of the leptin receptor (lepr-B) produces a mouse which is as obese as the POMC-driven knockout, demonstrates that VMN neurones are at least as important as POMC neurones in mediating the effects of leptin. We have shown that PACAP is co-localised with steroidogenic factor 1 (SF1; a surrogate marker

of leptin sensitivity) in the VMN and that loss of lepr-B in these cells reduces PACAP expression. Furthermore, $PACAP_{6-38}$, a PACAP antagonist, reduced the acute effects of central leptin *in vivo*. Together, these data provide strong evidence that PACAP cells are a target for leptin.

Finally, the role of leptin signalling in the VMN was further explored by examining synaptic architecture. Specific deletion of lepr-B in the VMN results in adult onset obesity and we found that this is preceded by increased inhibitory and decreased excitatory inputs onto VMN cells. These changes may result in hyper-inhibition of the VMN, and hence, contribute to the aetiology of obesity in this strain.

Declaration

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

Zoe Hawke

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Abbreviations

µCi	microcurie(s)
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
^{125}I	iodine 125
2-DG	2-deoxyglucose
^{33}P	phosphorus 33
^{35}S	sulphur 35
AA	acetic anhydride
AGRP	agouti-related protein
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP	area postrema
Arc	arcuate nucleus
AS	antisense
BAC	bacterial artificial chromosome
BAT	brown adipose tissue
BBB	blood brain barrier
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BDNF	brain-derived neurotrophic factor
bp	base pairs
BSS	behavioural satiety sequence
c	central
cAMP	cyclic AMP
CART	cocaine and amphetemine activated transcript
CCK	cholecystokinin
CeA	central amygdala
CLAMS	comprehensive laboratory animal monitoring system
cm	centimetre(s)
CNS	central nervous system
cpm	counts per minute
Cre	cre-recombinase
CREB	cAMP response element binding protein
CRH	corticotrophin releasing hormone
CSF	cerebrospinal fluid
DAB	diaminobenzidine
d-ATP	2'-deoxyadenosine 5'-triphosphate
db	diabetes gene

DIG	digoxigenin
DIO	diet-induced obese(ity)
DIO-R	diet-induced obesity-resistant
dm	dorsomedial
DMN	dorsomedial nucleus
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ES	embryonic stem
FFA	free fatty acid
flox	flanked with loxP
FLP	flippase
g	gram(s)
GABA	γ-aminbutyric acid
GAD	glutamic acid decarboxylase
GE	glucose-excited
GFP	green fluorescent protein
GI	glucose-inhibited
GLP-1	glucagon-like peptide 1
h	hour
HED	high-energy diet
HPA	hypothalamo-pituitary-adrenal
HPT	hypothalamo-pituitary-thyroid
HSL	hormone sensitive lipase
i.c.v.	intracerebroventricular
i.m.	intramuscular
i.p.	intraperitoneal
IR	immunoreactivity
ISHH	*in situ* hybridisation histology
JAK	janus activated kinase
kb	kilobase(s)
kcal	kilocalories
kg	kilogram(s)
KO	knockout
l	litre(s)
lepr	leptin receptor
LH	lateral hypothalamus
L-NAME	N^G-nitro- L-arginine methyl ester
loxP	locus of crossover in P1

M	molar
MAPK	mitogen-activated protein kinase
MCH	melanin concentrating hormone
MC-R	melanocortin receptor
MeA	medial amygdala
mEPSC	miniature excitatory postsynaptic current
mg	miligram(s)
$MgCl_2$	magnesium chloride
mHab	medial habenula
mIPSC	miniature inhibitory postsynaptic current
ml	mililitre(s)
mm	milimetre(s)
mRNA	messenger RNA
MTII	melanotan II
NA	nucleus accumbens
NaCl	sodium chloride
NBT	Nitro blue tetrazolium chloride
NC	normal chow
Neo	neomycin
ng	nanogram(s)
NGS	normal goat serum
nmol	nanomoles
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NPY	neuropeptide Y
NS	not significant
NTP	nucleotide triphosphate
NTS	nucleus of the solitary tract
ob	obese gene
°C	degrees centigrade
OD	optical density
p	phosphorylated
PAC_1	PACAP-1 receptor
PACAP	pituitary adenylate cyclase-activating polypeptide
PAG	periaqueductal grey
PB	phosphate buffer
PBS	phosphate buffered saline
PBT	phosphate buffer with triton
PCR	polymerase chain reaction
PeV	periventricular nucleus

PFA	paraformaldehyde
phMRI	pharmacological magnetic resonance imaging
PI3K	phosphatidylinositol 3 kinase
pmol	picomoles
POMC	proopiomelanocortin
PRV	pseudorabies virus
PTP1B	protein tyrosine phosphatise 1B
PVN	paraventricular nucleus
P-YY	peptide-YY
rATP	2'-deoxycytidine 5'-triphosphate
rCTP	2'-deoxyadenosine 5'-triphosphate
recombineering	recombination-driven genetic engineering
rGTP	2'-deoxyguanidine 5'-triphosphate
RNA	ribonucleic acid
RNAi	RNA-interference
RQ	respiratory quotient
RT	room temperature
RT-PCR	reverse-transcription PCR
rUTP	2'-deoxyuridine 5'-triphosphate
S	sense
SEM	standard error of the mean
SF1	steroidogenic factor 1
SNS	sympathetic nervous system
SOCS	suppressor of cytocine signalling
SON	Supra-optic nucleus
SSC	standard sodium citrate
STAT	signal transducers and activators of transcription
T_b	core-body temperature
TBS	tris-buffered saline
TdT	terminal deoxynucleotydyl transferase
TE	Tris-EDTA buffer
TEA	triethanolamine hydrochloric acid
TRH	thyrotrophin-releasing hormone
TrkB	tyrosine kinase-B
U	units
UCP1	uncoupling protein 1
VCO_2	carbon dioxide production
VGAT	vesicular GABA transporter
VGlut	vesiculat glutamate transported
VIP	vasoactive intestinal polypeptide

vl	ventrolateral
VMN	ventromedial nucleus
VO_2	oxygen consumption
VPAC	VIP/PACAP receptor
VTA	ventral tegmental area
WAT	white adipose tissue
wt	wildtype
Y-R	NPY receptor
α-MSH	alpha MSH
β-AR	beta-adrenoreceptor

CHAPTER 1

GENERAL INTRODUCTION

1.1 BODY WEIGHT REGULATION

Despite what are often wide disparities in body weight within a population, individuals typically display impressive stability of body weight, which persists over long periods of time. This tight control of energy balance is achieved by co-ordinated adjustments of energy intake and/or energy expenditure which compensate for any short-term imbalance. As such, the brain and periphery are constantly conversing; the periphery notifies the brain of its immediate metabolic requirements (McMinn et al., 2000) and the brain responds through its control of neuronal pathways involved in energy balance. These pathways affect behaviour, food intake, physical activity, sympathetic nervous system (SNS) activation and metabolism (Schwartz et al., 2000; Spiegelman and Flier, 2001; Woods and D'Alessio, 2008).

Over the past two decades, we have gained tremendous insight into the neuroanatomical and physiological basis behind the central regulation of energy homeostasis. Metabolic sensing neurones, which are the major integrators of both humoral and neural inputs from the periphery, have been discovered, and their molecular phenotypes for the large part characterised. Unlike most neurones, these cells utilise glucose and other indicators of energy status as signalling molecules to regulate their membrane potential and firing rate. Moreover, they express key genes which encode neuropeptides, receptors and neurotransmitters which drive a coherent behavioural and endocrine output in response to energy balance stimuli (reviewed in Levin, 2006; and Schwartz et al., 2000). The central energy balance circuits are of particular interest in the current climate of prevalent obesity as they represent promising targets for pharmacological alteration of body fat stores.

Peripherally-produced peptides act in the brain, principally in the hypothalamus and brainstem, to indicate the nutritional state of the animal (reviewed in Luckman and Lawrence, 2003). The gastrointestinal (GI) tract in particular, provides extensive short-term hormonal feedback to the central energy balance circuits. Among others, these hormones include ghrelin, a potent appetite stimulant, and the post-prandially released satiety factors cholecystokinin (CCK), peptide- YY (P-YY) and glucagon-like peptide 1 (GLP-1) from the stomach and intestines (reviewed in Dhillo and Bloom, 2004; Mendieta-Zeron et al., 2008; and Murphy et al., 2006). Dietary metabolites such as glucose and free-fatty acids (FFAs) also act as signalling molecules in the brain, as do insulin and glucagon released from the pancreas (Schwartz et al., 2000). White adipose tissue (WAT) is also a major source of humoral signals that reflect lipogenesis,

storage, and lipolysis and feed back to the brain. Hence, the long-held view that fat is merely a passive storage depot has been abandoned over the past 15 years, and it is now recognised as a dynamic secretory organ (reviewed in Wozniak et al., 2008). This revolution began with the discovery of leptin in 1994 (Zhang et al., 1994), and since then a multitude of factors including adiponectin, resistin and adipsin have been found to be released from adipocytes. These signalling molecules are believed to regulate energy balance over the longer term by matching energy intake to fat stores. Thus, countless signals generated in the processes of feeding, ingestion, energy metabolism and storage throughout the body converge on the central regulatory circuits which maintain energy homeostasis.

Adaptation to negative and positive energy balance

Considerable progress has been made towards defining the cellular events controlling energy balance, but these have tended to focus on the response to negative energy balance and weight loss. Food deprivation or restriction causes a number of behavioural and metabolic changes, many of which have been shown to involve the adipostatic hormone leptin. As such, leptin replacement in fasted animals is sufficient to prevent many of the physiological consequences of negative energy balance, including hyperphagia, decreased energy expenditure, immunosuppression, reproductive disfunction and changes in the expression levels of key genes in the brain (Ahima et al., 1996; Lord et al., 1998) Thus, it is widely accepted that evolutionarily conserved adaptive mechanisms are in place which guard against body weight loss and promote survival (reviewed in Mercer and Speakman, 2001).

Considerably less is known about the physiological processes which resist weight gain, and indeed why these systems appear to fail in individuals who readily become obese. Involuntary overfeeding paradigms, involving either oral gavage or intragastric infusion, produce marked reductions in voluntary food intake and increases in energy expenditure which persist for many days beyond the cessation of treatment (Harris et al., 1986; Harris and Martin, 1989; Rothwell and Stock, 1980; Seeley et al., 1996). Similar adaptations have also been reported in rodents maintained on highly palatable, high-energy diets (HED), although there is noticeable heterogeneity in the magnitude of these responses between individuals (Enriori et al., 2007; Koza et al., 2006; Young et al., 1982). Thus, it appears though weight-gain-preventing mechanisms do appear to exist, in many cases they prove insufficient to prevent obesity. While the mechanisms behind this variability are currently unclear, there is evidence that energy expenditure

correlates negatively with obesity level and as such could be a major contributor (Lowell and Spiegelman, 2000; Wijers et al., 2008; Xiao et al., 2007).

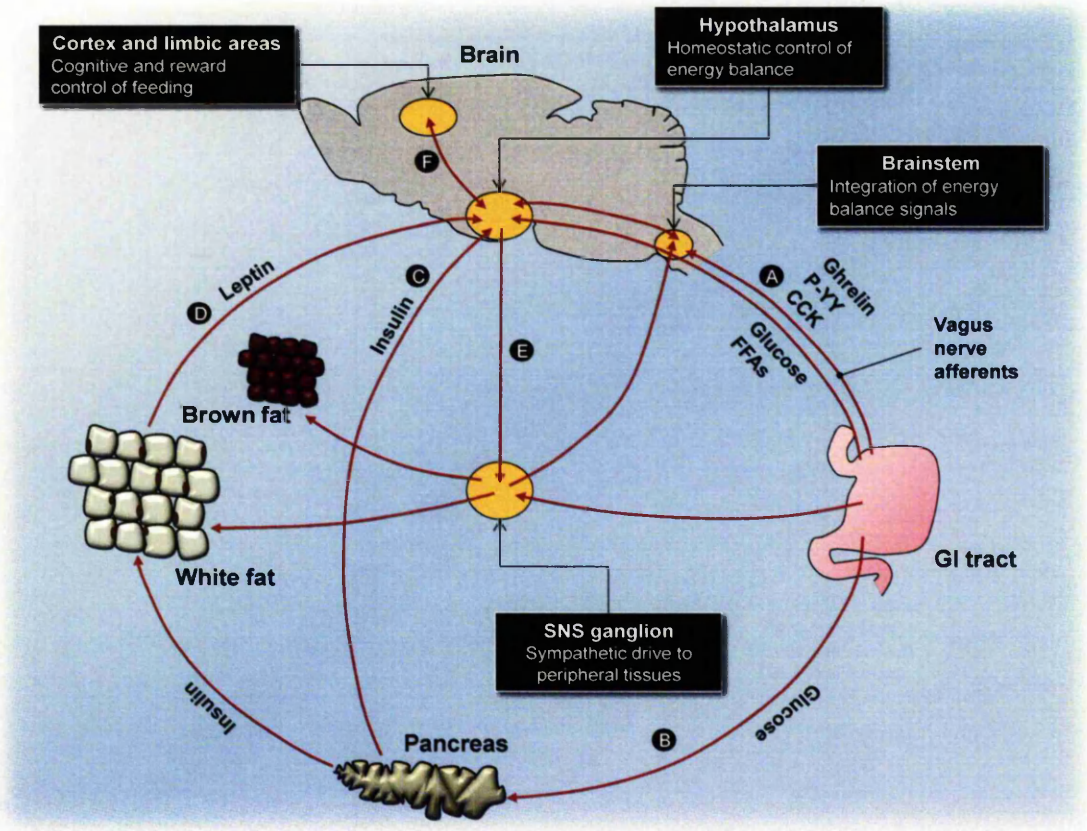


Figure 1.1 Control of energy balance

Schematic illustration showing the major factors released from peripheral tissues that act on the energy balance circuitry of the brain. **A.** The gastro-intestinal (GI) tract is the main source of humoral signals to the brain, including ghrelin, P-YY and CCK as well as glucose and free fatty acids (FFAs) from food. The GI tract also signals to the brain via activation of the vagal connection to the brainstem, which works with the hypothalamus to control energy balance. **B.** Circulating glucose also stimulates insulin release from the pancreas that increases nutrient uptake by fat and **C.** reduces food intake by actions in the hypothalamus. **D.** The main hormonal signal from fat is leptin, which acts on key neruones in the hypothalamus. **E.** A major output of the hypothamic circuits is to the sympathetic nervous system, which drives energy expenditure by activation of brown and white fat and feeds back to the brainstem. **F.** Homeostatic pathways of the brain also interact with higher centres such as the cortex and limbic system, which control the hedonic aspects of feeding such as reward.

While typical models of energy balance appear to focus primarily on the control of appetite and feeding, control of energy expenditure may be equally important in the development and advancement of obesity. A negative correlation between energy expenditure and weight gain has been demonstrated in several overfeeding paradigms in humans (Bouchard et al., 1990; Levine et al., 1999). Furthermore, hypothalamus-lesioned rodents, normally characterised by intense hyperphagia, still manifest obesity when pair-fed to controls, due to chronic depression of their energy expenditure (Himms-Hagen, 1989). In the rodent, a key organ associated with the regulation of body weight is the brown adipose tissue (BAT), located in discrete interscapular and intercostal depots. BAT is the major thermogenenic centre and is typically associated with the thermogenic response to cold; however, diet is now recognised as another potent regulator of BAT thermogenesis. Starvation can reduce energy expenditure by up to 40%, while excessive or HED-feeding cause compensatory increases in thermogenesis, termed 'adaptive thermogenesis' (Lowell and Spiegelman, 2000). BAT cells are characterised by multilocular lipid droplets, a large number of mitochondria, dense innervations by sympathetic nerves, and expression of uncoupling protein 1 (UCP1). UCP1 is expressed in the mitochondrial membranes and in response to sympathetic activation mediates the uncoupling of respiration, resulting in heat production in the absence of work (Cannon and Nedergaard, 2004). Sympathetic nerve activity of BAT is reduced in several models of genetic obesity including the leptin-deficient *ob/ob* mouse (Himms-Hagen, 1989), and transgenic mice in which BAT is ablated develop obesity, despite normal food intake (Lowell et al., 1993). Similarly, mice which lack all three β-adrenergic receptors (β-AR), the primary mediators of SNS signalling, are highly prone to obesity as a result of reduced sympathetic outflow to BAT (Bachman et al., 2002). Sympathetic drive to brown fat thus represents a major component of energy balance regulation and should not be overlooked. As well as being markedly downregulated as part of the starvation response, it also appears equally important in the aetiology of obesity.

Leptin has a stimulatory effect on sympathetic drive to BAT, when injected either peripherally or centrally (Collins and Surwit, 1996; Haynes et al., 1997), and increases UCP1 messenger RNA (mRNA) and protein levels (Scarpace et al., 1997). Furthermore, many (if not all) of the neuronal factors which impinge on food intake also have effects on energy expenditure when administered centrally. For example, administration of orexigenic factors like neuropeptide-Y (NPY) (Billington et al., 1991) and orexin A (Yasuda et al., 2005), produce corresponding decreases in SNS activation of BAT, while anorexigenic peptides α-melanocortin stimulating hormone (α-

MSH) (Yasuda et al., 2004) and corticotrophin-releasing hormone (CRH) (Irwin et al., 1992) increase its sympathetic drive. Thus, it appears that common circuitry is responsible for the co-ordinated control of food intake and energy expenditure by BAT-mediated thermogenesis.

1.2 REGULATORY CENTRES OF THE BRAIN

The hypothalamus emerged as one of the critical sites for the regulation of energy balance as a result of rodent brain lesion studies in the 1940s and 1950s. Selective destruction of the arcuate nucleus (Arc), paraventricular nucleus (PVN), dorsomedial nucleus (DMN) or ventromedial nucleus (VMN) of the hypothalamus was found to cause obesity with immediate hyperphagia (Anand and Brobeck, 1951; Gold, 1973). Conversely, lesion of the lateral hypothalamus (LH) had the opposite effect, resulting in hypophagia and weight loss (Anand and Brobeck, 1951). Thus, it became widely accepted that populations of neurones in different areas of the hypothalamus have opposing effects on energy balance. Since these early studies, the molecular correlates of these effects have been largely identified, particularly with the discovery of specific neuropeptidergic neuronal populations. The major sensing areas are thought to be the Arc and VMN, which in turn project to the PVN, DMN and LH. However, cells in these latter areas are also capable of responding to peripheral signals such as glucose and leptin.

The arcuate nucleus

Following the discovery of leptin in the mid 1990s (Zhang et al., 1994), the Arc emerged as an attractive candidate for the 'heart' of the central energy balance circuitry. The leptin receptor was found to be richly expressed in this nucleus, and subsequently, the Arc was found to be essential in mediating the anorexigenic response to leptin (Takeda et al., 2002). Its position, immediately adjacent to the blood-brain-barrier (BBB) deficient median eminence is ideal for that of a metabolic sensor (Cone et al., 2001) and Arc neurones are responsive to a remarkably wide array of hormones and nutrients including glucose, insulin, ghrelin and P-YY (reviewed in Dhillo and Bloom, 2004). Phenotypic characterisation of Arc cells (Cheung et al., 1997; Elias et al., 1999; Mercer et al., 1996a) revealed neuropeptidergic populations with discrete spatial distribution that are oppositely regulated by metabolic signals such as leptin.

In rodents, the ventromedial subdivision of the nucleus is characterised by dense expression of NPY, a powerful orexigenic peptide. Chronic central administration of NPY produces extreme hyperphagia and increased fat mass (Zarjevski et al., 1993), while blocking endogenous NPY expression using antisense oligonucleotides decreases food intake and body weight (Kalra and Kalra, 2000). NPY release from the Arc is also nutritionally regulated, as negative energy balance results in elevations in

hypothalamic NPY (Dube et al., 1992; Kalra et al., 1991). In addition to its potent effects on feeding, NPY is also involved in the control of energy expenditure and storage. Central administration produces a reduction in SNS activity in BAT, and also promotes fat storage in WAT (Billington et al., 1991; Egawa et al., 1991). NPY binds to the Y_1-Y_6 receptors, which are widely expressed in the brain, and which have been linked with a number of physiological processes. The effects of NPY on energy balance are mediated by Y_1R and Y_5R, which are richly expressed in the Arc, PVN, LH and DMN (Herzog, 2003; Inui, 1999). However, knockout models reveal that the role of NPY and its receptors in the regulation of feeding and energy expenditure may be far more complex than first assumed. Neither knockout of NPY nor the Y_1 or Y_5 receptors produces the expected lean phenotype, or indeed alters the physiological responses to food deprivation (reviewed in Thorsell and Heilig, 2002). These observations suggest that NPY and its receptors are not critical for normal maintenance of energy balance and demonstrate clearly the level of redundancy that is present in this system. For example, NPY in the Arc is tightly co-expressed with another orexigenic neuropeptide agouti-related peptide (AgRP) which may well compensate for lack of NPY signalling in these animals.

A role for AgRP was first established following the observation that mice that ubiquitously over express AgRP or Agouti are hyperphagic and exhibit severe obesity (Ollmann et al., 1997). AgRP, a hypothalamic protein with high sequence homology to the pigment protein Agouti, is powerfully orexigenic when administered centrally to rodents (Ollmann et al., 1997; Shutter et al., 1997), and like NPY, is significantly upregulated by stimuli such as fasting, as well as during pregnancy and lactation (Rocha et al., 2003; Sorensen et al., 2002; Wilson et al., 1999). Furthermore, AgRP injected animals which are not allowed to over-consume still manifest increased fat mass, presumably due to alterations in energy expenditure (Small et al., 2001). Conversely, RNA interference (RNAi) against Arc AgRP increases metabolic rate and reduces body weight, without affecting food intake (Makimura et al., 2002). AgRP acts as an endogenous antagonist at the adenylate-cyclase-coupled melanocortin receptors (MCR) (Ollmann et al., 1997), the agonist of which is produced by a separate population of Arc cells.

The ventral aspects of the Arc are primarily occupied by catabolic neurones that express proopiomelanocortin (POMC), and cocaine and amphetamine-regulated transcript (CART). POMC is the precursor to α-MSH which binds to MC_3R and MC_4R, both of which are expressed in the brain (Mountjoy et al., 1994; Roselli-Rehfuss et al.,

1993). In contrast to NPY, expression of POMC and CART is markedly reduced during negative energy balance and is rapidly restored by re-feeding or leptin administration (Kristensen et al., 1998; Mizuno et al., 1998a; Swart et al., 2002). While the physiological relevance of CART is still relatively poorly understood, the bi-directional 'melanocortin system' is believed to have a pivotal role in controlling energy balance, both by effects on feeding and on energy expenditure (reviewed in Shimizu et al., 2007). Genetic knockout of POMC or the MC_4R produces a morbidly obese phenotype, while the phenotype of MC_3R null mice is far less severe (reviewed in Butler and Cone, 2002). In reflection of their function, MC_4Rs are found in hypothalamic nuclei involved in energy homeostasis, including the Arc, VMN, and PVN (Mountjoy et al., 1994) and in neurones that lie upstream of the sympathetic pathways to adipose tissues (Song et al., 2005; Song et al., 2008).

The paraventricular nucleus

The PVN is a major target of Arc projections and functions as a relay centre that projects to autonomic and endocrine effectors. Cells of the PVN are innervated by both AgRP/NPY, and POMC fibres, leading to the suggestion that they act as 'second-order' neurones, downstream of the Arc (Cowley et al., 1999). In fact, PVN neurones are highly sensitive to a wide range of peptides which have been implicated in feeding. Intra-PVN injection of NPY is an extremely powerful appetite stimulant, but CCK, leptin, insulin, orexin-A also affect food intake when administered into this nucleus (reviewed in Ferguson et al., 2008). This suggests that cells of the PVN are capable of integrating opposing signals from the periphery, the Arc, and elsewhere in the brain.

The PVN has been implicated in a number of autonomic functions, including regulation of the hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-thyroid (HPT) axes, water homeostasis, and SNS activity in addition to its role in controlling appetite (reviewed in Ferguson et al., 2008). It was first implicated in energy homeostasis following the observations that its stimulation inhibits food intake, while lesion results in hyperphagic obesity syndrome. Consistent with these findings, a number of catabolic cell types have been identified in the parvocellular portion of the PVN, including CRH and thyrotrophin-releasing hormone (TRH) (Hashimoto et al., 1982; Lechan and Jackson, 1982). These cells are densely innervated by both NPY/AgRP and melanocortin inputs from the Arc (Fekete et al., 2000a; Legradi and Lechan, 1999) and exert anorectic and weight-reducing effects when administered centrally (Morley and Levine, 1982; Vijayan and McCann, 1977). Moreover, their mRNA expression is markedly down regulated in response to food deprivation, an effect which can be

blocked by administration of leptin or α-MSH (Fekete et al., 2000a; Legradi et al., 1997a). Similar to fasting, intracerebroventricular (i.c.v.) administration of either NPY or AgRP markedly reduces the expression of these PVN neuropeptides.

One of the major outputs of the PVN involves projections to areas of the brainstem and spinal cord which drive sympathetic outflow. Trans-synaptic retrograde tracing studies have identified the PVN as a major upstream regulator of neuronal activation of peripheral fat tissues (Bamshad et al., 1998; Bamshad et al., 1999). There is evidence that both CRH and TRH may contribute to this function, since both peptides increase body temperature, BAT activation, oxygen consumption and lipid mobilisation (reviewed in Lechan and Fekete, 2006; and Richard et al., 2000). Moreover, the effects of TRH can be attenuated by sympathetic denervation of BAT, or by co-administration of β-adrenergic antagonists, suggesting that they are partially mediated by central autonomic pathways (Chi and Lin, 1983; Shintani et al., 2005). As previously discussed, sympathetic activation of fat tissues is now widely believed to be a major contributor to body weight regulation.

The lateral hypothalamus

The LH is also believed to be involved in 'second-order' signalling and receives dense inputs from the Arc. The LH was originally proposed as the brain's 'feeding centre' following the discovery that its stimulation results in increased food intake (Anand and Brobeck, 1951). Subsequently, discrete populations of neurones were discovered in this nucleus that express the orexigenic neuropeptides melanin-concentrating hormone (MCH) and orexin (also known as hypocretin). MCH rapidly increases food intake when administered centrally to rats, and its expression in the LH is up-regulated during negative energy balance (Qu et al., 1996). Furthermore, transgenic deletion of MCH (Shimada et al., 1998) or its receptor MCH-1 (Marsh et al., 2002) result in lean phenotypes, whereas MCH over expression causes obesity (Ludwig et al., 2001). This is in contrast to similar manipulations of NPY, and demonstrates that not all orexigenic pathways display redundancy.

There are two orexin peptides, orexin A and orexin B, which are products of the 130 amino-acid prepro-orexin gene, expressed in LH neurones distinct from those which produce MCH (Broberger et al., 1998). Like MCH, prepro-orexin is markedly upregulated by food deprivation and orexin A and, to a lesser extent, orexin B increase food intake when administered centrally (Sakurai et al., 1998). Orexin neurones exert their effects by actions at the excitatory orexin-1 and orexin-2 G-protein-coupled

receptors by projections to virtually all brain areas including the PVN, Arc, brainstem, cerebral cortex and preganglionic sympathetic neurones. Orexin knockout mice exhibit both hypophagia, but also narcolepsy due to orexin's key role in arousal (Chemelli et al., 1999; Hara et al., 2005). It is thought that the upregulation of orexin during fasting may therefore promote both arousal and hunger in order to induce food-seeking behaviour.

Both MCH and orexin cells of the LH are densely innervated by NPY/AgRP and POMC neurones from the Arc (Broberger et al., 1998). In turn, NPY/AgRP cells of the Arc are heavily contacted by orexin terminals (see Figure 1.2) and orexin application stimulates these cells both in calcium imaging and electrophysiological assays (Muroya et al., 2004; van den Top et al., 2004). Thus, it appears orexin and NPY work together to drive food intake. Cells of the LH are also responsive to humoral signals such as leptin and ghrelin. Almost all orexin neurones (Funahashi et al., 2000) and a relatively small number of MCH neurones (Hakansson et al., 1998) express the lepr, while ghrelin potently activates orexin, but not MCH cells (Lawrence et al., 2002; Toshinai et al., 2003). In addition to sensing hormonal signals, cells of the LH also sense changes in physiological glucose levels. The LH was first proposed as a 'sugar sensor' following the work of Anand and Oomura in the 1960's who demonstrated that injection of glucose, either peripherally or centrally decreased the firing of cells in this region. In the brain, glucose-excited (GE) neurones increase, whereas glucose-inhibited (GI) neurones decrease their firing rate as glucose levels rise, with a primary sensitivity range between 0.5–3.5 mM (Silver and Erecinska, 1994). Approximately 20% of LH cells respond to changes in extracellular glucose concentration, and the majority of these are GI. Recent studies have shown that GI neurones of the LH that increase their firing in response to low glucose also express orexin (Burdakov and Alexopoulos, 2005; Yamanaka et al., 2003). It appears, therefore, that the designation of the LH as a 'hunger centre' over 50 years ago was surprisingly accurate, as this area is able to integrate short and long-term peripheral 'hunger' signals, as well as those relayed from the Arc, in order to bring about feeding.

The ventromedial nucleus

The VMN was first associated with energy balance more than 50 years ago, but the mechanisms by which it regulates appetite and body weight are still largely unknown (McClellan et al., 2006; Schwartz et al., 1999). Many studies have demonstrated that rodents with bilateral lesions of the VMN are immediately hyperphagic and become morbidly obese to a greater extent than PVN-lesioned animals (Anand and Brobeck,

1951). However, it has been suggested that lesion sites in early studies may also include the Arc, casting doubt over the importance of the VMN in energy balance (reviewed in King, 2006). Moreover, following the discovery of NPY/AgRP and POMC/CART neuronal circuits research shifted from the VMN to the Arc, with many models of energy balance regulation excluding the VMN altogether (Berthoud and Morrison, 2008; Broberger, 2005). However, chemical lesion, gene therapy and pharmacological studies have supported the notion that the VMN inhibits feeding and increases energy expenditure, and thus is an important regulator of body weight (Bagnasco et al., 2002; Satoh et al., 1999; Tejwani and Richard, 1986). Furthermore, it was recently shown that VMN-agenesis by knockout of the transcription factor steroidogenic factor 1 (SF1) produces an obese phenotype (Majdic et al., 2002; Zhao et al., 2004), providing the first concrete evidence that the VMN is essential for normal body weight.

Another possible reason why interest in the VMN waned is that afferent projections from the Arc to the VMN are relatively sparse compared with those to nuclei such as the LH and PVN. However, the VMN does express substantial levels of MC_4R and NPY receptors. Furthermore, injection of NPY into the VMN potently elicits feeding, while NPY suppression prevents VMN-lesion induced hyperphagia (Dube et al., 1995; Jolicoeur et al., 1995). However, work by the Lowell laboratory has revealed that VMN and Arc-mediated effects on energy balance may be largely independent (Balthasar et al., 2004; Dhillon et al., 2006). Unexpectedly, they showed that mice with SF1-driven knockout of leptin receptor (lepr) are equally obese as POMC-driven-lepr-knockouts, and moreover, crossing the two strains produces a doubly-obese animal. This would suggest that convergence of Arc and VMN energy balance pathways is minimal, and also implicates the VMN as a first order target for leptin.

While neuronal subtypes remain to be fully characterised, it has been demonstrated that subpopulations of VMN cells are responsive to many peripheral factors, including leptin, ghrelin, insulin and glucose which convey information about body energy stores. Leptin (and insulin) causes mainly excitatory responses in VMN neurones, presumably by activation of catabolic cell types; however in overfed obese rats leptin has the opposite effect (Davidowa and Plagemann, 2000). Subsets of VMN neurones also change their firing rate following alterations in extracellular glucose concentration, which tightly reflects changes in the plasma (Oomura, 1983; Silver and Erecinska, 1998). Approximately 30% of neurones within the VMN are glucose sensitive, with the majority being classed as GE (Silver and Erecinska, 1998; Song et al., 2001). Local glucoprivation by application of the non-metabolisable glucose-analog, 2-deoxyglucose

(2-DG), into the VMN area triggers increased neuroendocrine and sympathoadrenal output which is characteristic of systemic hypoglycemia (Borg et al., 1995).

As well as its role in feeding behaviour and glucose metabolism, the VMN is also critically involved in thermogenesis (Perkins et al., 1981), female reproductive cycles and the lordosis response (Pfaff and Sakuma, 1979a, b). This supports the theory that the VMN is composed of a number of distinct subpopulations, similar to the situation characterised in the Arc. Satoh and colleagues (1999) showed that leptin injection into the VMN, but not other hypothalamic nuclei potently increases sympathetic drive to peripheral tissues. Furthermore, both VMN-lesioned and SF1-Cre $lepr^{flox/flox}$ animals have compromised adaptive thermogenesis responses, implicating the VMN in the sympathetic thermogenic response to HED (Dhillon et al., 2006; Himms-Hagen, 1989).

Despite these observations, the cellular and molecular correlates of VMN function remain relatively poorly understood. This is largely due to the fact that unlike the Arc, PVN and LH, few specific markers for VMN neurones are currently available. Recent studies have addressed this problem by employing molecular biology to identify genes that are enriched in the VMN compared with other hypothalamic nuclei (Kurrasch et al., 2007; Segal et al., 2005). In terms of neuropeptides, pituitary adenylate cyclase-activating polypeptide (PACAP) and brain-derived neurotrophic factor (BDNF) are robustly expressed in the VMN. Both these peptides have been implicated in energy balance, reducing food intake when administered centrally. Further work is required however, if these are to be characterised as true mediators of energy balance.

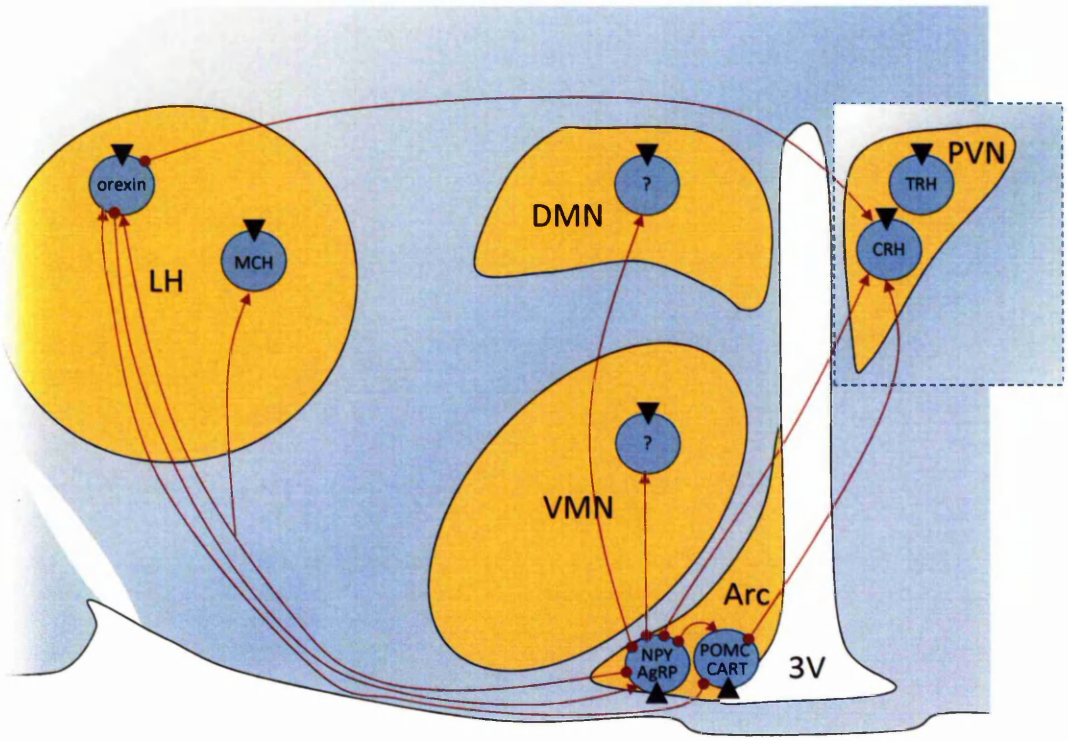


Figure 1.2 Hypothalamic energy balance circuits

Schematic showing the major intra-hypothalamic connections involved in the control of food intake. Black triangles represent lepr-B. LH= lateral hypothalamic area, DMN=dorsomedial nucleus, VMN=ventromedial nucleus, PVN=paraventricular nucleus, Arc=arcuate nucleus, MCH=melanin-concentrating hormone, NPY=neuropeptide-Y, AgRP=Agouti-related peptide, POMC=pro-opiomelanocortin, CART=cocaine amphetamine-regulated transcript, CRH=corticotrophin-releasing hormone, TRH=thyrotrophin-releasing hormone, '?'=unknown cell type

Other brain regions which influence energy balance

The hypothalamus is not the sole contributor to energy homeostasis, and several other regions have been implicated in energy sensing and feeding drive. There are extensive reciprocal connections between the hypothalamus and brainstem, which receives information from the gut both directly from humoral signals, and via vagal afferents from the GI tract. The main processor of energy balance inputs is the nucleus of the solitary tract (NTS) which, like the Arc, is situated adjacent to a region of compromised BBB, the area postrema. The NTS is understood to be a key regulator of meal initiation and termination and cells in this region are activated by feeding, gut distention, intra-gastric nutrient infusion and CCK (Luckman and Lawrence, 2003). Hence, the NTS appears key to short-term energy balance regulation.

In addition to energy deficit, the 'rewarding' sensation of feeding can itself act as an orexigenic stimulus, even in the absence of homeostatic need. The study of the role of reward centres, such as the nucleus accumbens (NA), in feeding and their interaction with classic energy balance systems has expanded in recent years. Indeed, many researchers believe hedonic feeding to be the main underlying cause of human obesity. The dopaminergic, opioid and endocannabinoid systems have all been implicated in feeding drive by reciprocal interactions with hypothalamic networks (reviewed in Berthoud, 2004). This communication means that the sensation of reward can itself be influenced by energy status, and factors such as leptin act on reward systems as well as homeostatic circuitry (Fulton et al., 2000). These studies highlight the fact that while investigation of the hypothalamic energy balance pathways is useful, the reality is that the decision to feed is far more complex, especially when the food is palatable.

Plasticity in energy balance circuits

Altering energy state has marked effects on neuronal firing and expression levels of neuropeptides in nuclei of the hypothalamus. For example, POMC is down regulated, while NPY is up regulated in animals subjected to a period of food deprivation. Clearly these plastic changes are designed to allow the circuits to produce different outputs depending on the afferent signals from the periphery. However, recent evidence demonstrates that the hypothalamus also displays what is coming to be known as 'soft-wiring', that is, the synaptic inputs onto key neurones are reorganised in response to metabolic stimuli (reviewed in Horvath, 2005). While such plasticity has been described in other systems as early as the 1980s, it had not been considered that such events

could contribute to the output of the hypothalamic energy balance pathways. Observations by several groups (Horvath and Gao, 2005; Pinto et al., 2004; Sternson et al., 2005) suggest that these changes may be a regulatory component under physiological conditions, while abnormalities in synaptic wiring may partly underlie the pathology of genetic obesity. Leptin-deficient *ob/ob* mice display marked abnormalities in the ratio of excitatory to inhibitory inputs onto both NPY/AgRP and POMC/CART cells, which are rapidly normalised by leptin treatment, even before the effects on food intake (Pinto et al., 2004). Similar re-wiring has subsequently been described following ghrelin treatment or food deprivation, raising the question of whether this might represent a more general regulatory response (Horvath and Gao, 2005). In support of this, alterations in synaptic architecture are not restricted to Arc neurones, since both orexin and dopamine cells undergo similar changes according to metabolic stimuli.

Since these changes appear to precede any behavioural and endocrine output in these studies it is tempting to infer that these changes must influence neuronal activity, however this has yet to be proven. It has been suggested, however, that such changes may alter the 'set point' or likelihood that a neurone will fire when it is stimulated by some other input. For example, an excitatory input is more likely to stimulate an action potential if it is surrounded by fewer GABAergic inputs. This raises the possibility that differential synaptic wiring could be a pre-determinant of obesity, depending on the sensitivity of the hypothalamic feeding circuits to metabolic factors. Another current unknown is the underlying mechanism which causes these changes to occur. It is possible, considering the obese phenotype of the basomedial hypothalamus-deficient BDNF mouse (Unger et al., 2007), and its well-studied role in synaptic plasticity in the hippocampus (reviewed in Hennigan et al., 2007), that actions of hypothalamic BDNF could be involved.

1.3 THE HORMONE LEPTIN

Unquestionably, the most significant discovery for energy balance research was the hormone leptin and its receptor, mutations of which underlie the morbidly obese phenotypes of the *ob/ob* (Zhang et al., 1994) and *db/db* (Lee et al., 1996) mouse strains respectively. *ob/ob* and *db/db* mice are characterised by hyperphagia, increased adiposity, decreased thermogenesis, hyperglycaemia and insulin resistance. Leptin replacement in *ob/ob* mice completely reverses the metabolic phenotype of the *ob/ob* mouse (Pelleymounter et al., 1995), and subsequently this hormone promised to be the long-sought peripheral feedback signal which would cure the obesity epidemic. Unfortunately such predictions never materialised, though enormous insights into energy homeostasis have been gained from the study of leptin and its physiological effects.

Leptin is secreted into the bloodstream primarily by WAT, and in the long term plasma leptin levels strongly correlate with the degree of adiposity. However, leptin levels can also be altered rapidly, for example during nutritional deprivation or cold exposure, before fat mass is significantly changed (Ahima et al., 1996; Hardie et al., 1996). Decreased leptin during fasting triggers adaptive decreases in energy expenditure, reproductive function and increased appetite, and these responses can be prevented if leptin levels are maintained at control levels (Ahima et al., 1996). Although leptin is generally believed to exert its effects on body weight over the long term, acute administration of leptin has potent appetite-reducing effects both in obese and lean animals (Halaas et al., 1995; Pelleymounter et al., 1995; Rentsch et al., 1995). In addition to these effects on food intake, leptin also impinges on energy expenditure by increasing BAT-mediated thermogenesis. Leptin injection either centrally or systemically increases sympathetic nerve activity to BAT and expression of UCP1 (Collins and Surwit, 1996; Haynes et al., 1997; Scarpace et al., 1997). It is still unknown whether the actions of leptin on energy intake and expenditure are mediated by the same, or by separate central targets.

The leptin receptor

The lepr is a member of the cytokine receptor family and possesses a single transmembrane domain. Alternative splicing and post-translational modifications result in multiple isoforms which can be divided into 'long', 'short' and 'secreted' (Tartaglia et al., 1995). The long form (lepr-B) possesses a long intracellular domain, which is required for activation of its intracellular signalling cascade and the effects of leptin on

energy intake and expenditure. Peripherally administered leptin in normal mice rapidly induces hypothalamic lepr-B signalling through three pathways: the PI3K (phosphatidylinositol 3 kinase) pathway, the JAK2-STAT3 (janus kinase 2- signal transducers and activators of transcription 3) pathway and the MAPK (mitogen-activated protein kinase) pathway (see Figure 1.3). The obese phenotype of the *db/db* mouse has been attributed to a mutation in the intracellular domain of lepr-B which prevents these pathways from being activated (Lee et al., 1996). Roles for the other lepr isoforms remain to be fully elucidated, although there have been suggestions that the short forms may be involved in transport of leptin across the BBB (Banks et al., 2002).

Leptin resistance and obesity

Since leptin mutations in humans are extremely rare (Montague et al., 1997), the overwhelming majority of obese humans have elevated leptin levels and very few respond favourably to exogenous leptin (Heymsfield et al., 1999). Therefore, a state of 'leptin resistance', rather than 'leptin deficiency' appears to be the major contributor to the lack of compensatory responses in obese subjects. It is thought that leptin resistance develops as a direct result of the hyperleptinaemia (White et al., 2008), although the mechanism remains elusive. A number of mechanisms have been suggested for leptin resistance, following examination of diet-induced obese (DIO) rodents which are resistant to the weight-reducing effects of peripherally administered leptin. The BBB leptin transport system is now believed to be a site of so-called 'peripheral leptin resistance', since DIO animals respond better (although less well than controls) to centrally, rather than peripherally administered leptin (Banks, 2008; Van Heek et al., 1997; Widdowson et al., 1997). The mechanism for this defect is thought to arise from a naturally-evolved starvation response, whereby hypertriglycerideaemia impedes leptin entry to the brain. The pathologically high triglyceride levels which manifest in obese individuals may therefore be a key cause of chronic leptin resistance (Banks, 2006).

There is also strong evidence that a second, later-onset central leptin resistance occurs at the level of the lepr-B intracellular signalling. Despite leptin activating various intracellular cascades, leptin resistance is almost exclusively characterised as a reduced activation of STAT3. El-Hashimi and colleagues (2000) were the first to show that prolonged exposure to HED renders even central administration of leptin ineffective at activating STAT3. Progress has since been made in identifying the intracellular molecules which could mediate this phenomenon; SOCS-3 (suppressor of

cytocine signalling-3) and PTP1B (protein tyrosine phosphatise 1B). SOCS-3 is a negative feedback signal which binds lepr-B and prevents STAT3 phosphorylation (Bjorbaek et al., 1999). Similarly, PTP1B inhibits the JAK2-STAT3 pathway by dephosphorylating JAK2, preventing activation of STAT-3 (Zabolotny et al., 2002) (see Figure 1.3). Genetic deletion of either of these signalling molecules produces mice which are lean, leptin hypersensitive and DIO-resistant (DIO-R) (Elchebly et al., 1999; Klaman et al., 2000; Mori et al., 2004). New evidence suggests that mediobasal hypothalamic PTP1B (White et al., 2008), and Arc SOCS-3 (Munzberg et al., 2004) are upregulated in obese individuals that fail to respond to leptin. Currently however, there is little direct evidence to imply that these molecules may be causal to the development of obesity, and indeed whether they represent realistic targets for medical intervention strategies.

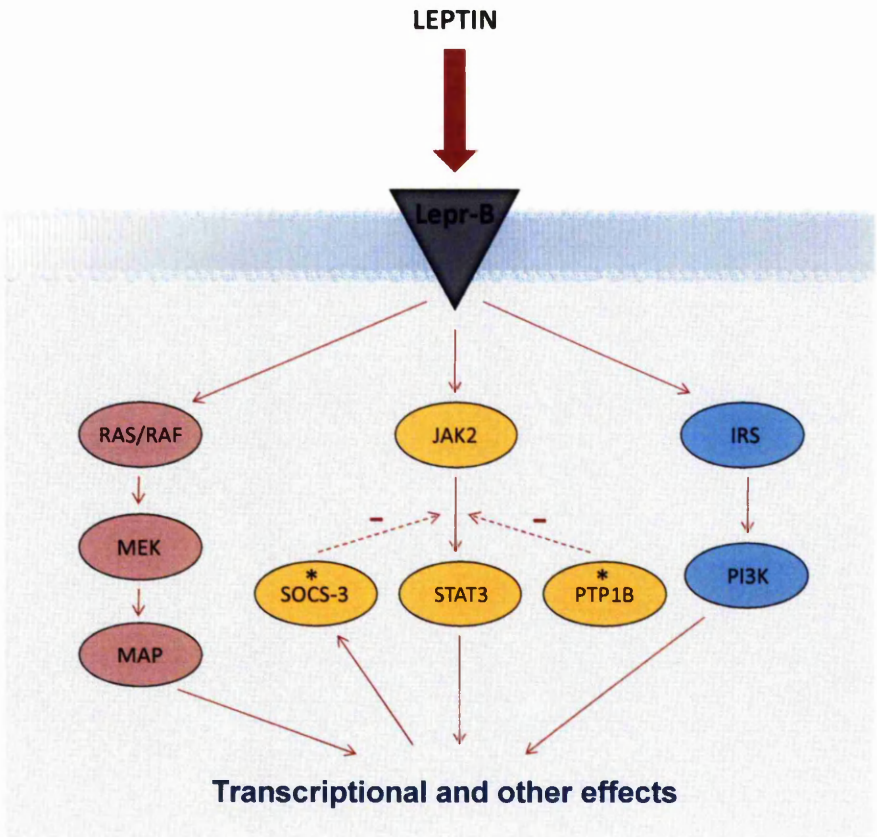


Figure 1.3 Leptin receptor signalling cascade

There are three feed-forward signal propagation pathways which are activated by leptin binding to lepr-B: the MAPK (mitogen-activated protein kinase) pathway, the JAK2-STAT3 (janus kinase 2- signal transducers and activators of transcription 3) pathway and the PI3K (phosphatidylinositol 3 kinase) pathway. The activation of SOCS-3 acts as a negative feedback of the JAK-2-STAT3 pathway by binding to lepr-B and preventing STAT3 activation. PTP1B, which is induced by leptin also negatively-regulates this pathway by dephosphorylating JAK2.

Leptin targets

The signalling form of the leptin receptor, lepr-B, is expressed in several brain regions, including in particular the hypothalamic Arc, PVN, VMN, DMN and LH (Mercer et al., 1996b) (see Figure 1.2). Although different forms of the leptin receptor have also been localised to a number of peripheral tissues, the effects of leptin on energy balance have been demonstrated to be mediated entirely by central targets. Injection of leptin into the lateral ventricle potently reduces food intake and increases sympathetic outflow to BAT and WAT. Furthermore, central-nervous system (CNS)-specific deletion of leprs produces marked obesity, whereas genetic reconstitution of lepr expression in the brain of *db/db* mice reverses their obesity (Cohen et al., 2001; Kowalski et al., 2001). However, the relative importance of leptin action at different central targets is currently unknown.

The attraction of the Arc as the primary mediator

To date, the majority of studies have focussed on the role of leptin signalling in the Arc, while the role of leptin signalling in extra-arcuate targets has been largely ignored. Undeniably, the Arc is fundamental for eliciting leptin's effects on body weight, as Arc-lesioned *ob/ob* animals fail to lose weight in response to exogenous leptin (Takeda et al., 2002) and replacement of lepr expression in the Arc of lepr-deficient rats reduces their obesity. Leptin has been shown to inhibit anabolic NPY/AgRP cells and stimulate catabolic POMC/CART cells in the Arc, via activity at lepr-B (Cowley et al., 2001; Nagamori et al., 2003). Furthermore, expression levels of POMC, AgRP and NPY mRNAs are disregulated in the *ob/ob* mouse, and these levels can be normalised by exogenous leptin (Mercer et al., 1997; Mizuno et al., 1998a; Mizuno and Mobbs, 1999) A hierarchical role for the Arc in the response to leptin was put forward by Munzberg (2008), following the observation that peripheral leptin induces pSTAT-3 more rapidly and with greater sensitivity in the Arc than other sites. While this evidence is persuasive, genetic deletion of lepr-B selectively on POMC neurones did not result in as extreme a phenotype as expected (Balthasar et al., 2004). These mice do develop obesity, but only mildly (around 20% that of *db/db*), raising the possibility that other first-order leptin responsive neurones also contribute to leptin's effects on body weight. Considering also that $NPY^{-/-}$ crosses to the *ob/ob* strain only partly ameliorate obesity (Erickson et al., 1996), and AgRP-driven lepr-B deletion only gives a mild obesity phenotype (Dhillon et al., 2006), these additional targets are almost certainly located outside the Arc, refuting the old model. As such, and considering that no practical therapeutic targets for the treatment of obesity and other metabolic diseases have thus far been identified in the Arc, interest is now shifting to extra-arcuate sites.

The VMN is essential for leptin signalling

It has long been acknowledged that leptin receptors are expressed in other hypothalamic nuclei (Mercer et al., 1996b), and that these are readily engaged by exogenous leptin, as shown by robust induction of c-Fos expression (an immediate early gene which denotes neuronal activation) and STAT-3 phosphorylation (Elmquist et al., 1997; Hubschle et al., 2001). Dhillon et al., (2006) recently showed that SF1 cells of the VMN are first-order leptin responsive neurones, since 80 % of these cells increase their firing rate in response to exogenous leptin. Furthermore, deletion of lepr-B selectively in these VMN cells results in obesity comparable to that of POMC-driven deletion. These data imply that, far from being subordinate to the Arc, the VMN may impose just as much influence on central leptin signalling. Interestingly, and unlike the POMC lepr-B mutants, these mice also display a marked inability to adapt to HED with increased SNS drive to BAT and decreased food intake. As such, their obesity is massively exacerbated by HED-feeding. It is conceivable then that defective leptin signalling in the VMN may pre-dispose to increased adiposity, by improper control of both energy intake and expenditure, in an obesogenic environment. While SF1 is a useful marker of leptin-responsive cells, the functional phenotype of these neurones remains unknown. Future research must therefore be directed at identifying genes which delineate subsets of VMN neurones, and the downstream consequences of their activation.

1.4 SUMMARY

The processes that control feeding behaviour, energy expenditure and ultimately body weight are extremely complex and involve interactions between peripheral organs and hypothalamic and brainstem nuclei, as well as inputs from sensory and reward centres of the brain. Researchers have come a long way in recent years in elucidating the networks that control energy balance, but harsh lessons regarding oversimplification should be learned from recent transgenic models, that refute the Arc as the most important regulator. Furthermore, since pharmacological targeting of the Arc neuoropeptidergic populations has been fruitless in terms of therapy for pathological energy imbalance, research is now shifting to extra-arcuate sites. The fat-derived hormone leptin is still considered one of the most important regulators of energy balance and recently the VMN was demonstrated to be an essential mediator of leptin's effects on body weight. However, unlike other hypothalamic nuclei, the VMN remains poorly characterised in terms of its cellular phenotypes. Further investigation into the mechanisms by which the VMN regulates energy balance, particularly in reponse to leptin signalling is therefore required.

1.5 PhD AIMS AND OBJECTIVES

The initial objective of my PhD was to identify neuronal markers for the VMN and to characterise their expression levels in response to positive and negative energy balance stimuli. Particular emphasis was given to the regulatory responses to leptin, by examination of leptin-naïve and leptin-treated *ob/ob* mice. Following this initial 'screening' process, we then went on to further assess the interaction between leptin and a promising VMN-marker, PACAP, using *in vivo* pharmacology and histological studies. The interactions between PACAP and other established feeding circuits such as the CRH and melanocortin pathways were also considered. Finally, since the actions of leptin in the VMN are currently poorly understood, we also investigated the possibility that leptin-induced synaptic re-wiring in the VMN may be a mechanism for body weight control.

CHAPTER 2

GENERAL METHODS

2.1 GENERAL METHODS

Animals

Unless otherwise stated, experiments were performed using adult male CD1 mice (35-40g, Harlan, UK). Animals were maintained on a 12:12 light:dark cycle at 22±1 °C with 45±10 % humidity and had free access to standard rodent laboratory chow (Beekay, UK) and tap water. All experiments were performed in accordance with the Animals (Scientific Procedures) Act (1986).

Drugs

Compounds for investigation were administered via injection i.c.v. or intraperitoneally (i.p.). All drugs were obtained from commercial suppliers and were certified pyrogen and pathogen free. PACAP (Bachem, UK) and leptin (Peprotech, UK) were recombinant murine and were diluted in sterile saline vehicle and stored in aliquots at -20 °C. Central injection volume was 1 µl and peripheral injection volumes ranged between 80 and 100 µl.

2.2 *IN VIVO* TECHNIQUES

Implantation of guide cannulae

Animals were anaesthetised using 3-5 % isoflurane (Baxter International Incorporated, USA) and following initial knockdown, were maintained on 1-2 % isoflurane in oxygen (500 ml/min). Animals received an intramuscular (i.m.) injection of buprenorphine (0.03 mg/kg vetergesic®, Reckitt Benckister Healthcare, UK) at the start of surgery. During cannula implantation, the skull was immobilised in a stereotaxic frame using ear bars and an incisor bar, and depth of anaesthesia was monitored by loss of the foot-pinch reflex and by rhythmicity of breathing. Briefly, fur was removed from the surgical area and a 2 cm incision was made along the midline of the cranium, exposing the skull. A sterilised guide cannula was inserted to the depth of 1 mm through a 1.5 mm hole drilled 1 mm lateral and 0.2 mm posterior to bregma. The cannula was fixed in position using acrylic dental cement (Simplex Rapide, UK) adhered to a jewellers screw, positioned posterior to the cannula. The wound was then closed with suture (Mersilk, Ethicon, USA).

Implantation of remote radiotelemetry transmitters

Animals were anaesthetised as described above, and positioned supine. A 4x3 cm area of the lower abdomen was shaved and a 1.5 cm incision was made in the skin and underlying muscle, exposing the peritoneal cavity. A pre-calibrated sterile radio transmitter (TA-F20, Data Sciences International, USA) was inserted into the cavity and muscle and skin layers were closed with suture. Following surgery, animals were singly housed and were maintained on insulating heat pads until they had fully regained consciousness. Post-surgical animals were given 1 week to fully recover before being used in any experiment.

Daytime assessment of fast-induced re-feeding behaviour

Animals were food-deprived from 16:00 on the day before injection with *ad libitum* access to tap water. Between 10:00 and 11:00 the following day animals were injected i.c.v. with saline or drug into the lateral ventricle using a 5 µl Hamilton syringe attached to a flexible injection cannula. Minimal restraint was required as the animals had been pre-acclimated to handling. Weighed food was immediately returned to the food hopper and food intake was monitored at 1, 2, 4 and 24 hours post injection.

Assessment of nocturnal feeding behaviour

Animals were food-deprived for 2 hours prior to lights off (20:00) and received an i.c.v. injection of saline or drug between 19:45 and 20:15. Weighed food was immediately returned and food intake monitored at 1, 2, and 24 hours post injection. Body weight was measured at the time of injection, and 24 hours post-injection.

Measurement of core-body temperature by remote radiotelemetry

Chronically implanted radio transmitters were magnetically activated and were detected by individual receiving pads, positioned underneath the animal's cage. The signal was converted to temperature readouts by an external processor (Dataquest Art version 2.3 software, Data Sciences International, USA). Baseline temperature data were established 24 hours prior to injection and recording continued until 24 hours post-injection. Animals had free access to food and water throughout the experiment. 1 µl i.c.v. injections of saline or drug were given between 10:00 and 11:00 using minimal restraint.

Measurement of energy expenditure using indirect calorimetry

Animals had free access to chow and water throughout the experiment. The Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, USA)

setup was used, allowing for automated, non-invasive collection of metabolic data. Animals were pre-weighed and allowed to acclimatise to the CLAMS cages for 24 hours. Oxygen consumption (VO_2), carbon dioxide production (VCO_2) and respiratory quotient (RQ) of each animal were calculated by indirect calorimetry, using the Oxymax computer programme. Injections of saline or drug were given i.c.v. at 10:00 using minimal restraint.

Tissue fixation by transcardial perfusion

Animals were deeply anaesthetised using 5 % isoflurane in oxygen (500 ml/min) before transcardial perfusion through the left cardiac ventricle with heparinised (10,000 I.U/l) isotonic saline (0.9 % sodium chloride; NaCl) for 5 minutes, followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for a further 10-15 minutes. Brains were removed and post-fixed in 4 % PFA in 0.1 M PB for 24 hours at 4 °C, before cryoprotection in 30 % sucrose in 0.1 M PB for 48 hours at 4 °C. Brains were then frozen on dry ice and stored at -80 °C until required for immunohistochemical staining.

2.3 *IN SITU* HYBRIDISATION HISTOLOGY (ISHH)

^{35}S Oligonucleotide labelling for ISHH

Oligonucleotide probes (see Table 3.1) were end-labelled with ^{35}S-dATP by incubation with terminal deoxynucleotydyl transferase (TdT) enzyme. Each labelling reaction contained 100 ng nucleotide (Sigma-Aldrich, UK), 25 µCi ^{35}S-dATP (Perkin Elmer, USA) and 30 U TdT enzyme (Promega, UK) in a final volume of 10 µl. Reactions were incubated at 37 °C for 1 hour, then 90 µl Trisma-ethylenediaminetetraacetic acid (EDTA) buffer (TE; Sigma Aldrich, UK) was added and temperature increased to 65 °C to deactivate the enzyme. Unincorporated labelled nucleotides were removed by loading samples in MicroSpin™ G-25 columns (Amersham Biosciences, UK) and centrifuging for 2 minutes at 735 x gravity according to kit instructions. Care was taken throughout to avoid contamination with RNase.

Tissue preparation and ISHH

Brains for ISHH were removed under RNase-free conditions and immediately frozen on dry ice. 15 µm hypothalamic sections were then cut using a cryostat and thaw-mounted onto Polysine™ slides (Menzel and Glaser, UK) and dried using a hair dryer. Sections were fixed using 4 % PFA in 0.1 M PB and then washed twice in 0.1 M phosphate-

buffered saline (PBS). Sections were then treated with 0.25 % acetic anhydride in 0.1 M triethanolamine hydrochloric acid /0.9 % NaCl (TEA/AA) for ten minutes followed by dehydration in increasing concentrations of ethanol, and then air dried. Sections were hybridised at 37 °C overnight in a moist chamber, with 250 µl hybridisation buffer (HB: 4x standard sodium citrate (SSC; Promega, UK), 500 µg/ml salmon testes DNA, 2 % Denhardt's solution, 2.5 % dextran sulphate, 50 % de-ionised formamide; all Sigma-Aldrich, UK) containing $2.5x10^5$ cpm labelled oligonucleotide and 0.9 µl of β-mercaptoethanol per slide. Slides were briefly washed in 1x SSC to remove excess buffer. Sections then had three 30 minute washes in 1x SSC at 55 °C, followed by one hour at room temperature (RT) in the same buffer. Finally, slides were dipped in 300 mM ammonium acetate to remove salts, rinsed in 70 % ethanol and air dried. Slides were then exposed to Kodak Biomax® film and stored in the dark at 4 °C. Exposure time varied according to the probe used (see Table 3.1). Data were collected semi-quantitatively using Leica-Qwin imaging software to calculate region-specific optical density (OD). Relative gene expression was then calculated by normalising data to background and expressing as arbitrary units.

2.4 STATISTICAL ANALYSES

Data were expressed as mean ± standard error of the mean (SEM). Where only two groups are compared, an unpaired student's *t*-test was used. Where more than two groups were compared, a one-way analysis of variance (ANOVA) was performed, followed by Newman-Keuls (to compare all groups) or Dunnets (to compare to control only) *post hoc* tests. Where more than one variable was compared e.g. drug and genotype, a two-way ANOVA was used, followed by Bonferroni *post hoc* test to assess significant effects of either parameter. To examine the relationship between body weight on high enery diet and VMN gene expression, Pearson Correlation was performed. Statistical significance was taken as $P<0.05$. All statistical analyses were performed using Graphpad Prism version 4 software for Windows (Graphpad Software, La Jolla, USA).

CHAPTER 3

REGULATION OF VMN GENES

3.1 INTRODUCTION

The VMN is essential in the control of appetite, thermogenesis, glucose homeostasis and a number of other autonomic functions, yet despite this, few markers for specific classes of VMN neurones are available (Segal et al., 2005). This is in stark contrast to other hypothalamic nuclei involved in energy balance regulation, such as the Arc, PVN and LH where numerous neuropeptides have been identified and which delineate specific populations (reviewed in Kalra et al., 1999). Identification of such markers has allowed huge progress to be made in the understanding of signalling pathways, particularly in the Arc where catabolic POMC and anabolic NPY cells can be seen to respond in opposite ways to stimuli such as leptin (Elias et al., 1999). The initial aim of this thesis is to better characterise VMN cell types, in the context of energy homeostasis, and to identify potentially valuable neuronal markers, similar to those that exist elsewhere in the hypothalamus. In this chapter, we will use semi-quantitative ISHH to investigate whether expression of selected VMN-enriched genes, nNOS, KCC2, BDNF and PACAP, some of which have already been implicated in energy balance and glucose handling, are responsive to different metabolic stimuli in rats and mice.

The role of nNOS in regulating energy balance

Once known only for its ability to pollute the atmosphere, nitric oxide (NO) is now recognised as a major messenger molecule in a number of different body systems, including the CNS (Garthwaite et al., 1988; reviewed in Knott and Bossy-Wetzel, 2008). In the adult brain NO modulates synaptic transmission and plasticity, and acts retrogradely to induce presynaptic release of a variety of neurotransmitters (Segovia and Mora, 1998). The neuronal form of the enzyme nitric oxide synthase (nNOS) catalyses conversion of L-arginine to L-citrulline and NO and is widely expressed in the brain. Identification of a metabolic role for NO was first discovered by Morley and Flood (1991), and subsequent work has demonstrated that arginine analogs, which inhibit NO production reduce food intake when administered centrally, while L-arginine itself is orexigenic (Morley et al., 1995a; Squadrito et al., 1993). Furthermore, N^G-nitro- L-arginine methyl ester (L-NAME) also increases oxygen consumption and sympathetic activation of brown adipose tissue activation in a similar manner to leptin (De Luca et al., 1995; Sadler and Wilding, 2004). NO may also have a role in the glucose sensing properties of VMN neurones, as nNOS expression is negatively correlated with glucose concentration in cultured glucose-inhibited cells (Canabal et al., 2007). Together, these

in vivo results imply a role for NO in the central regulation of energy homeostasis and the response to glucoprivation.

The role of KCC2 in regulating energy balance

KCC2 is a potassium chloride co-transporter which is highly expressed in the adult rat brain, including in the VMN, and is understood to be important in evoking the inhibitory response to GABA. Since the VMN is particularly strongly innervated by GABAergic projections, it is perhaps not surprising that it expresses particularly high levels of KCC2 mRNA. Whilst changes in KCC2 expression have been primarily associated with shifts in excitability in the developing brain (Lu et al., 1999; Rivera et al., 1999), KCC2-dependent shifts from hyperpolarising to depolarising $GABA_A$-mediated responses have been observed under various pathophysiological epileptiform conditions in the adult CNS (Woo et al., 2002; Woodin et al., 2003). While such shifts have not been described under physiological stimuli, it is not unfeasible that changes in KCC2 expression could be involved in modulating GABAergic responses in intact adult systems, such as hypothalamic feeding circuitry. Indeed, knockout mouse models show that KCC2 expression is negatively regulated by BDNF (Rivera et al., 2004; Wardle and Poo, 2003), a VMN neuropeptide which has been strongly linked with regulation of body weight (Xu et al., 2003).

The role of BDNF in regulating energy balance

BDNF and its tyrosine-kinase receptor, TrkB, are widely expressed throughout the adult brain, in particular the hippocampus and hypothalamus. BDNF plays a crucial role in neuronal survival and differentiation, and has been associated with numerous central roles including regulation of food intake (Binder and Scharfman, 2004; Lebrun et al., 2006). BDNF has antiobesity and antidiabetic effects, causing a potent dose-dependent reduction in feeding following VMN injection (Wang et al., 2007). Furthermore, central BDNF administration increases energy expenditure, and ameliorates hyperinsulinaemia in both genetic *(db/db),* and acquired (DIO), models of obesity and leptin resistance (Nakagawa et al., 2003). The TrkB hypomorph ($trkB^{PLC/PLC}$) expresses TrkB at approximately 25 % normal levels and manifests adult onset obesity (Xu et al., 2003). Moreover, while BDNF knockout is lethal, an obese phenotype is observed in both $BDNF^{+/-}$ heterozygotes (Kernie et al., 2000)and in conditional knockout mice that have BDNF removed either post-natally (Rios et al., 2001), or selectively in the ventromedial hypothalamic region (Unger et al., 2007).

The role of PACAP in regulating energy balance

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the secretin/ glucagon/ vasoactive intestinal polypeptide family of peptides and was initially isolated for its cAMP-stimulating properties in cells of the anterior pituitary (Miyata et al., 1989). While PACAP has multiple centrally mediated functions (reviewed in Vaudry et al., 2000), several lines of evidence demonstrate a role for PACAP in the regulation of energy balance. Central PACAP administration reduces food intake in rodents (Mizuno et al., 1998b; Morley et al., 1992), chicks (Tachibana et al., 2003) and goldfish (Matsuda et al., 2005b) and causes hyperthermia in rodents (Masuo et al., 1995; Pataki et al., 2000, 2003). Furthermore, genetic ablation of PACAP signalling impairs lipid and carbohydrate metabolism, glucose handling and BAT thermogenesis (Adams et al., 2008; Gray et al., 2001; Gray et al., 2002; Hashimoto et al., 2000; Nakata et al., 2004). Since PACAP appears to affect energy balance pathways, it is of interest that PACAP mRNA is strongly expressed in neurones of the hypothalamus, and particularly in the VMN. Moreover, subpopulations of neurones in the Arc and PVN express PACAP receptors (Mounien et al., 2006; Nomura et al., 1996), suggesting that PACAP may modulate energy balance via action in these nuclei.

Objectives:

To test whether the expression levels of VMN genes are affected by 48-hour fasting

Food deprivation induces a number of physiological changes which allow an organism to conserve energy until food becomes available, including down regulation of BAT thermogenesis, heart rate, spontaneous locomotor activity, and reproductive function. States of negative energy balance are also characterised by low plasma leptin, insulin and glucose levels, high plasma ghrelin, and increased levels of free-fatty acids, due to increased oxidation of fat stores. The response to fasting is a straightforward paradigm for identification of genes involved in energy balance since alterations in hypothalamic pathways which promote replenishment of fat and lean mass are intensely up regulated in response to this stimulus. Following a fast, mRNA levels of orexigenic neuropeptides, such as AgRP and MCH (Qu et al., 1996; Wilson et al., 1999) are significantly increased, whereas anorexigenic POMC mRNA is decreased (Mizuno et al., 1998a) . Since the VMN is a key regulator of both appetite and energy expenditure, and is responsive to an array of metabolic signals, it is likely that fasting may induce changes in gene expression in this nucleus as it does elsewhere in the hypothalamus. We will use radioactive ISHH to investigate whether expression of nNOS, KCC2, BDNF

and PACAP in the VMN and elsewhere in the brain are responsive to a moderate food-deprivation stimulus in rats and mice.

To test whether the expression levels of VMN genes are affected by HED-feeding

DIO in rodents represents a state of positive energy balance roughly akin to that of common human obesity. Rodents (and humans) possess varying susceptibility to increased adiposity in an obesogenic environment, with some individuals showing natural resistance to weight gain (Enriori et al., 2007; Jebb et al., 2006; Koza et al., 2006; Levin et al., 1997). While a variety of mechanisms could contribute to this variability, several lines of evidence suggest that differences in the hypothalamic energy balance circuitry could be responsible. Recent characterisation studies on HED-fed mice reveals impairments of thermogenic adaptation, calorie intake control (Koza et al., 2006), glucose handling and the anorexigenic response to leptin (Enriori et al., 2007) in obese but not lean HED-fed mice. These features suggest defects at the level of the hypothalamus, although the molecular correlates remain to be identified.

Specific examination of VMN-enriched genes has thus far been overlooked and we propose that this may be a key region for uncovering early indicators of pre-disposition to obesity and the mechanisms behind the processes which guard against it. In support of this concept, the Lowell laboratory recently demonstrated that VMN-targeted mutagenesis of Lepr-B renders mice incapable of compensatory homeostatic adaptations and, as such, these animals are prone to severe obesity on HED (Dhillon et al., 2006). We will carry out radioactive ISHH in hypothalamic brain sections from rats and mice which have been maintained on either HED or standard laboratory chow. This should give us an insight into whether obesity-prone animals have abnormalities in VMN cell populations that could prevent them from regulating their energy balance in a normal homeostatic manner.

To test whether expression levels of VMN-enriched genes are affected by leptin

Leptin levels are altered during stimuli such as fasting and HED, and one of leptin's known major chronic effects is to induce changes in central gene expression. A major dissimilarity between naturally DIO-R and DIO animals is their responsiveness to leptin, and leptin resistance is known to be a major contributor to persistent weight gain. To better characterise whether leptin is a significant influence on VMN gene expression, we employed the leptin deficient *ob/ob* mouse model. *ob/ob* mice have marked abnormalities in hypothalamic expression levels of AgRP and POMC (Arvaniti et al., 2001), and these can be rectified by administration of exogenous leptin, eventually

resulting in normal energy balance and body weight. We will measure expression levels of our chosen genes in the VMN of *ob/ob* mice compared with heterozygous lean littermate controls. We will then investigate if sub-chronic treatment of *ob/ob* mice with exogenous, peripherally administered leptin can normalise expression levels before it fully ameliorates obesity.

3.2 METHODS

Animals and diets

Experiments were performed using male Sprague-Dawley rats (300-350g) or male CD1 mice (35-40g) unless otherwise stated.

A. **48-hour fasting:** Experimental animals were food-deprived for 48 hours (tap water *ad libitum*) before sacrifice. Controls had free access to chow and water. (rats n=6, mice n=7 per group)

B. **High-energy diet:** Animals were maintained on HED for a total of 8 weeks (rats: 4.73 kcal/g, 45 % from fat, D12451; mice: 5.24 kcal/g, 60 % from fat, D12492; Research Diets Inc., USA). Age-matched controls received normal chow (NC) for the duration of the experiment (rats n=6-18, mice n=8)

C. **Leptin deficiency and replacement:** 8-week old male *ob/ob*-C57BL/6 mice (Harlan, UK) were given daily i.p. injections of recombinant murine leptin (2 mg/kg), or vehicle (sterile isotonic saline) at 10:00 for 5 days. *ob/wt* lean controls received daily injections of saline (n=8). Mice were weighed daily throughout the experiment.

Semi-quantitative ISHH

Animals were killed by cervical dislocation between 10:00 and 12:00 and brains quickly removed and frozen on dry ice. *In situ* hybridisation of hypothalamic sections was carried out using ^{35}S-labelled oligonucleotide probes, as detailed in the general methods chapter using the probes listed in Table 3.1. Statistical analysis of resulting signal density in the regions indicated in Figure 3.1, was carried out as described in the general methods chapter. Representative sections are shown in Figure 3.2.

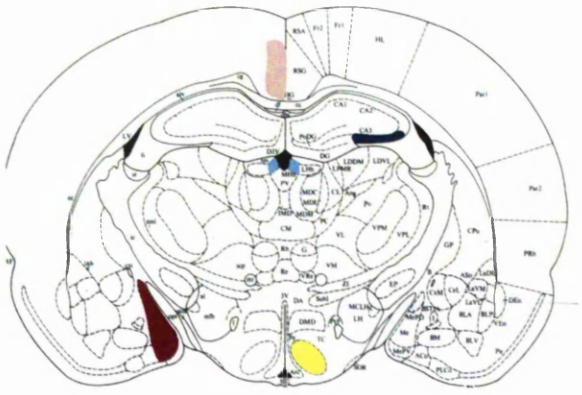


Figure 3.1 Regions of expression analysis
Schematic showing regions assessed for optical density of radioactive *in situ* hybridisation histological signal for each probe. Yellow= ventromedial nucleus of the hypothalamus, all genes; red=medial amygdala, nNOS; pink=Retrosplenal cortex, KCC2; green=CA3 hippocampus, BDNF; blue=medial habenula, PACAP. Adapted from Paxinos and Watson 1986. Figure 29.

Probe	Sequence	Reference	Exposure
nNOS	5'-AGAGTTCTGTCGCCTCTGTCTTGACGTCAACTTGGCGTCATCTGC-3'		
nNOS	5'-AGGCAGAGCAGGGCCACTCGGTTCAGCCGTGTGTCCGAATTT-3'	(Luckman et al., 1997)	4 weeks
nNOS	5'-CCTTTGTTGGTGGCATACTTGACATGGTTACAGATGTAGTTGAAC-3'		
KCC2	5'-TGGCTTCTCCTCGTTGTCACAAGCTGTCTCTTCGGG-3'		
KCC2	5'-TAGGAGGTCATATCACTGAAGACATAGGGGTGGTCC-3'	(Kanaka et al., 2001)	9 days
BDNF	5'-GGTCTGGTAGAAATATTGCTTCAGTTGGCCTTTTGATACCGGGAC-3'	(De Foubert et al., 2004)	8 weeks
PACAP	5'-CAGTCACTGTCTGGAGCTGGTGTCTGGAGAGAAGCC-3'	N/A	4 weeks

Table 3.1 Oligonucleotide probes
Oligonucleotide sequences used for *in situ* hybridisation histology of rat and mouse hypothalamic sections. KCC2 and nNOS probes were used as equal mass combinations.

3.3 RESULTS

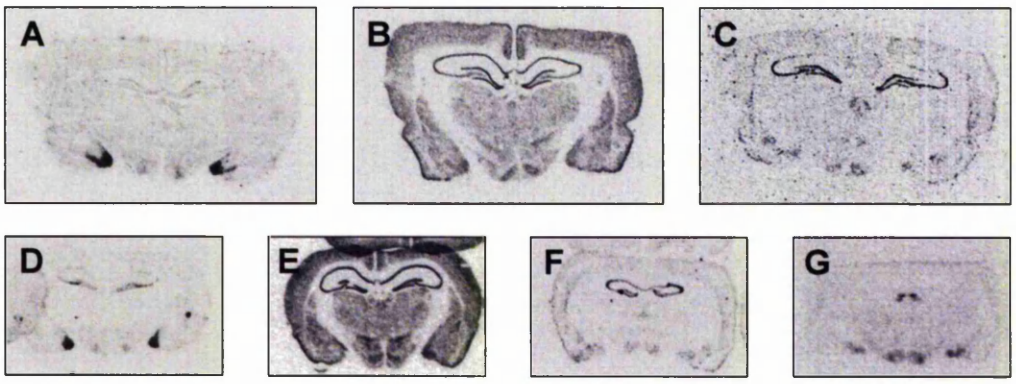


Figure 3.2 Representative autoradiographs

Representative coronal sections showing oligonucleotide binding in normal rat (**A.-C.**) and mouse (**D.-G.**) brain. **A. and D** nNOS, **B. and E.** KCC2, **C. and F.** BDNF, **G.** PACAP.

Effect of 48-hour fasting and 8 week HED on body weights of rats and mice

48-hour food deprivation caused a significant reduction in body weight in rats and mice (-17±1 % and -20±2 %, respectively; see Figure 3.3). Conversely, animals maintained on HED for 8 weeks were heavier than chow-fed controls (rat: 22±8 %; mouse: 21.5±2 %). As has been previously reported in cohorts of outbred animals, the extent of obesity varied, with an apparent bimodal distribution in both rats and mice, although our groups represent too small a sample to verify this (see Figure 3.4).

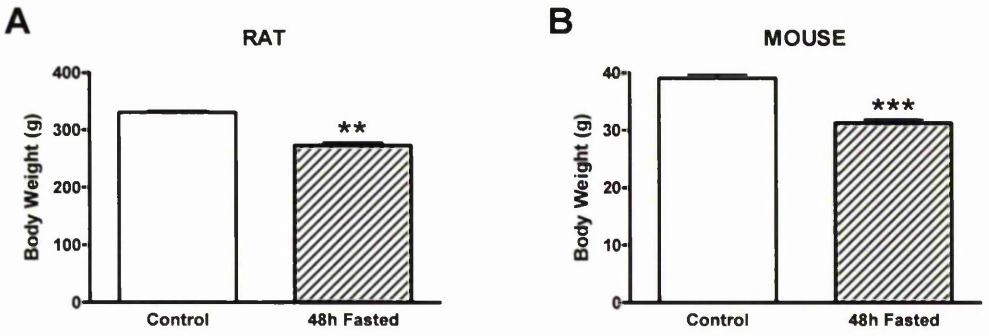


Figure 3.3 Body weight data: 48-hour fast

Final body weights of **A.** rats and **B.** mice following 48 hours of food-deprivation, or control chow *ad libitum*. Data are expressed as Mean ± SEM. **P<0.01, ***P<0.001; Student's unpaired *t*-test.

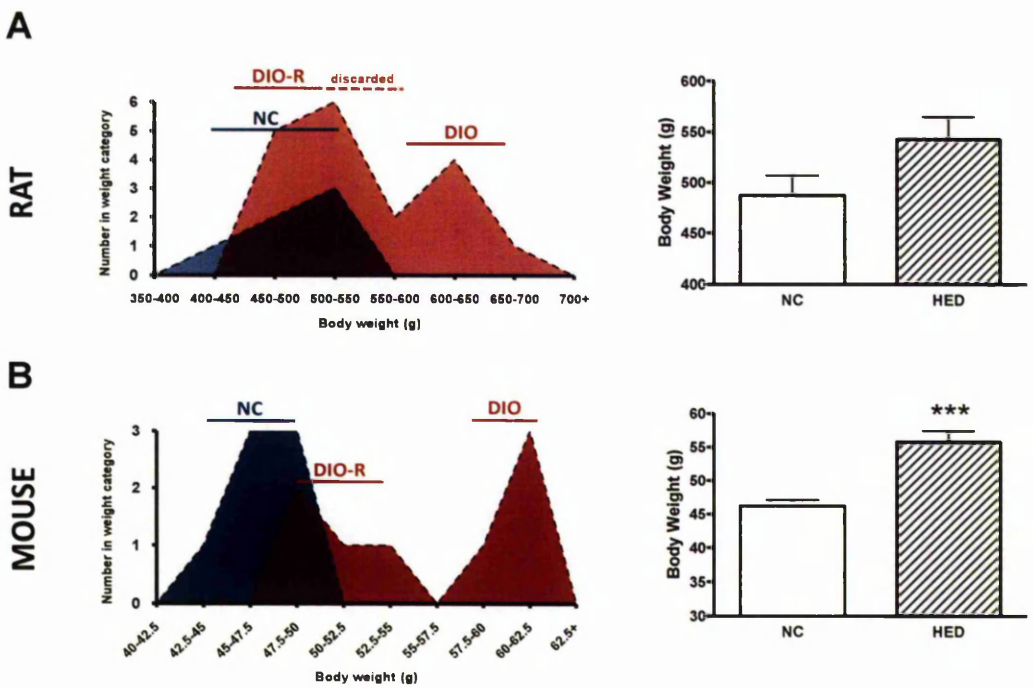


Figure 3.4 Body weight data: HED

Graphical representation of the final body weight distributions of rats and mice following 8 weeks on normal chow (blue) or high-energy diet (red). Graphs on the right show body weights of high-energy diet (HED)-fed versus normal chow (NC) groups, expressed as Mean ± SEM. ***P<0.001; Student's *t*-test.

Effect of 48-hour fasting and HED on VMN gene expression

Neuronal nitric oxide synthase (nNOS)

In the brain, high expression of nNOS mRNA was detected in the hypothalamic nuclei, including the ventrolateral VMN, the Arc and PVN, as well as the medial amygdala (MeA). Optical density of nNOS signal was analysed both in the VMN, and the MeA, as a comparison. 48-hour food-deprivation caused a significant reduction in nNOS expression in the VMN in both rats (-23±6 %) and mice (-27±2 %) compared with *ad libitum* fed controls. This effect was not observed in the MeA of either rats or mice (see Figure 3.5). In rats (-24±5 %), but not mice that were fed HED, there was a significant decrease in nNOS expression. However Pearson Correlation revealed no significant intra-group correlation between individual body weight and nNOS expression in the HED group. Again, no significant change in expression was observed in the MeA of either species (see Figure 3.6).

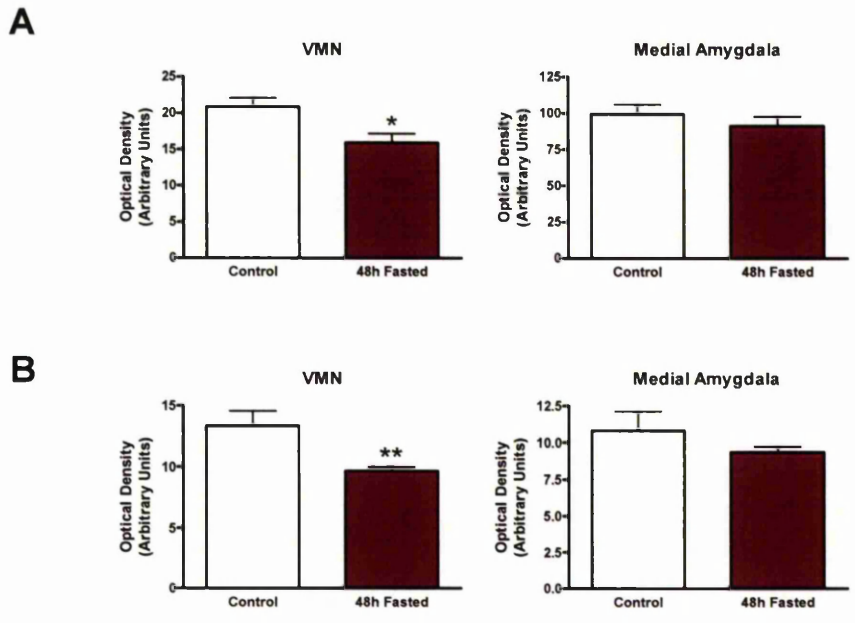


Figure 3.5 nNOS fasting

Effect of 48-hour food-deprivation on nNOS mRNA expression, as measured by optical density, in the VMN and medial amygdala of **A.** rats and **B.** mice. Data are expressed as Mean ± SEM. *P<0.05, **P<0.01; Student's unpaired *t*-test.

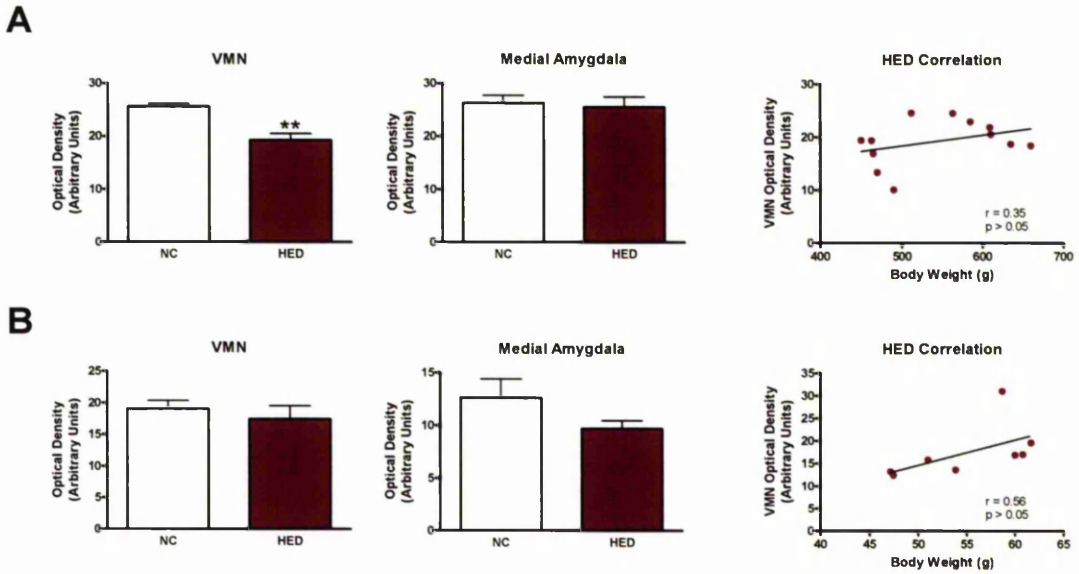


Figure 3.6 nNOS HED

Effect of high-energy diet on nNOS mRNA expression, as measured by optical density, in the VMN and medial amygdala of **A.** rats and **B.** mice. The right hand graphs show Body weights of the HED group plotted against relative VMN gene expression and analysed using Pearson correlation.

The potassium-chloride co-transporter KCC2

KCC2 is abundantly expressed in the brain, and we detected strong mRNA signal in the cortex, hippocampus, thalamus and hypothalamus, particularly the VMN, of both rat and mouse. As a region of relatively high expression, the retrosplenal cortex was used as a comparison to establish whether any expression changes observed are common throughout the brain. We found no changes in VMN or cortical expression of KCC2 in response to the food-deprivation stimulus, in neither rats nor mice (see Figure 3.7). However, both species showed a significant down-regulation in VMN KCC2 in response to HED, (rat: -17±3 %; mouse: -30±3 %). Pearson Correlation of body weight and VMN KCC2 expression revealed significant positive correlation in both species. Expression in the retrosplenal cortex was equivalent to control levels in HED rats and mice (see Figure 3.8).

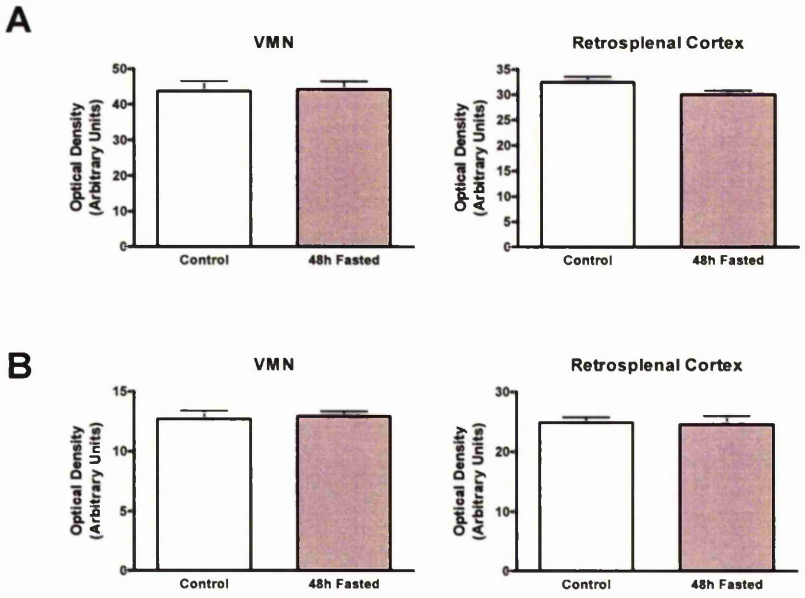


Figure 3.7 KCC2 fasting

Effect of 48-hour food-deprivation on KCC2 mRNA expression, as measured by optical density, in the VMN and retrosplenal cortex of **A.** rats and **B.** mice. Data are expressed as Mean ± SEM.

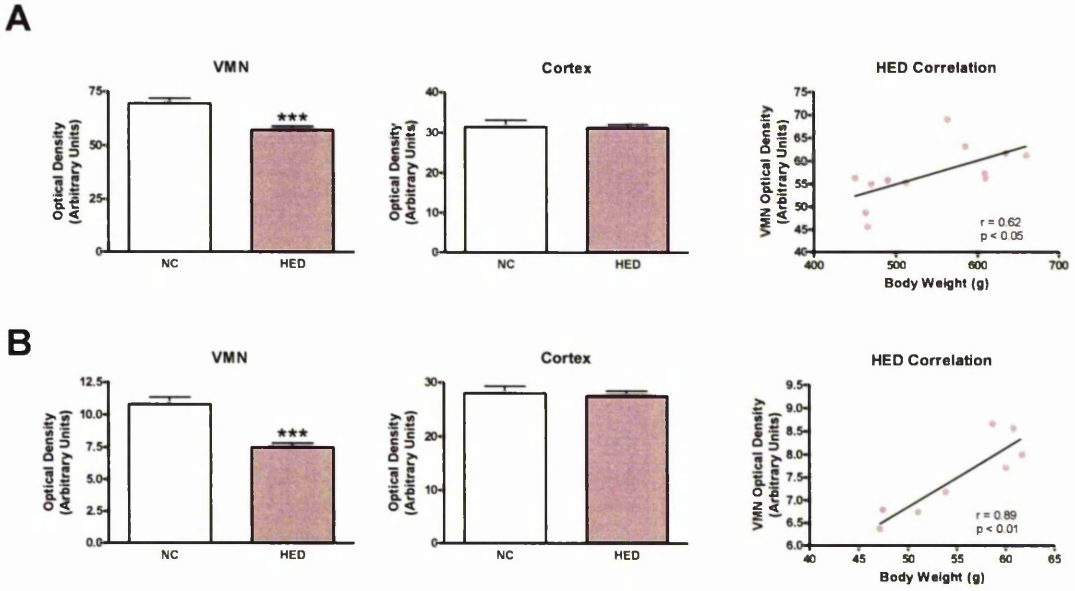


Figure 3.8 KCC2 HED

Effect of high energy diet on KCC2 mRNA expression, as measured by optical density, in the VMN and retrosplenal cortex of **A.** rats and **B.** mice. The right hand graphs show Body weights of the HED group plotted against relative VMN gene expression and analysed using Pearson correlation.

Brain-derived neurotrophic factor (BDNF)

BDNF mRNA expression was most strongly detected in the hippocampus, selected as our comparison region, where its effects on neuronal plasticity and memory have been extensively studied (reviewed in Mangina and Sokolov, 2006; and Rattiner et al., 2005). We also found relatively strong signal in the cortex, medial amygdala and VMN of both rat and mouse. BDNF mRNA levels in the rat VMN were not significantly affected by 48-hour food-deprivation, whereas in the mouse they were significantly decreased compared with non-fasted controls (-19±6 % and -52±5 %, respectively). No changes were detected in hippocampal BDNF expression in either species using this paradigm (see Figure 3.9). HED-feeding caused no significant changes in BDNF expression in rats compared with controls. However, Pearson Correlation revealed a significant negative correlation between body weight and BDNF expression in the HED-fed rats. HED-fed mice showed a significant up regulation in VMN BDNF expression (+34±15 % compared to controls) which again displayed a negative correlation between body weight and expression level. Furthermore, HED-fed mice showed a significant down regulation in hippocampal BDNF expression (-29±3 %) compared with chow-fed controls (see Figure 3.10).

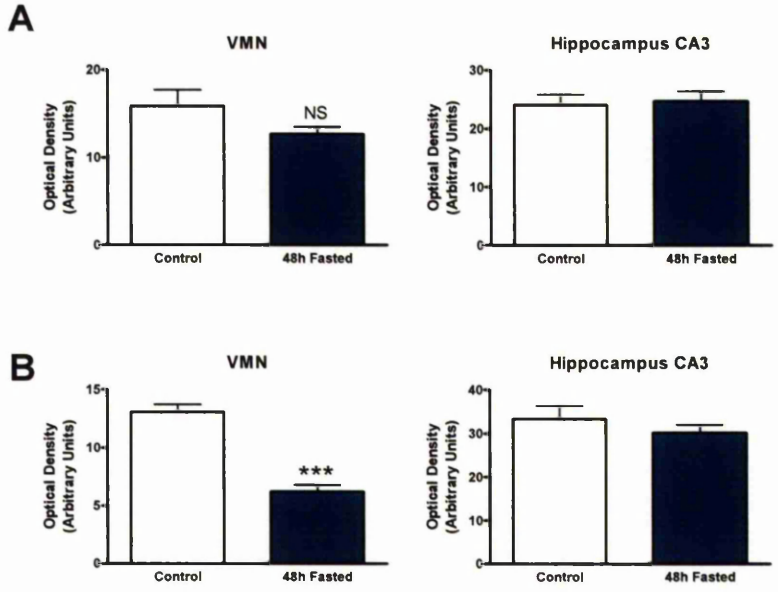


Figure 3.9 BDNF fasting

Effect of 48-hour food-deprivation on BDNF mRNA expression, as measured by optical density, in the VMN and CA3 region of the hippocampus of **A.** rats and **B.** mice. Data are expressed as Mean ± SEM. ***P<0.001; Student's unpaired *t*-test.

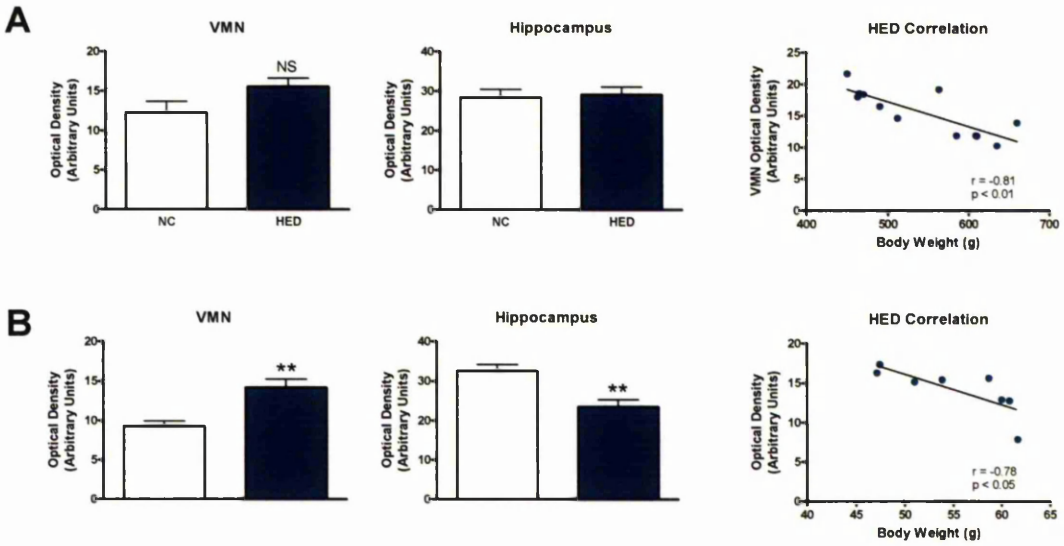


Figure 3.10 BDNF HED

Effect of high-energy diet on BDNF mRNA expression, as measured by optical density, in the VMN and CA3 region of the hippocampus of **A.** rats and **B.** mice. The right hand graphs show Body weights of the HED group plotted against relative VMN gene expression and analysed using Pearson correlation.

Pituitary adenylate cyclase- activating polypeptide (PACAP)

Due to unavoidable events, we were unable to perform PACAP *in situ* hybridisation histology on rat tissue. Therefore, data presented here are from mouse experiments only. PACAP has a distinctive mRNA expression pattern in the mouse brain, with particularly strong signal in the VMN, habenula and MeA. We used the medial habenula (mHab) as a comparison region when analysing changes in VMN PACAP expression. Following 48-hour food-deprivation, we found a significant down regulation in PACAP expression in the VMN (-44±7 %) compared with non-fasted controls, which was not observed in the mHab (see Figure 3.11). Conversely, following 8-week exposure to HED PACAP expression in the VMN was significantly elevated (+52±14 %) over chow-fed controls, and Pearson Correlation revealed a strong negative correlation between individual body weight and VMN PACAP expression in the HED group. No effect on PACAP expression was observed in the mHab of either HED-fed group compared with chow-fed controls (see Figure 3.12).

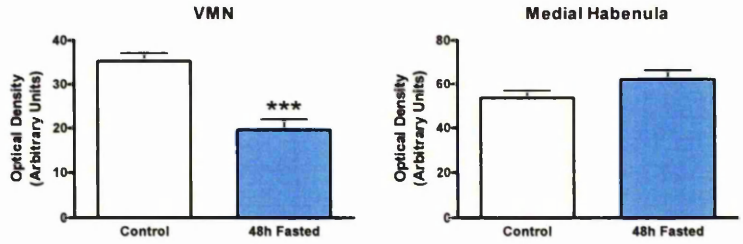


Figure 3.11 PACAP fasting

Effect of 48-hour food-deprivation on PACAP mRNA expression, as measured by optical density, in the VMN and medial habenula of mice. Data are expressed as Mean ± SEM. ***P<0.001; Student's unpaired *t*-test.

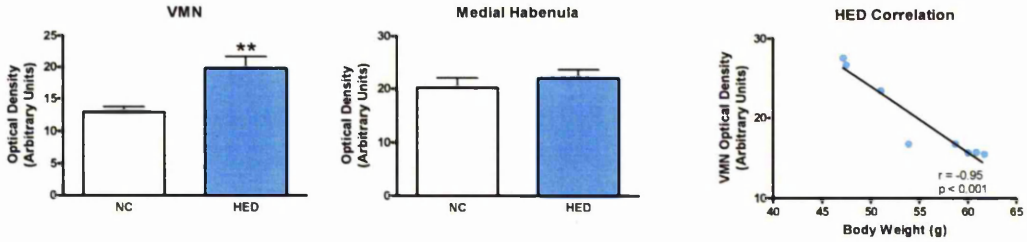


Figure 3.12 PACAP HED

Effect of high-energy diet on PACAP mRNA expression, as measured by optical density, in the VMN and medial habenula of mice. The right hand graphs show Body weights of the HED group plotted against relative VMN gene expression and analysed using Pearson correlation.

Effect of leptin treatment on body weight in leptin-deficient *ob/ob* mice

At the start of the experiment obese *ob/ob* mice were on average 56±2 % heavier than their lean, heterozygous littermates, which were used as controls. Leptin-injected *ob/ob* mice lost body weight steadily over the course of the experiment, and on day 5 were significantly lighter than the *ob/ob* group receiving saline (-10±3 %). However, 5-day leptin treatment was not sufficient to completely rectify the obese phenotype of *ob/ob* mice and they remained significantly heavier than lean controls (see Figure 3.13).

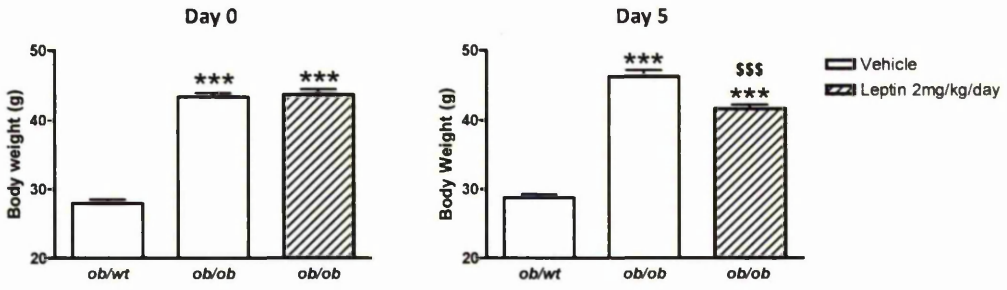


Figure 3.13 Body weight data: *ob/ob* leptin replacement

Body weight data before and after 5-day treatment. *ob/ob* mice received daily i.p. injections of leptin 2 mg/kg/day, or Vehicle (isotonic saline). *ob/wt* lean littermate controls also received i.p. saline (n=8). Data are expressed as Mean ± SEM; One-way ANOVA followed by Newman-Keuls *post hoc* test to compare all groups. ***P<0.01. *compared with *ob/wt*, $compared with *ob/ob* + Vehicle.

Effect of leptin deficiency and replacement on VMN gene expression

VMN nNOS mRNA expression of *ob/ob* mice was not significantly different from that of *ob/wt* controls. 5-day leptin replacement caused a non-significant reduction in VMN nNOS of *ob/ob* mice. Analysis of nNOS mRNA expression in the MeA revealed no significant differences between the three groups (see Figure 3.14). BDNF mRNA expression was not significantly different between any of the three groups; either in the VMN or the hippocampus (see Figure 3.15). VMN PACAP expression was significantly reduced in *ob/ob* mice compared with lean littermates, and expression was restored to control levels by treatment with exogenous leptin. No differences were found in PACAP expression in the mHab between any of the three groups (see Figure 3.16).

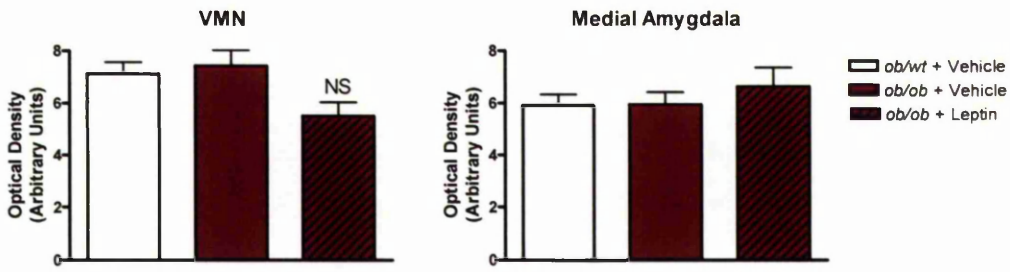


Figure 3.14 nNOS *ob/ob* leptin replacement

Effect of leptin deficiency (*ob/wt* + Vehicle vs *ob/ob* + Vehicle) and leptin replacement (*ob/ob* + leptin) on nNOS mRNA expression, as measured by optical density, in the VMN and medial amygdala of mutant mice. Data are expressed as Mean ± SEM. One-way ANOVA followed by Newman-Keuls *post hoc* test to compare all groups.

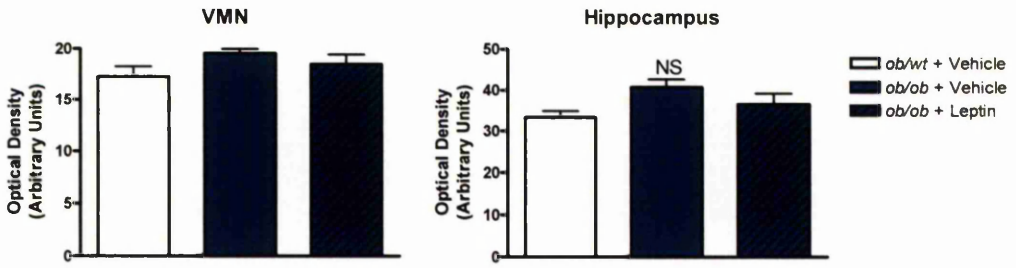


Figure 3.15 BDNF *ob/ob* leptin replacement

Effect of leptin deficiency (*ob/wt* + Vehicle vs *ob/ob* + Vehicle) and leptin replacement (*ob/ob* + leptin) on BDNF mRNA expression, as measured by optical density, in the VMN and CA3 region of the hippocampus of mutant mice. Data are expressed as Mean ± SEM. One-way ANOVA followed by Newman-Keuls *post hoc* test to compare all groups.

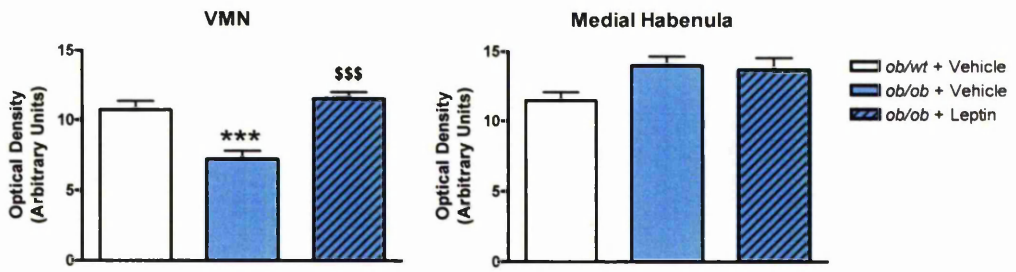


Figure 3.16 PACAP *ob/ob* leptin replacement

Effect of leptin deficiency (*ob/wt* + Vehicle vs *ob/ob* + Vehicle) and leptin replacement (*ob/ob* + leptin) on PACAP mRNA expression, as measured by optical density, in the VMN and medial habenula of mutant mice. Data are expressed as Mean ± SEM. ***P<0.001; One-way ANOVA followed by Newman-Keuls *post hoc* test to compare all groups. *compared with *ob/wt*, $compared with *ob/ob* + Vehicle.

3.4 DISCUSSION

nNOS

NO is an interesting signalling molecule, and the specific functions of the discrete populations of neurones which secrete it are yet to be fully clarified. Numerous studies have examined hypothalamic expression of nNOS in response to a variety of metabolic stimuli, but the results are difficult to interpret. The *in vivo* data cited earlier would suggest that NO may itself have orexigenic and metabo-suppressive properties, and as such, might be expected to increase following a fast. Canabal et al., (2006) recently showed that downward ramping of extracellular glucose (as would occur *in vivo* during fasting) acutely increases NO-production selectively in GI cells in hypothalamic cell cultures. Interestingly however, we recorded a significant down regulation of nNOS expression in the VMN in response to food-deprivation, as has been previously described in the PVN and supraoptic nucleus (SON) (Isse et al., 1999). Similar decreases in NADPH diaphorase (which reflect NOS levels), and in NO metabolites (Otukonyong et al., 2000; Yamada et al., 2002) have also been shown, demonstrating that *in vivo,* certain hypothalamic NO pathways appear to be down regulated during negative energy balance. Our data shows that HED-feeding, like fasting, decreases VMN nNOS mRNA expression in rats, and this finding correlates with the previously recorded reduction in nNOS immunodetection (Sadler and Wilding, 2004). Individual susceptibity to DIO does not appear to strongly correlate with nNOS expression however; perhaps suggesting that diet, rather than obesity may be causing these changes. Although it is impossible to infer a clear role for nNOS in energy homeostasis from these data, they further implicate NO as a plastic component of the central energy balance circuitry.

One of the major inputs into this central circuitry is the fat-derived hormone leptin and it has been suggested that leptin and NO may interact. In the leptin deficient *ob/ob* mouse hypothalamic nNOS mRNA expression and central citrulline concentrations are elevated compared with lean littermates and these parameters can be normalised by repeated leptin administration (Morley et al., 1995b). Furthermore, exogenous leptin prevents nNOS induction in response to decreasing glucose in hypothalamic cells maintained *in vitro* (Cannon and Nedergaard, 2004). However, histological characterisation of the VMN reveals that lepr-B and nNOS are synthesised in regionally distinct neuronal populations, making a first-order interaction unlikely in this nucleus. In agreement with this, we found no change in nNOS expression in the *ob/ob* VMN, although interestingly, we did find that exogenous leptin caused a modest decrease in

expression. Whether this is a consequence of leptin signalling *per se*, or a secondary consequence to other leptin effects is currently unclear. Sugimoto and colleagues (2003) previously demonstrated that NO metabolite levels are unaltered in the mouse hypothalamus during leptin-induced hypophagia, arguing against an acute interaction between the two factors.

While our results are in line with the current understanding that NO is likely to partake in the control of energy balance, the unique nature of nNOS signalling complicates their interpretation. Centrally-released NO is capable of retrogradally potentiating the release of a plethora of neurotransmitters, and it is important to consider that VMN nNOS cells are likely to show heterogeneity in terms of their afferent inputs. To put this in context, it was recently found that cultured medio-basal hypothalamic cells ubiquitously express the major NO receptor, soluble guanylyl cyclase and therefore all represent potential targets. Neurones of the VMN are primarily, but not exclusively innervated by GABAergic projections and as such assessing directional changes in VMN nNOS levels as a whole may be misleading. Moreover, the ventrolateral portion of the VMN, where nNOS cells are localised also governs sexual function, which is secondarily affected in conditions such as food deprivation. It is important to consider that changes observed in this region of the hypothalamus may not reflect an exclusively metabolic role.

KCC2

Although not previously associated with energy balance regulation, KCC2 is abundantly expressed in the VMN and is known to be essential for the postsynaptic inhibitory response to GABA. As a GABA target, neuronal populations of the VMN express both $GABA_A$ and $GABA_B$ receptors, while levels of the GABA-synthesis enzyme glutamic acid decarboxylase (GAD) are negligible. Transgenic knockout of the SF1 gene severely disrupts the normal GABA neurone exclusion during VMN development, and this may contribute to the morbid obesity which later manifests in these mice (Dellovade et al., 2000; McClellan et al., 2006). Many studies have established a role for GABA in the acute responses to glucoprivation, a state similar to that of fasting. Several groups have shown that in rats, glucoprivation by insulin or 2-DG induces food intake, and also increases GABA release in the VMN (Lenin Kamatchi et al., 1986; Specter et al., 1996). Furthermore, hyperphagic responses to glucoprivation can be blocked by injection of GABA antagonists into the VMN, but not other parts of the hypothalamus. It is conceivable that GABA sensitivity could be increased when blood glucose is low, in order to suppress catabolic cell types more

efficiently. However, we found no change in KCC2 expression in either fasted rats or mice, suggesting that while GABA signalling itself may well be modified, acute changes in VMN KCC2 expression are not involved in the physiological response to fasting. Alternatively, it is possible that the 'normal' state represents maximal inhibitory response to GABA, rendering up regulation of KCC2 mRNA impossible, or of no benefit.

Considering that expressional regulation of KCC2 in the adult CNS has only been previously reported under seizure-like conditions, we were pleased to find that in HED-fed rats and mice VMN KCC2 mRNA was robustly down regulated. This trend was more prominent in the DIO-R individuals, leading to the notion that long-term decreases in VMN GABA sensitivity in response to HED could contribute to the compensatory hypophagia observed in DIO-R animals. As this study is the first of its kind, it is only possible at this stage to speculate which upstream messengers may be influencing KCC2 expression in HED-fed animals. It is unlikely that leptin is responsible for these changes, since fasting, which causes a rapid drop in circulating leptin has no effect on KCC2. Furthermore, DIO animals are almost certainly leptin resistant, but still show a significant decrease in KCC2 levels. BDNF, acting via its receptor Trk-B, is known to negatively regulate KCC2 mRNA levels in the embryonic brain (Aguado et al., 2003; Rivera et al., 2004), so is a promising candidate. BDNF is strongly expressed in the VMN and Arc (which projects to the VMN) and although it's downstream effects are yet to be determined, has been shown to be essential for normal body weight (reviewed in Lebrun et al., 2006). We have investigated the effects of different energy states on BDNF levels, to further clarify this relationship.

BDNF

Several studies have already shown that BDNF is nutritionally regulated in the rodent VMN, being significantly elevated by i.c.v. leptin (Komori et al., 2006) or i.p. glucose (Unger et al., 2007), and reduced by a 48-hour fast (Unger et al., 2007; Xu et al., 2003). We found that BDNF mRNA was significantly down regulated in the VMN of mice, but not rats following 48-hour food deprivation. Conversely, HED-feeding increased BDNF in mice, but not rats. In both species however, a strong negative correlation was observed between HED body weight and VMN BDNF expression, leading us to postulate that a decrease in BDNF in obese animals may contribute to their higher energy intake. Although no significant changes in VMN BDNF expression were seen in rat, non-significant trends were directionally equivalent to the statistically

significant differences in mouse in both experimental paradigms. This could imply that BDNF is more important in the control of energy balance in mice than it is in rats, where other neuropeptides/neurotransmitters may predominate. More likely, it may be due to differences in the way in which each species responds to the stimuli. Mice are affected more severely by fasting due to their higher basal metabolic rate, meaning that a 48-hour fast represents a stronger stimulus than it does in rats. Similarly, in the second experiment, mice were fed a more energy-dense diet, and developed a more marked obesity than did rats, with some individuals almost doubling their original body weight.

The direction of these expressional changes is consistent with BDNF's action as a catabolic peptide. Furthermore they support the concept that BDNF is induced in the VMN in response to leptin, since leptin levels fall rapidly upon food deprivation, and increase during HED-feeding. The lack of significant up regulation of BDNF in DIO mice may be due to the development of leptin resistance on account of their obesity. Indeed, the literature supports a role for leptin in the regulation of VMN BDNF expression, with evidence of both direct and indirect action. Xu et al., (2003) provided evidence that BDNF neurones are activated downstream of the MC_4R. However, more recently, Komori et al., (2006), demonstrated that following leptin i.c.v., VMN BDNF cells contain phosphorylated STAT3, a component of leptin-induced intracellular signalling, indicating direct activation. Previous reports have shown that the leptin deficient *ob/ob* mouse has abnormally elevated levels of BDNF expressing cells in the dorsomedial subdivision of the VMN (Komori et al., 2005). In contrast to this, we found no difference in relative BDNF mRNA expression in *ob/ob* compared with lean controls. Neither did we find any effect of 5 day leptin replacement. These data do not however rule out a regulatory role of leptin in VMN BDNF expression; and only suggest that, at least in the *ob/ob* model, other factors may be controlling BDNF levels.

PACAP

PACAP expression is particularly strong in the VMN, although its regulation in the brain has not been well studied, especially in terms of energy balance. Centrally administered PACAP affects both food intake and energy expenditure in rodents (Mizuno et al., 1998b; Morley et al., 1992; Pataki et al., 2000), and so clarification of whether VMN PACAP expression is modulated in response to metabolic stimuli is justified. In a very recent study, Mounien and colleagues (2008) found 48-hour food-withdrawal markedly reduces PACAP mRNA in total hypothalamic samples, measured using reverse-transcription polymerase chain reaction (RT-PCR). Concurrently, we also

found that expression of PACAP mRNA in the VMN of outbred mice is significantly down regulated following 48-hour of food deprivation, as would be expected for a catabolic peptide.

Interestingly, in animals maintained on HED, only those which were resistant to obesity showed major changes in VMN PACAP expression. The strong negative correlation between PACAP expression and body weight on HED suggests that rather than being a response to dietary content *per se*, there are intrinsic differences in the hypothalamic circuits of obesity prone and obesity resistant mice. PACAP is known to decrease feeding when administered centrally to rodents, therefore higher endogenous PACAP in the VMN of lean HED-fed mice is a very interesting finding. It may well be that VMN PACAP plays an important role in the compensatory responses to HED, which serve to guard against increased adiposity. Matsuda et al., (2005a) reported similar up regulation of central mRNA expression of both PACAP and its receptor after 7 days of excessive feeding in goldfish. The amino acid sequence of PACAP is extremely conserved across vertebrate species, indicating a strong functional constraint during the course of evolution. Our data may implicate a role for PACAP as an intrinsic defence mechanism designed to protect against chronic states of positive energy balance. Equally, the lack of PACAP up regulation in obese mice may be a key contributor to their capacity for weight gain and may either precede or be a result of leptin resistance.

Unlike for BDNF and nNOS genes, we recorded significant differences in VMN expression of PACAP in *ob/ob* mice compared with lean littermates. Like fasted wildtype mice, leptin deficient *ob/ob* mice had around 40 % lower expression than controls, perhaps contributing to their hypometabolism, and chronic hyperphagia. The fact that levels were low despite the obvious obese phenotype, suggests that leptin levels and not body weight may be important in determining PACAP expression. Indeed, we were able to restore VMN PACAP levels in *ob/ob* mice by exogenous leptin treatment, despite body weight remaining relatively high. This suggests that leptin, acting either directly or indirectly, may be involved in the induction of PACAP expression, similar to other catabolic signalling molecules POMC and CRH elsewhere in the hypothalamus. In light of this, the relationship between leptin and PACAP and subsequent downstream targets will form the major focus of the remainder of this thesis.

3.5 SUMMARY

- 48-hour fasting causes expressional changes of VMN-enriched genes, decreasing nNOS, BDNF and PACAP, but no change in KCC2

- HED causes expressional changes of VMN-enriched genes, some of which are dependent on susceptibility to obesity. nNOS and KCC2 are decreased in HED-fed animals, whereas BDNF and PACAP are increased, particularly in DIO-R animals.

- *ob/ob* mice have significantly lower expression levels of VMN PACAP, but normal nNOS and BDNF mRNA expression.

- Repeated administration of exogenous leptin significantly increases VMN PACAP in *ob/ob* mice.

- Changes in expression are restricted to the VMN, except in the case of BDNF which is significantly lower in the hippocampus of DIO mice.

3.6 CONCLUSIONS

Gene expression of peptides, transporter molecules and neurotransmitters in the VMN is affected by changes in energy state in both rats and mice. This highlights the complex nature of energy balance regulation, and that the focus on neuropeptides may be too narrow. That aside, we found robust regulation of the peptide PACAP in the VMN which showed a positive correlation with predicted peripheral leptin levels, with the exception of DIO mice which are likely to be leptin resistant. The role of VMN PACAP in energy balance forms the basis of the remainder of this thesis, as it appears the most promising candidate for a VMN metabolic marker.

CHAPTER 4

IN VIVO EFFECTS OF PACAP

4.1 INTRODUCTION

PACAP is a pleiotropic peptide which has been linked with a range of central processes (Vaudry et al., 2000). However, considering its distinctive expression pattern in the brain, particularly with regards to its strong expression in the VMN, it is possible that PACAP may be involved in controlling energy balance. In the previous chapter we demonstrated using semi-quantitative ISHH that PACAP expression in the VMN is regulated according to nutritional status in directions appropriate for a catabolic peptide. Furthermore, numerous groups have reported on the anorexigenic effects of centrally administered PACAP *in vivo* (Matsuda et al., 2005b; Mizuno et al., 1998b; Morley et al., 1992; Tachibana et al., 2003). In terms of energy expenditure, one of the most striking features of PACAP knockout mice is their inability to maintain core-body temperature due to a defect in BAT thermogenesis (Gray et al., 2002). This may be at least partly due to the loss of central pathways since PACAP is reported to cause a hyperthermic response when given i.c.v. in rats (Pataki et al., 2000) and mice (Masuo et al., 1995). However, so far, little has been reported regarding PACAP's effects on energy expenditure *per se*. In this chapter we have clarified the effects of PACAP on food intake, core-body temperature, oxygen consumption, carbon dioxide production and respiratory quotient in mice. PACAP was administered into the lateral ventricle in all experiments, in order to best mimic release from energy balance-responsive PACAP neurones in the VMN of the hypothalamus.

Objectives:

To test whether i.c.v. PACAP alters food intake by altering appetite

Due to the dose discrepancies in the current literature it was decided to investigate the effects of very low doses of PACAP on food intake. PACAP has been reported to reduce feeding in rats, chicks and goldfish; however alterations in locomotor activity and other behaviours such as grooming have also been observed when PACAP is administered in the nmol range (Adamik and Telegdy, 2004; Masuo et al., 1995). In order to class PACAP as an anorectic peptide, it is important to show that effects on food intake are due to physiological changes in appetite, as opposed to induction of sickness or drowsiness, or due to competitive preference for other behaviours. Analysis of the behavioural satiety sequence (BSS) looks at the sequence of behaviour of singly-housed animals following a meal (often by re-introduction to food following a fast) and how this sequence can be altered by administration of agents which control food intake (Antin et al., 1975; Halford et al., 1998). Factors which control feeding via a

physiologically relevant homeostatic mechanism do not alter the overall sequence of behaviours, and in the case of true satiety factors may accelerate the sequence. Aversive or sedative agents, on the other hand, cause marked disruption of the BSS. We will carry out BSS analysis to assess whether this is the case in these PACAP feeding studies.

To examine the effects of PACAP on energy expenditure and energy efficiency

Many factors which affect appetite have subsequently been shown to also affect energy expenditure (Billington et al., 1991; Wang et al., 2007; Yasuda et al., 2005; Yasuda et al., 2004). Given that the VMN is believed to be important in controlling energy expenditure, particularly via BAT thermogenesis (Halvorson et al., 1990; Perkins et al., 1981; Thornhill and Halvorson, 1993), it is reasonable to postulate that PACAP cells may also play a part in this function. Exogenous PACAP administration increases both core-body temperature (Pataki et al., 2000) and spontaneous locomoter activity (Masuo et al., 1995) in rodents, but direct measurement of energy expenditure has yet to be performed. Core-body temperature (T_b) is often used as a simple, surrogate method for estimating energy expenditure, since the two can often be closely correlated. However, oxygen consumption (VO_2) is a far more accurate measure of actual energy expenditure. Further to this we aim to record carbon dioxide production which, when compared with VO_2 can be used to estimate which fuels are being metabolised and the resulting metabolic efficiency. The ratio of CO_2 produced to O_2 consumed is termed the respiratory quotient (RQ), and generally fluctuates between 1.0 (pure carbohydrate oxidation; highest efficiency) to 0.7 (pure fat oxidation; lowest efficiency). Catabolic factors such as CRH and α-MSH decrease the value of RQ by promoting fat metabolism, whereas central administration of anabolic peptides increases it (Currie et al., 2001; Egawa et al., 1991; Hwa et al., 1997). We will use simultaneous non-invasive remote radiotelemetry and indirect calorimetry continuous recording systems to examine the effects of a single i.c.v. injection of PACAP on T_b, VO_2, VCO_2 and RQ.

To assess the relative roles of PACAP receptors in acute energy balance

Binding assays show that PACAP acts through the PACAP-specific PAC_1 receptor, but also binds with high affinity at the VPAC receptors which recognise the related peptide vasoactive intestinal peptide (VIP) (Gourlet et al., 1995). In mammals it is likely that PACAP exerts many of its effects through the PAC_1 receptor, demonstrated by the striking phenotypic similarities between PACAP and PAC_1KO mice (Hashimoto et al., 2000; Hashimoto et al., 2001), but not $VPAC_1$, or $VPAC_2$ knockout mice (Bechtold et

al., 2008; Hughes et al., 2004). We will use three approaches to try to ascertain which receptor(s) mediate the central metabolic effects of exogenous PACAP. Firstly, we will target PAC_1 and $VPAC_2$ by central administration of the N-terminus trunctated analog $PACAP_{6-38}$, which acts as a competitive antagonist at both receptors. We will initially test whether $PACAP_{6-38}$ affects food intake alone and subsequently whether co-injecting it with PACAP can block the expected feeding or thermogenic effects. Our second approach is to examine the *in vivo* effects of equimolar doses of VIP (which only binds $VPAC_1$ and $VPAC_2$) on food intake, T_b, VO_2, VCO_2 and RQ. If PACAP-mediated effects on energy balance heavily involve either of the VPAC receptors, we would expect VIP to have similar effects. Thirdly, and perhaps the most direct approach, we aim to look at the effects of PACAP injection in three transgenic mouse strains in which each of the three receptors, PAC_1, $VPAC_1$ and $VPAC_2$, have been knocked out.

4.2 METHODS

PACAP and $PACAP_{6-38}$ dose responses: feeding

Fast-induced re-feeding experiments were performed according to the protocol detailed in the general methods chapter. An injection cannule was inserted into the guide cannula to a depth of 2 mm below the skull surface and animals received a single 1 µl i.c.v. injection using minimal restraint. PACAP doses: 0, 15, 30 or 60 pmol all in sterile isotonic saline (n=5-6 per group); $PACAP_{6-38}$ doses: 0, 100, 300 or 600 pmol all in sterile isotonic saline (n=3-4 per group).

VIP dose response: feeding

A fast-induced re-feeding experiment was performed with animals receiving a single 1 µl i.c.v. injection containing 0, 30 or 60 pmol VIP or PACAP in isotonic saline (n=6 per group).

Behavioural satiety sequence

Mice were singly-housed in transparent observation cages for 3 days prior to the experiment to ensure sufficient acclimatisation. Animals were food-deprived from 16:00 on the day before injection, and at 10:00 the following day were injected i.c.v. with 1 µl 0, 15, 30 or 60 pmol PACAP in isotonic saline (n=4 per group). Food was immediately returned and behaviour was observed and recorded at 30 second intervals for 90 minutes post injection. Behaviour categories were established beforehand and were as follows: feeding, drinking, active, inactive, grooming, resting (See Table 4.1 for details). Behaviours were then pooled in 5 minute bins for graphic representation.

Behaviour	Classification
Feeding	Animal gnawing or holding food in paws
Drinking	Animal licking spout of the water bottle
Active	Includes rearing, sniffing, digging, climbing and moving around cage.
Inactive	Animal is motionless, with eyes open
Grooming	Animal is licking or scratching
Resting	Animal is motionless curled up in its nest, with eyes closed

Table 4.1 BSS behaviours

Criteria used to define each behaviour type for analysis of the behavioural satiety sequence. Only a single behaviour could be selected for each observation event.

Nocturnal administration of PACAP and pair feeding

A night-time feeding experiment was performed as detailed in the general methods section. Animals were given a single 1 µl i.c.v. injection of 0, or 30 pmol PACAP in sterile isotonic saline using minimal restraint (n=5 per group). The following evening, a third group (n=5) was subjected to a 2-hour pre-fast and saline injection and was then presented with the mean quantity of chow as had been consumed by the PACAP-injected animals the previous 24 hours. Body weight was measured at the time of injection, and 24 hours post injection.

Measurement of PACAP or VIP-induced metabolic effects

Radiotelemetry and CLAMS protocols were followed as detailed in the general methods section. Mice received a single 1 µl i.c.v. injection containing 30 pmol PACAP, 30 pmol VIP or saline only (n=8 per group).

Co-administration of PACAP and $PACAP_{6-38}$

Fast-induced re-feeding (n=6 per group) and radiotelemetry (n=4-6 per group) experiments were performed with mice receiving a single 1 µl i.c.v. injection containing either 30 pmol PACAP only, 300 pmol $PACAP_{6-38}$ only or 30 pmol PACAP + 300 pmol $PACAP_{6-38}$ in isotonic saline. Controls received saline only.

PACAP administration to receptor knockout mice

$VPAC_2$ knockout (KO) mice were a kind gift from the Hugh Piggins Laboratory (University of Manchester) and $VPAC_1$ and PAC_1 KO mice breeding stocks were obtained from the European Mutant Mouse Archive (Harwell UK), as part of an initiative by The Wellcome Trust. At the time of thesis submission, experiments with the latter two strains were ongoing. 12-week old male $VPAC_2$ KO mice and age-matched C57BL/6 controls were i.c.v. cannulated as described in the general methods chapter. A nocturnal feeding experiment was performed, with animals receiving a single 1µl i.c.v. injection containing 0 or 30 pmol PACAP in isotonic saline (n=5 per genotype). Mice then rested for 1 week following which CLAMS analyses were carried out as described in the general methods chapter, with mice receiving a single i.c.v injection of either saline or 30 pmol PACAP.

4.3 RESULTS

Effect of PACAP and $PACAP_{6-38}$ on food intake

PACAP administered i.c.v. significantly reduced fast-induced re-feeding at 60 and 30 pmol doses (-82±11 % and -36±9 % respectively at 1 hour), but not at 15 pmol. Effects were seen at 1, 2 and 4 hours post injection, with the highest dose producing persistent anorexia over 24 hours. $PACAP_{6-38}$ alone did not significantly alter fast-induced re-feeding at 30 or 300 pmol doses. Injection of 3 nmol caused a small but significant decrease in food intake at 1 hour post injection (-21±3 %), although no significant effect was observed thereafter (see Figure 4.1).

BSS behavioural analysis

BSS analysis of saline-injected animals revealed a classical behaviour pattern; initially animals spent the majority of time feeding to compensate their negative energy balance, interspersed with exploratory activity and bouts of drinking. Following this, mice increased the amount of time spent grooming and exploring. Finally behaviour shifted to rest and at the end of the observation time all control mice were asleep. These behaviours are hence referred to in this chapter as 'natural' behaviours. Notably, saline-injected mice spent almost no time 'inactive' suggesting that this is not a natural behaviour. Mice injected with 15 or 30 pmol also followed this pattern of behaviour; with 30 pmol PACAP significantly reducing time spent feeding, whilst maintaining integrity of the BSS pattern. 60 pmol PACAP also reduced time spent feeding. However this dose was found to cause marked disruption of the BSS (see Figure 4.2). In fact, statistical analysis revealed decreases in all natural behaviours, with much of the time spent 'inactive'. Based on feeding and BSS dose responses, 30 pmol was selected as optimal for subsequent experiments, as the highest dose to produce robust anorexia without disrupting the pattern of the BSS.

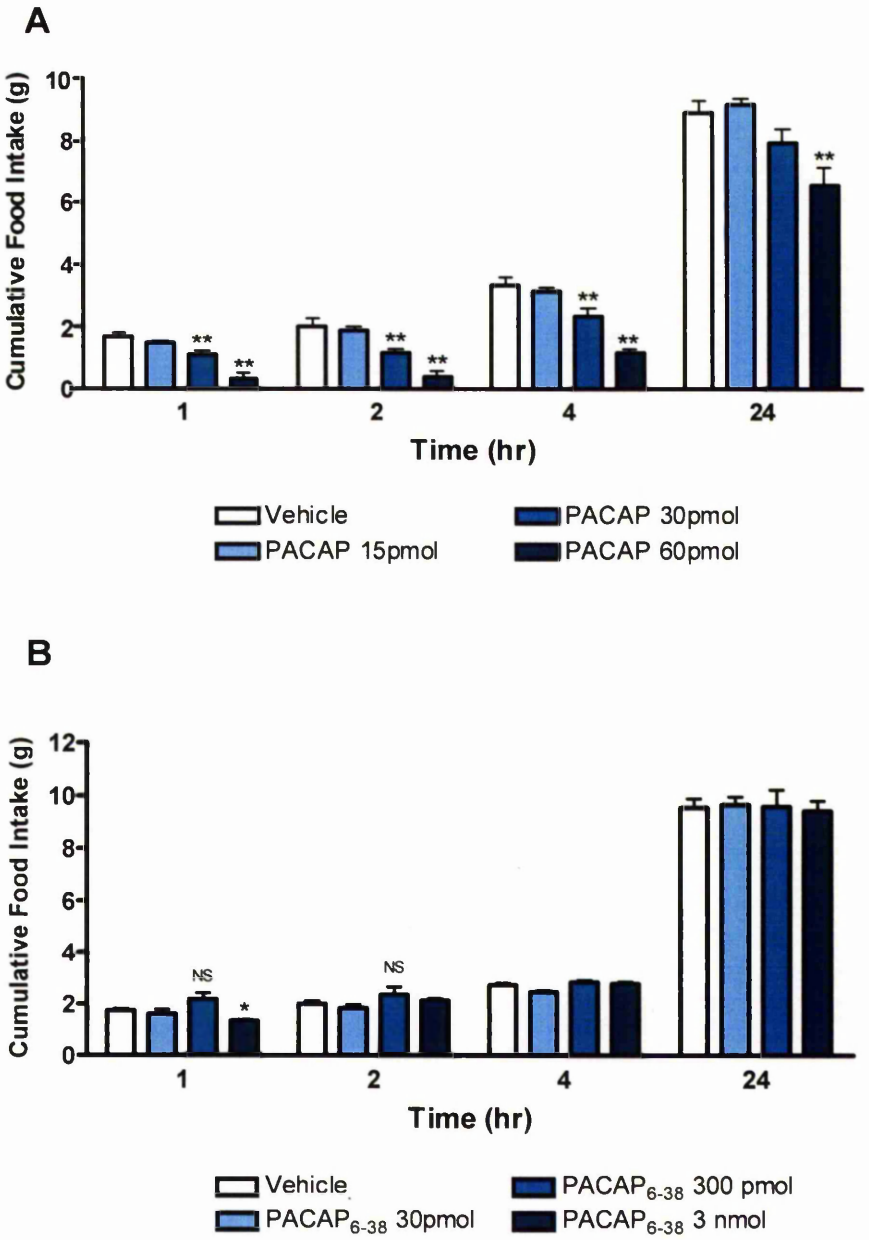
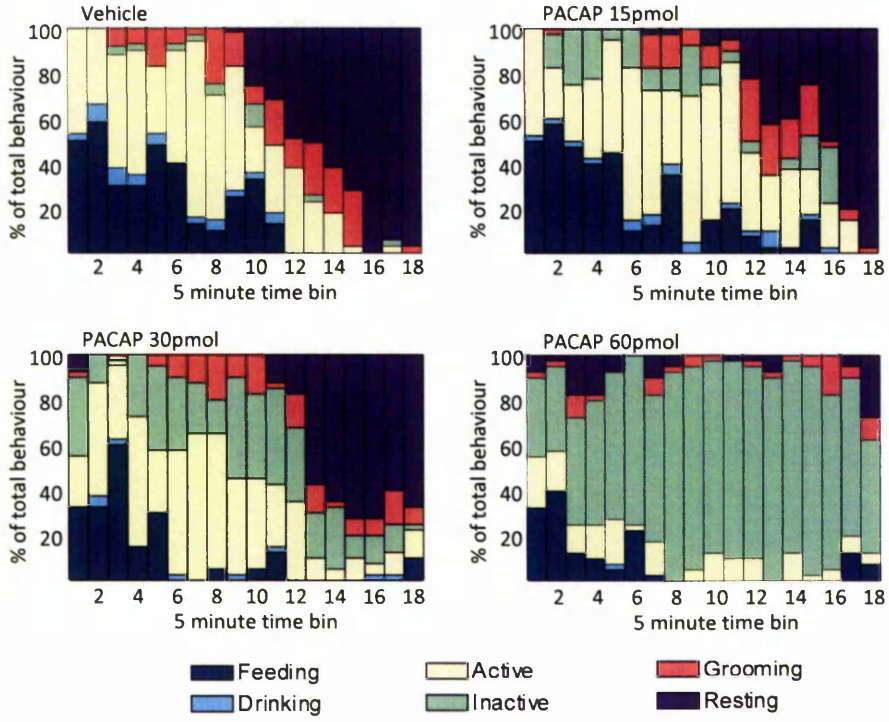


Figure 4.1 PACAP and $PACAP_{6-38}$ dose response experiments

The effect of different doses of **A.** PACAP and **B.** $PACAP_{6-38}$ on fast-induced re-feeding at 1, 2, 4, and 24 hours post injection. Mean ± SEM. * P<0.05, **P<0.01 all compared with vehicle; One-way ANOVA with Dunnet's *post hoc* test.

A



B

	Feeding	Drinking	Activity	Inactivity	Grooming	Resting
Saline	19±4 %	2±0.2 %	32±4 %	2±1 %	10±2 %	33±7 %
PACAP 15 pmol	20±3 %	2±0.5 %	37±9 %	10±4 %	8±2 %	22±6 %
PACAP 30 pmol	9±2 %*	1±0.9 %	33±5 %	23±4 %**	9±1 %	26±2 %
PACAP 60 pmol	8±4 %*	2±0.2 %	9±4 %**	71±9 %***	4±2 %	6±2 %*

Figure 4.2 PACAP BSS

Behavioural effects of i.c.v. administration of 15, 30 or 60 pmol PACAP compared with saline controls. Animals were fasted overnight and presented with food immediately after injection. Behaviours were scored every 30 seconds for 90 minutes and are expressed as **A.** mean % of total behaviour in 5 minute time bins. **B.** Mean % of total behaviour over the full 90-minute observational period. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Data were analysed using one way ANOVA with Dunnet's *post hoc* test

Effect of PACAP i.c.v. and pair feeding on body weight

30 pmol PACAP decreased normal nocturnal food intake more potently than is seen in the fasted, daytime paradigm. PACAP-injected animals consumed significantly less food than saline controls at 1, 2 and 24 hour time points and lost on average 11±3 % of their body weight. In contrast, vehicle-injected pair-fed mice, who received the same amount of chow as the PACAP-injected mice, lost only 3±1 % body weight (see Figure 4.3). Reduced food intake, therefore, cannot fully account for PACAP-induced weight loss, and since 30 pmol PACAP does not increase activity, this prompted us to investigate PACAP's effects on energy expenditure.

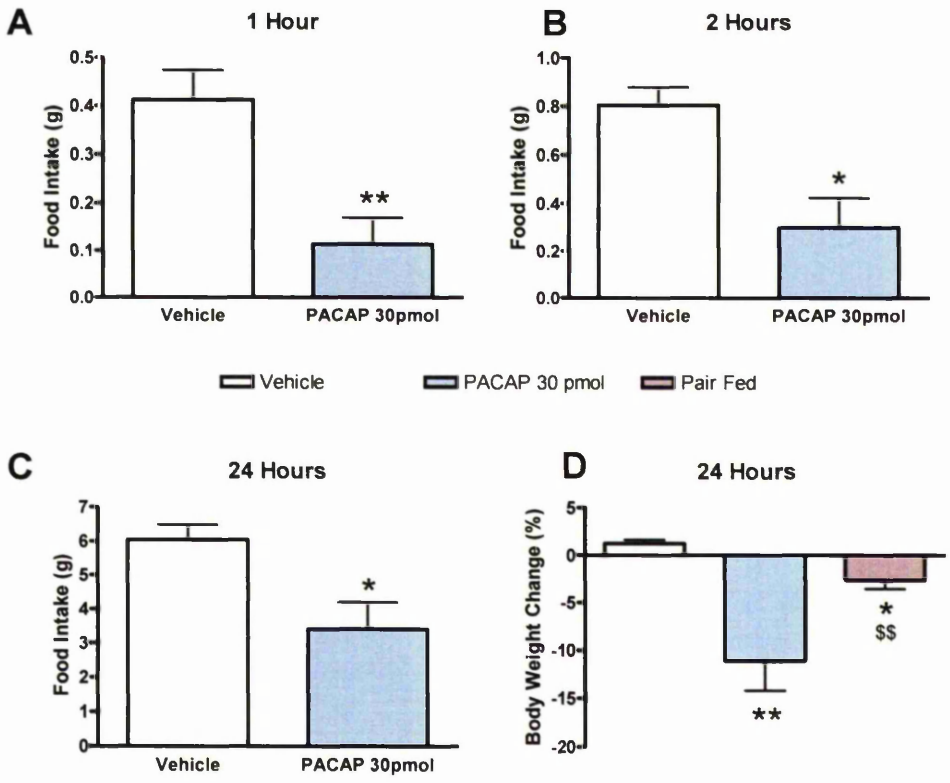


Figure 4.3 Nocturnal PACAP administration and pair feeding

A. - C. Effect of i.c.v. 30 pmol PACAP on nocturnal food intake at 1, 2 and 24 hours post injection and **D.** body weight 24 hours post injection, compared with saline control. **D.** Effect of pair feeding mice, with equivalent of mean consumption of PACAP-injected group, on body weight. *P<0.05, **P<0.01. Feeding data were analysed using unpaired student's t-test. Body weight data were analysed using a one-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle control, $compared with 30 pmol PACAP-injected groups.

Effect of PACAP on core-body temperature and metabolic rate

Nocturnal rodents have a well-characterised circadian pattern of VO_2, VCO_2 and T_b; with all three peaking during the active dark phase. We chose to inject mice during the mid morning to ensure a low baseline in all parameters in our control group. As has been observed previously, mice injected i.c.v. with saline frequently undergo a transient increase in VO_2, VCO_2 and T_b immediately following injection, with levels returning to baseline within 1 hour. 30 pmol PACAP-injected mice however, show a more persistent increase in T_b and VO_2, which is discrete from this initial spike and remains elevated over that seen in vehicle-treated mice for several hours (see Figures 4.4 and 4.5). Analysis of the period between 1 and 6 hours post injection (to eliminate the initial spike) shows a significant elevation in both mean T_b (+1±0.2 °C compared to controls) and VO_2 (+339±54 mg/kg/h) in the PACAP-injected group. At around 6 hours post injection T_b and VO_2 of vehicle-treated animals begins to increase in anticipation of lights off. Since the 24-hour mean level of neither parameter was significantly different from controls, we can assume that any effects of a single dose of PACAP on energy expenditure are short term. VCO_2 levels were not affected by PACAP administration (see Figure 4.6) and as such, a transient decrease in RQ to approximately 0.85 is observed in this group (see Figure 4.7), returning to control levels by lights off.

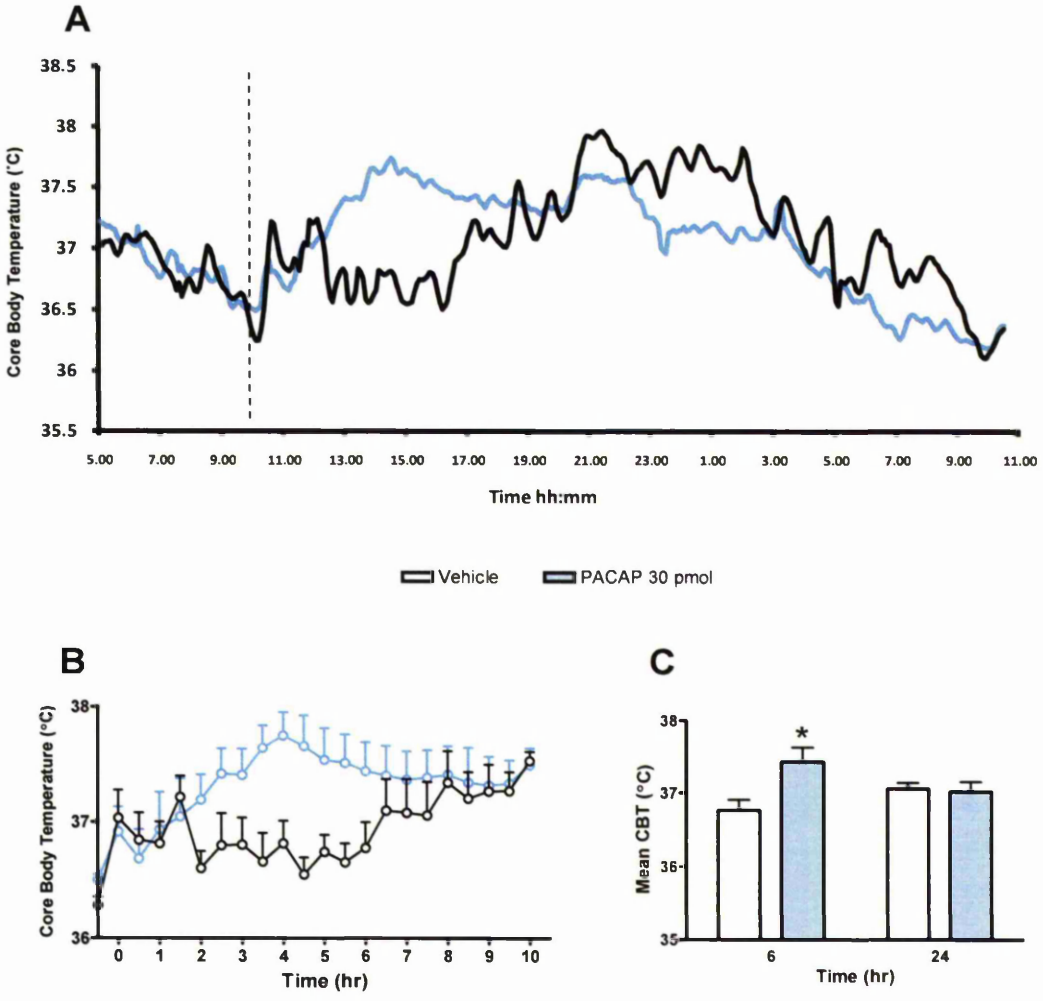


Figure 4.4 PACAP: Core-body temperature

Effect of i.c.v. 30 pmol PACAP (n=8) on core-body temperature (T_b; °C), compared with isotonic saline (n=8). **A.** Mean Tb 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. Error bars have been left off to aid clarity. **B.** Mean ± SEM Tb 10 hours post injection. **C.** Mean ± SEM Tb during 1-6 hours post injection and 1-24 hours post injection. *P<0.05. Data were analysed using an unpaired Student's *t*-test at each time point.

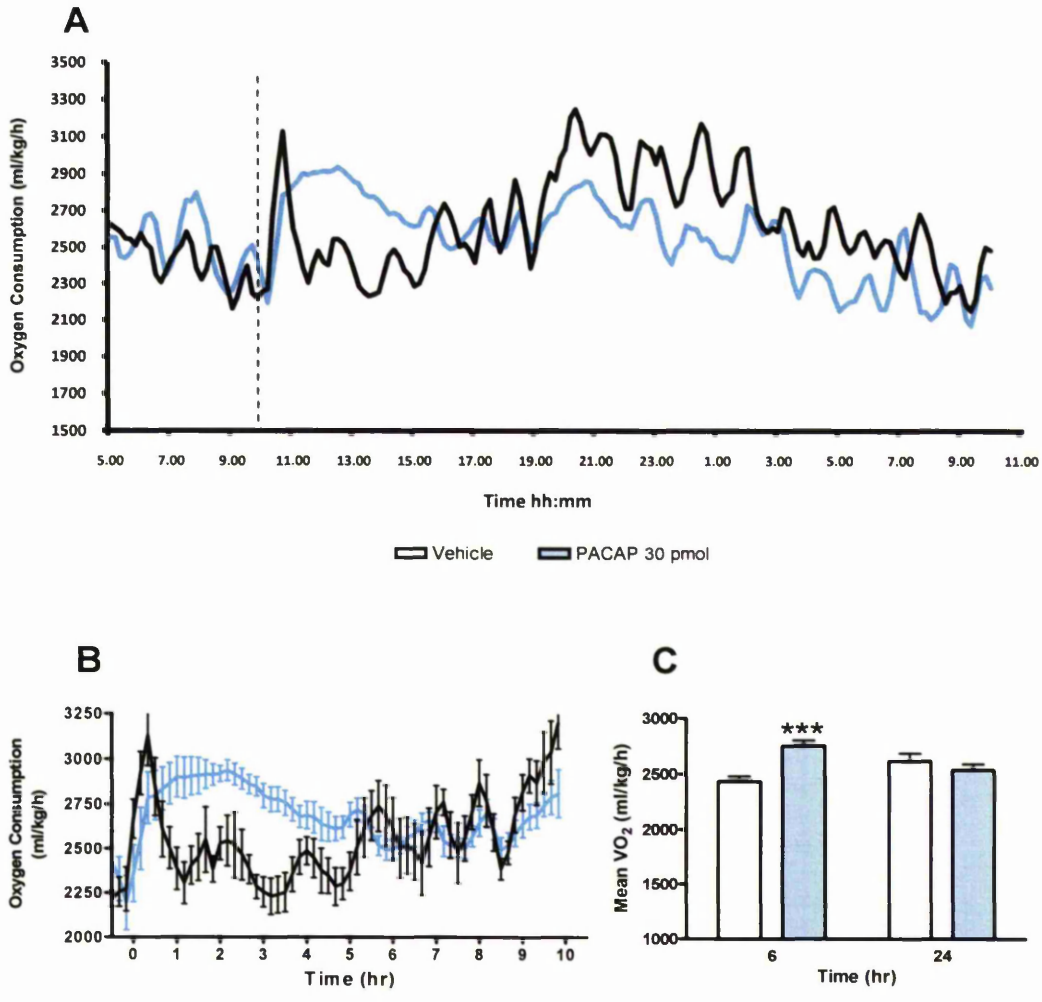


Figure 4.5 PACAP: Oxygen consumption

Effect of i.c.v. 30 pmol PACAP (n=8) on oxygen consumption (VO_2; ml/kg/h), compared with isotonic saline (n=8). **A.** Mean VO_2 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM VO_2 10 hours post injection. **C.** Mean ± SEM VO_2 during 1-6 hours post injection and 1-24 hours post injection. ***P<0.001. Data were analysed using an unpaired Student's *t*-test at each time point.

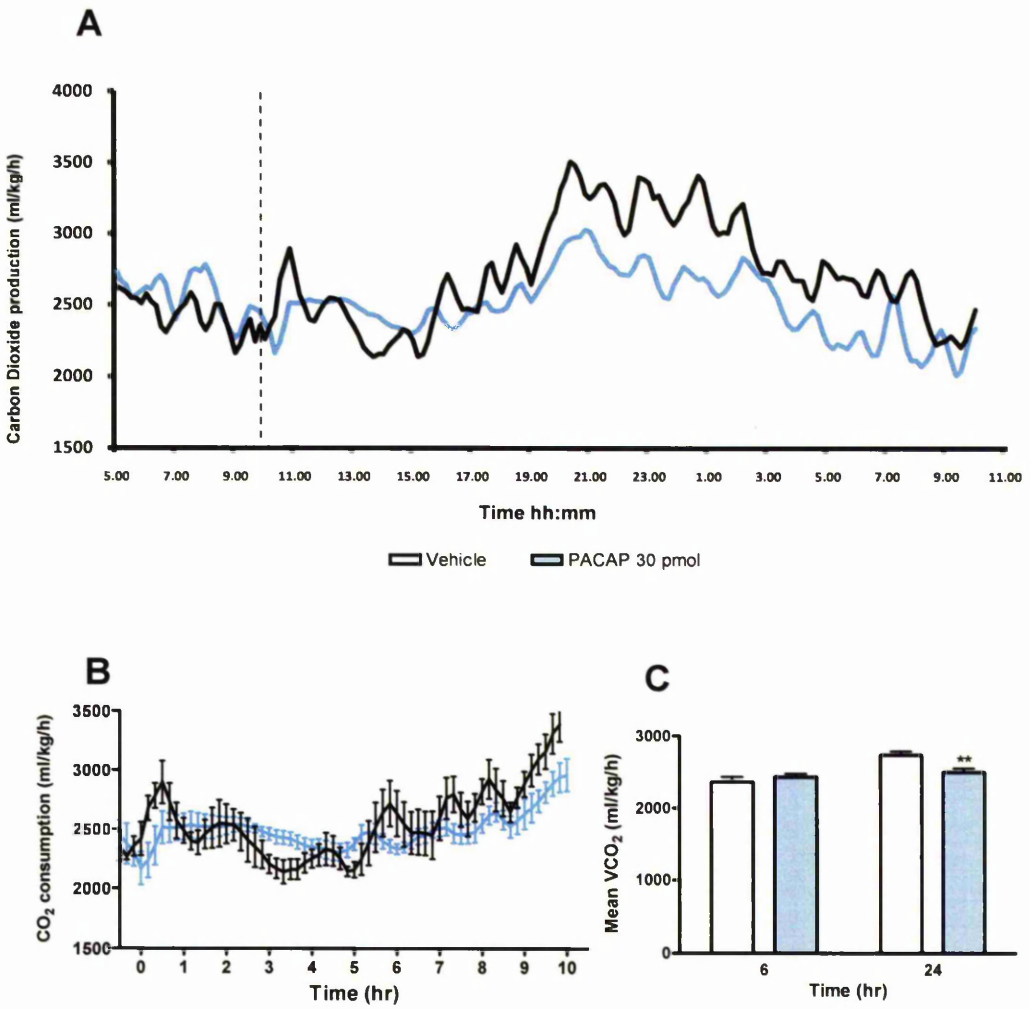


Figure 4.6 PACAP: Carbon dioxide production

Effect of i.c.v. 30 pmol PACAP (n=8) on carbon dioxide production (VCO_2; ml/kg/h), compared with isotonic saline (n=8). **A.** Mean VCO_2 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM VCO_2 10 hours post injection. **C.** Mean ± SEM VCO_2 during 1-6 hours post injection and 1-24 hours post injection. Data were analysed using an unpaired Student's *t*-test at each time point.

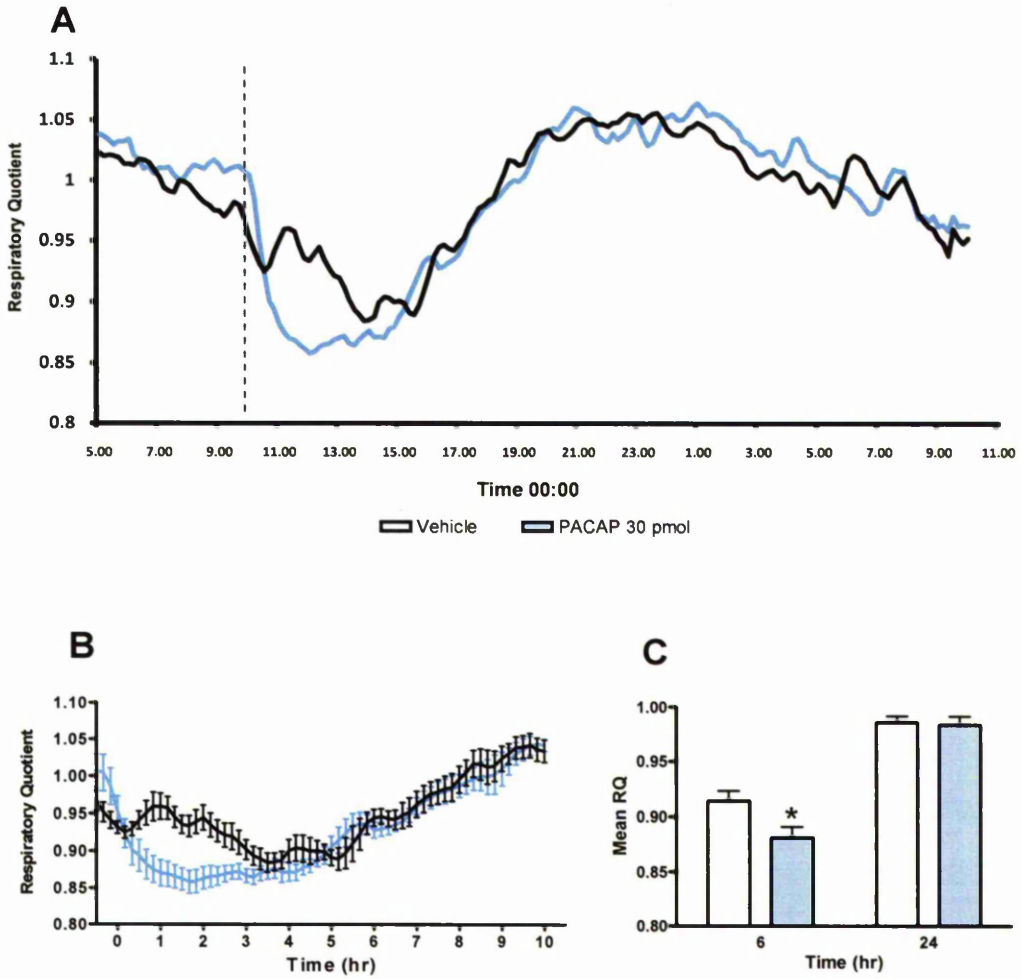


Figure 4.7 PACAP: Respiratory quotient

Effect of i.c.v. 30 pmol PACAP (n=8) on respiratory quotient (RQ), compared with isotonic saline (n=8). **A.** Mean RQ 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM RQ 10 hours post injection. **C.** Mean ± SEM RQ during 1-6 hours post injection and 1-24 hours post injection. *P<0.05. Data were analysed using an unpaired Student's *t*-test at each time point.

Blocking PACAP-induced anorexia and hyperthermia with $PACAP_{6-38}$

We examined the antagonistic effects of the PACAP analog $PACAP_{6-38}$ on the hypophagic and hyperthermic responses to 30 pmol PACAP. A dose of 300 pmol $PACAP_{6-38}$ was selected to antagonise PACAP binding since our initial dose response revealed this to be the highest dose which does not significantly alter food intake when given alone. Furthermore, binding assays show that $PACAP_{6-38}$ has approximately 10-fold lower PAC_1 and $VPAC_2$ affinity than does PACAP (Gourlet et al., 1995). We found that in all mice receiving the co-injection, 300 pmol of $PACAP_{6-38}$ completely prevented the anorexia observed when 30 pmol PACAP was administered alone. This effect was significant at 1, 2 and 4 hours post injection, while by 24 hours post injection no groups were significantly different (see Figure 4.8). As before, 300 pmol $PACAP_{6-38}$ had no effect alone. In addition to blocking feeding effects, $PACAP_{6-38}$ also antagonised the hyperthermic response to PACAP. As previously observed, at 1-6 hours post injection, i.c.v. 30 pmol PACAP caused a significant increase in mean T_b compared with controls. However, the mean T_b of mice given the co-injection of PACAP + $PACAP_{6-38}$ was indistinguishable from the control group, and significantly lower than those receiving PACAP alone. $PACAP_{6-38}$ had no effect on T_b when administered alone (data not shown). Over the full 24 hours there was no significant difference between any of the four groups (see Figure 4.9).

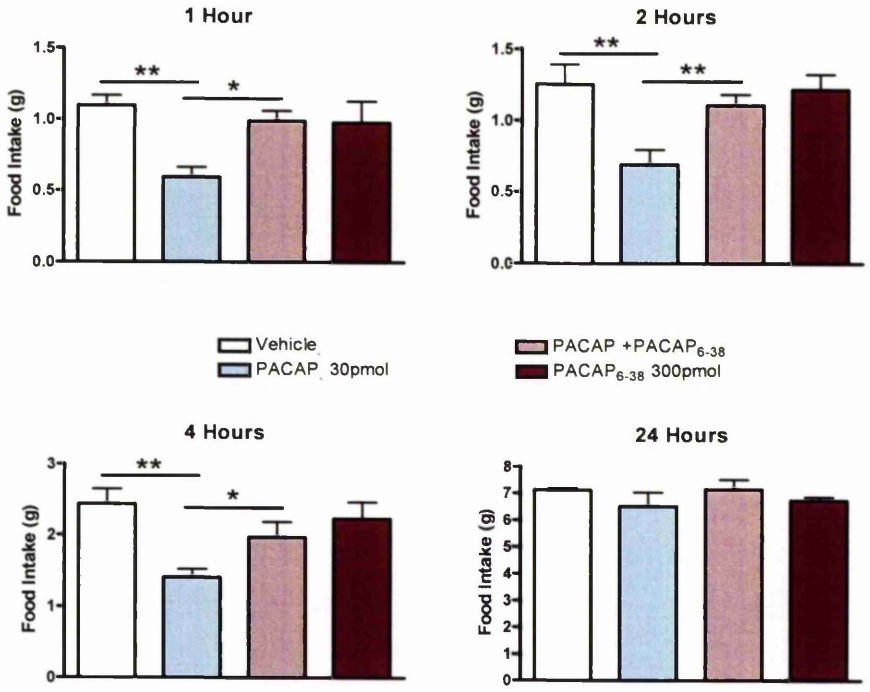


Figure 4.8 PACAP and $PACAP_{6-38}$ co-administration: Food intake

The effect of co-administration of $PACAP_{6-38}$ on i.c.v. PACAP-induced hypophagia at 1, 2, 4 and 24 hours post injection. Groups: Vehicle (sterile isotonic saline; n= 6), 30 pmol PACAP (n=6), 30 pmol PACAP + 300 pmol $PACAP_{6-38}$ (n=6), 300 pmol $PACAP_{6-38}$ (n=6) Mean ± SEM. * P<0.05, **P<0.01; one-way ANOVA with Newman-Keul's *post hoc* test to compare all groups.

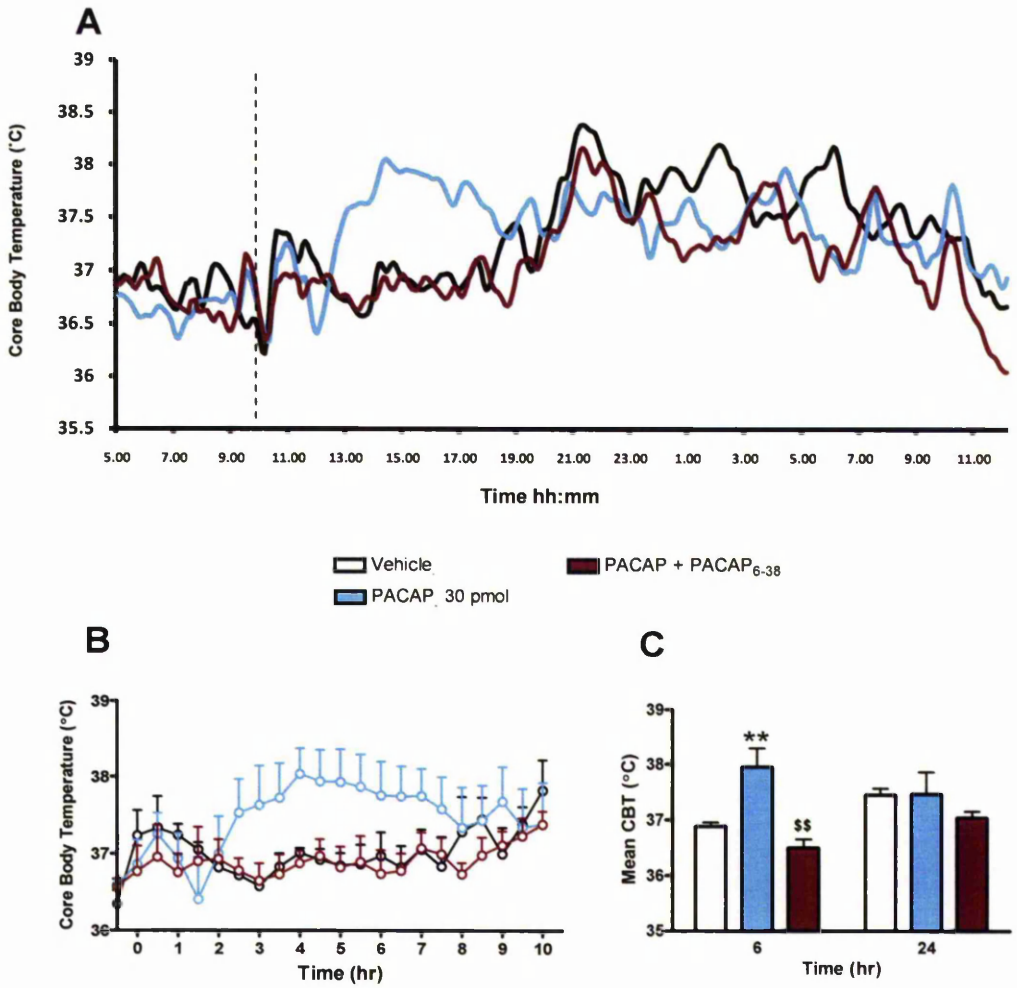


Figure 4.9 PACAP and $PACAP_{6-38}$ co-administration: Core-body temperature

Effect of co-administration of $PACAP_{6-38}$ on i.c.v. 30 pmol PACAP-induced hyperthermia. Groups: vehicle (sterile isotonic saline; n= 4), 30 pmol PACAP (n=6), 30 pmol PACAP + 300 pmol $PACAP_{6-38}$ (n=5), 300 pmol $PACAP_{6-38}$ (n=5; data not shown). **A.** Mean T_b 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM T_b 10 hours post injection. **C.** Mean ± SEM T_b during 1-6 hours post injection and 1-24 hours post injection. **P<0.01; one-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle control, $compared with PACAP-injected group.

Effect of VIP on food intake, core-body temperature and energy expenditure

VIP given i.c.v. was significantly less effective at producing hypophagia than equimolar doses of PACAP at 1, 2 and 4 hours post injection. As previously described, both 60 and 30 pmol i.c.v. PACAP doses produced robust anorexia, whereas 30 pmol VIP did not alter food intake at any time point, and 60 pmol VIP caused significantly smaller decreases in food intake at 1 and 2 hours post injection. By 4 hours there was no significant effect of either VIP dose, while animals injected with either PACAP dose had still eaten less than controls. At 24 hours significant effects on cumulative food intake could only be seen in mice receiving 60 pmol PACAP (see Figure 4.10).

In contrast with PACAP, 30 pmol VIP caused no change in T_b or VO_2 compared with saline control, either between 1-6 hours or over the full 24-hour experimental period (see Figures 4.11 and 4.12). VIP-treated mice did, however, undergo a non-significant decrease VCO_2 levels, which was most apparent over the first 1-6 hours (see Figure 4.13). This resulted in a decrease in RQ to approximately 0.88, which although not as pronounced as following PACAP injection, was significantly lower than controls between 1-6 hours post injection, returning to control levels by lights off (see Figure 4.14).

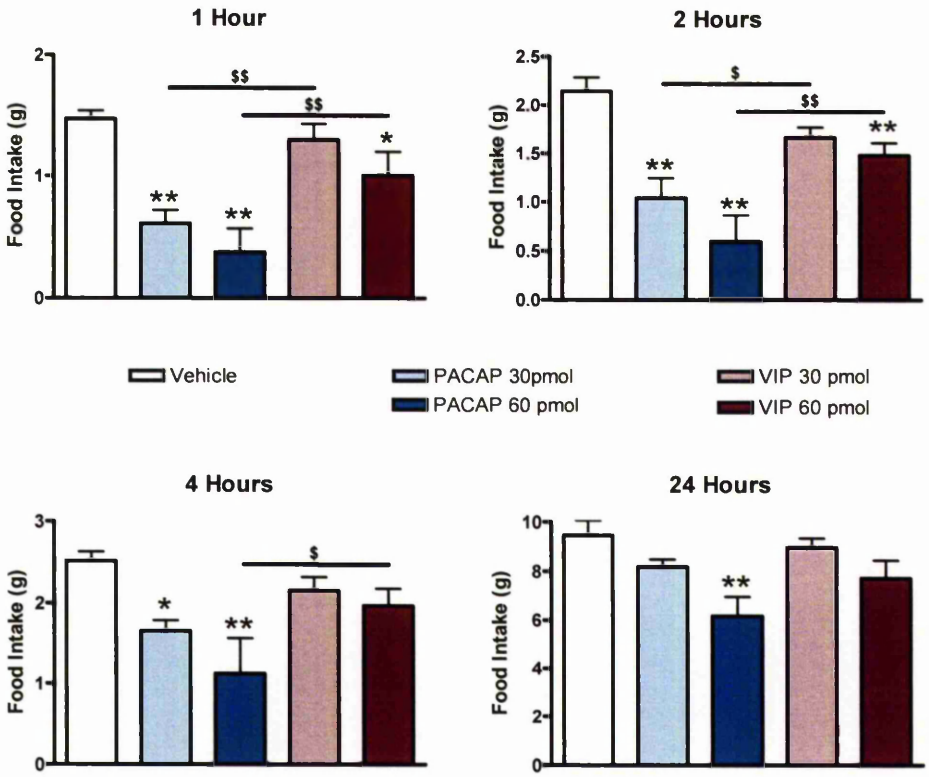


Figure 4.10 VIP dose response: Food intake

Effect of equimolar doses of VIP and PACAP on fast-induced refeeding at 1, 2, 4 and 24 hours post injection. Groups: 30 pmol PACAP (n=6), 60pmol PACAP (n=6), 30 pmol VIP (n=7), 60 pmol VIP (n=6), isotonic saline control (n=6). Mean ± SEM. * P<0.05, **P<0.01 One-way ANOVA followed by Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle control, $compared with equivalent dose PACAP.

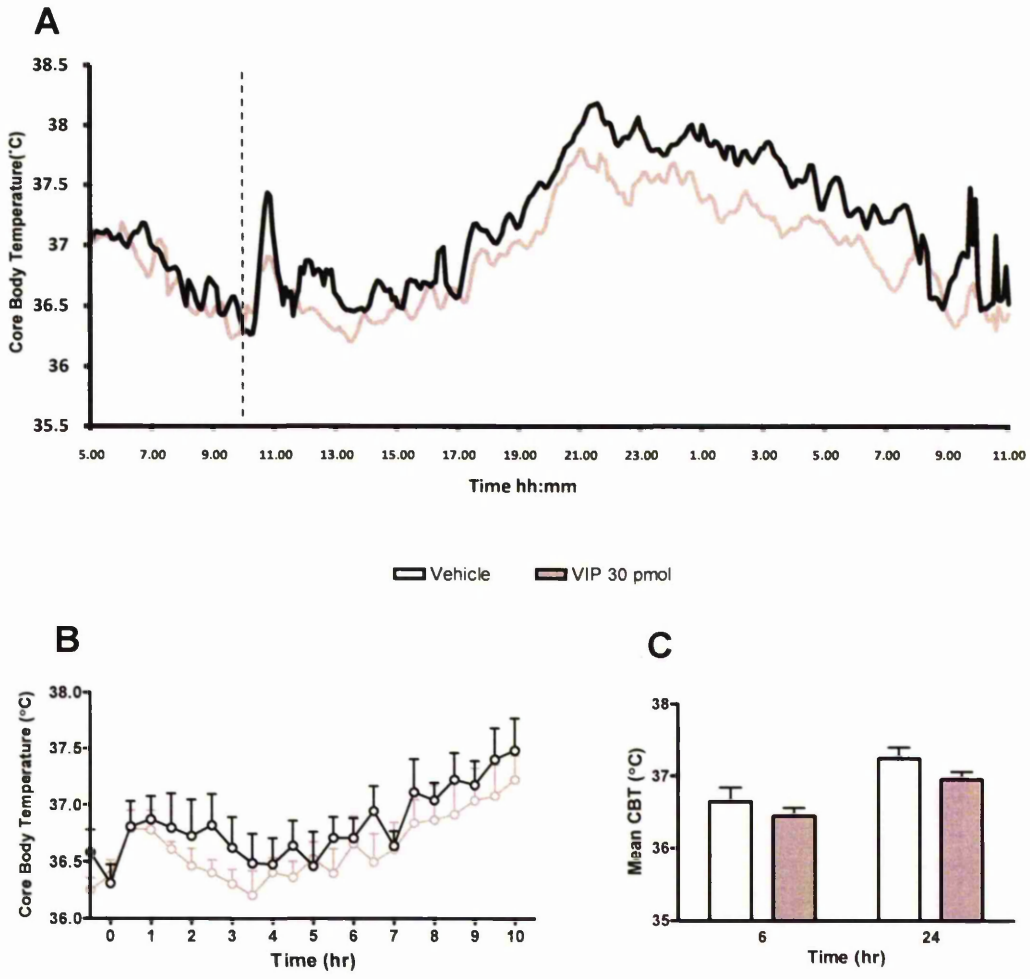


Figure 4.11 VIP: Core-body temperature

Effect of i.c.v. 30 pmol VIP (n=8) on core-body temperature (T_b; °C), compared with isotonic saline (n=8). **A.** Mean Tb 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM Tb 10 hours post injection. **C.** Mean ± SEM Tb during 1-6h post injection and 1-24 hours post injection. Data were analysed using unpaired Student's *t*-test at each time point.

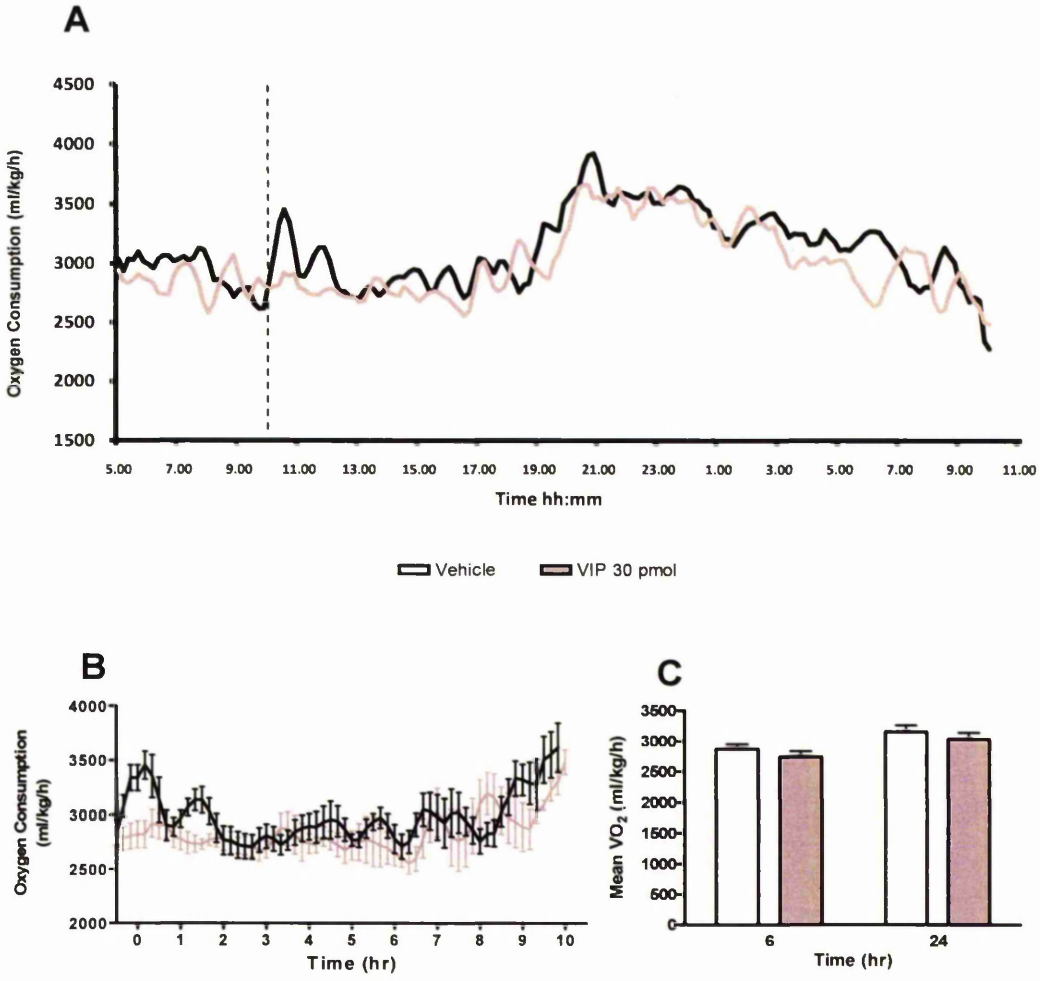


Figure 4.12 VIP: Oxygen consumption

Effect of i.c.v. 30 pmol VIP (n=8) on oxygen consumption (VO_2) (ml/kg/h), compared with isotonic saline (n=8). **A.** Mean VO_2 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM VO_2 10 hours post injection. **C.** Mean ± SEM VO_2 during 1-6 hours post injection and 1-24 hours post injection. Data were analysed using unpaired Student's *t*-test at each time point.

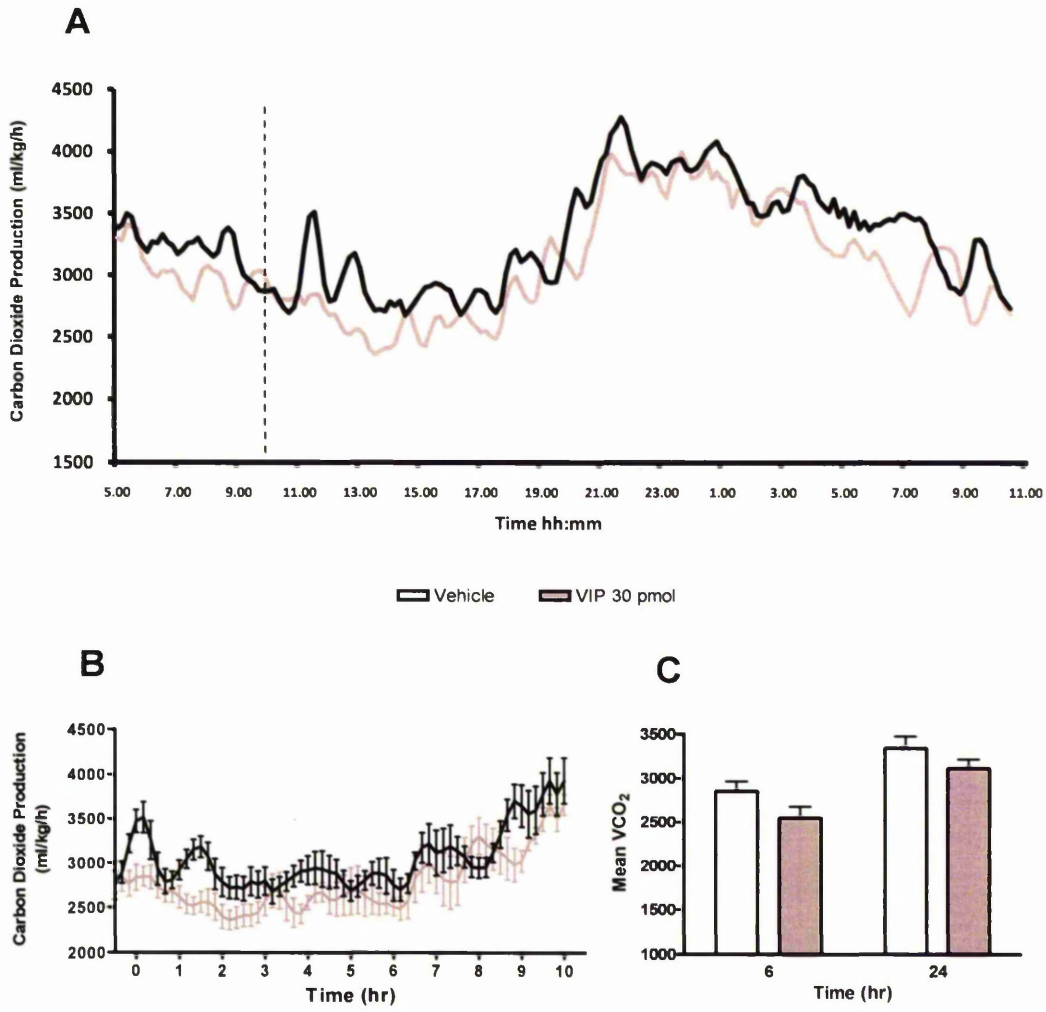


Figure 4.13 VIP: Carbon dioxide production

Effect of i.c.v. 30 pmol PACAP (n=8) on carbon dioxide production (VCO_2) (ml/kg/h), compared with isotonic saline (n=8). **A.** Mean VCO_2 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line. **B.** Mean ± SEM VCO_2 10 hours post injec tion. **C.** Mean ± SEM VCO_2 during 1-6 hours post injection and 1-24 hours post injection. Data were analysed using unpaired Student's *t*-test at each time point.

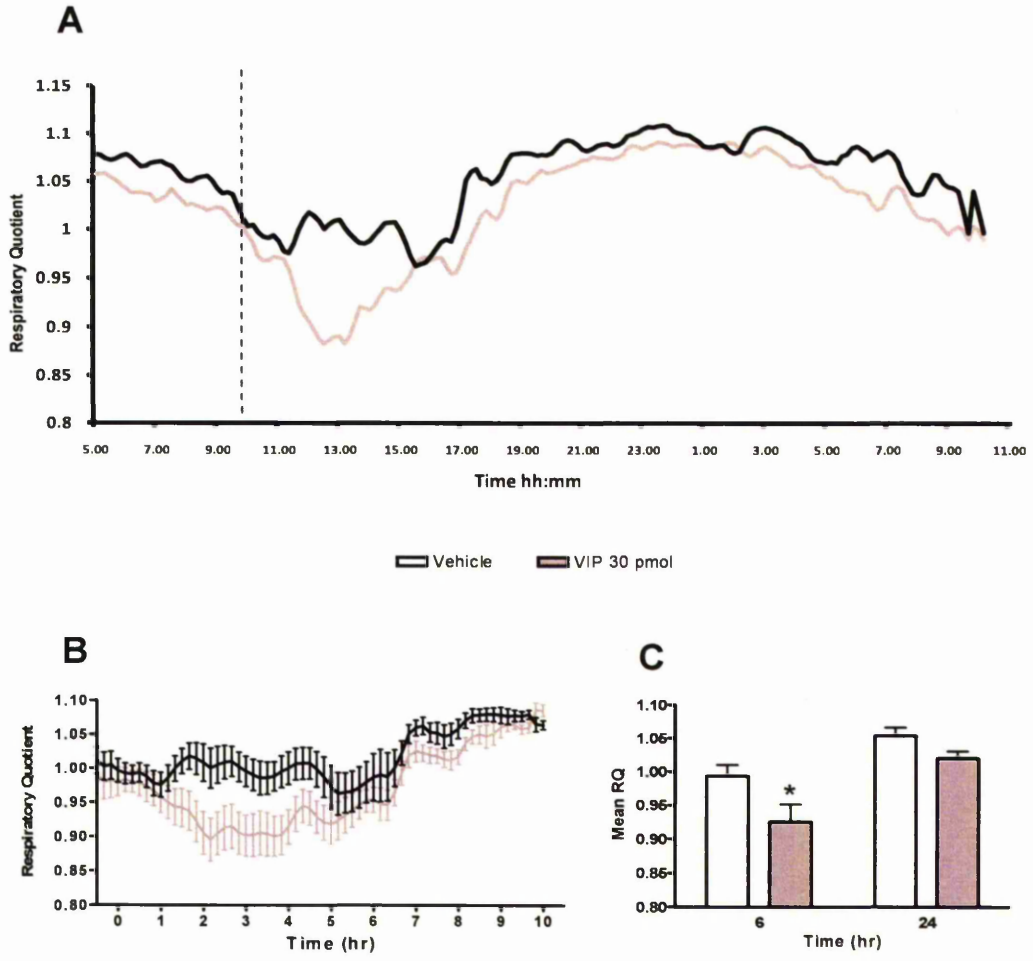


Figure 4.14 VIP: Respiratory quotient

Effect of i.c.v. 30 pmol PACAP (n=8) on respiratory quotient (RQ), compared with isotonic saline (n=8). **A.** Mean RQ 5 hours pre and 24 hours post injection. Injection is indicated by the dotted line.**B.** Mean ± SEM RQ 10 hours post injection. **C.** Mean ± SEM RQ during 1-6 hours post injection and 1-24 hours post injection. *P<0.05. Data were analysed using unpaired Student's *t*-test at each time point.

Effects of PACAP on $VPAC_2$ KO mice

$VPAC_2$ knockout mice have altered metabolism

Due to mechanisms yet to be fully characterised, $VPAC_2$ KO mice have marked abnormalities in their circadian metabolic profile when maintained under 12:12 light dark cycle (for details see Bechtold et al., 2007). Visual examination of CLAMS traces for these animals reveals a temporal advance in the decline of nocturnal VO_2 and VCO_2 levels, and a distortion in respiratory quotient rhythms compared with wildtype mice. These factors were eliminated as far as possible by the use of two-way ANOVA analysis, which takes into account effects of drug regardless of starting baseline.

Effect of PACAP on food intake of $VPAC_2$ KO mice

Owing to their abnormal metabolic profile, injections were performed at lights off when VO_2, VCO_2 and RQ of $VPAC_2$ KOs most closely resemble that of wildtype controls. $VPAC_2$ KO mice used were significantly heavier than age-matched C57BL/6 controls at the start of the experiment (+9±0.3 %). However, 30 pmol PACAP was effective at reducing nocturnal food intake in $VPAC_2$ KO mice to an equivalent degree to that observed in wildtype controls (wt: -68±11 %, KO: -58±17 % at 1 hour post injection), and two-way ANOVA analysis revealed no effect of genotype and no interaction between drug and genotype at any time point. Similarly, genotype had no significant effect on the extent of weight loss over the 24-hour period following PACAP injection (wt: -10±3 %, KO: -7±3 %; see Figure 4.15).

Effect of PACAP on metabolic rate of $VPAC_2$ KO mice

As previously shown, 30 pmol PACAP i.c.v. caused an acute increase in VO_2, no change in VCO_2, and a decrease in RQ in wildtype mice. PACAP caused a significant increase in VO_2 of $VPAC_2$ KO mice over 1-6 hours post injection, which was equivalent in magnitude to the effect seen in wildtypes (see Figure 4.16). VCO_2 levels were not significantly affected by PACAP administration in either strain (see Figure 4.17). Interestingly, although baseline RQ is significantly different between the two strains at 1-6 hours, PACAP caused RQ to decrease to equivalent levels in the $VPAC_2$ KO and wildtype groups (see Figure 4.18). The abnormal metabolic baseline of $VPAC_2$ KO mice was accounted for by the use of two-way ANOVA analysis, and there was no significant interaction between genotype and drug for any of the parameters tested.

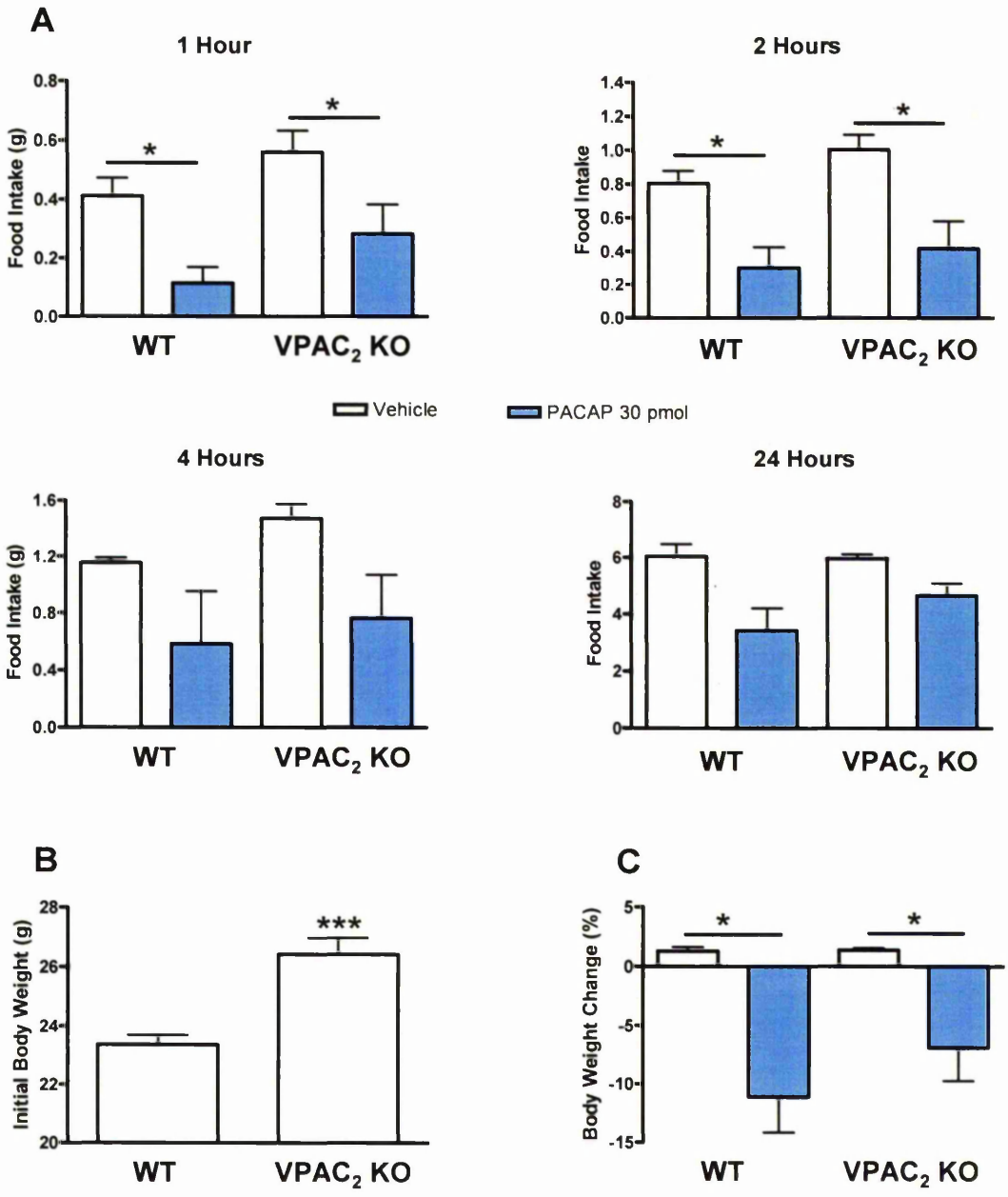


Figure 4.15 PACAP in $VPAC_2$ KO: Food intake and body weight

A. Effect of i.c.v. 30 pmol PACAP/saline on nocturnal food intake of $VPAC_2$ KO mice compared with C57BL/6 controls at 1, 2, 4 and 24 hours post injection. **B.** Initial body weights of $VPAC_2$ KO mice and age-matched C57BL/6 controls. **C.** Effect of 30 pmol PACAP/saline on body weight change of $VPAC_2$ KO mice compared with C57BL/6 controls 24 hours post injection. *P<0.05, ***P<0.001. Initial body weight data were analysed using a Student's t-test. Feeding and change in body weight data were analysed using two-way ANOVA with Bonferroni *post hoc* test.

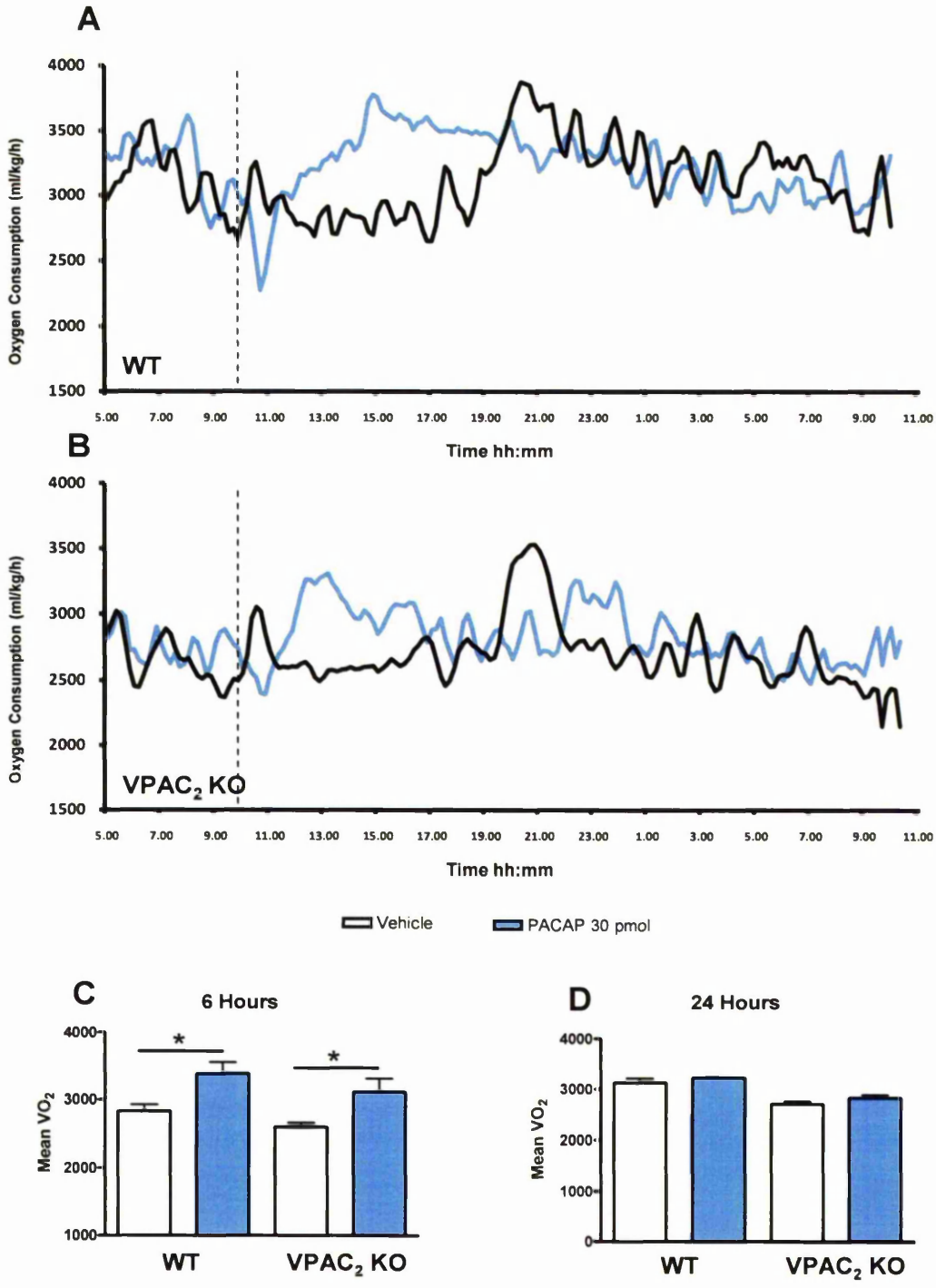


Figure 4.16 PACAP in $VPAC_2$ KO: Oxygen consumption

Mean VO_2 5 hours pre and 24 hours post injection in **A.** WT C57BL/6 controls and **B.** $VPAC_2$ KO mice. Error bars are left off for clarity. Injection is indicated by the dotted line. Mean ± SEM VO_2 during **C.**1-6 hours post injection and **D.** 1-24 hours post injection for each genotype. *P<0.05. Data were analysed using a two-way ANOVA with Bonferroni *post hoc* test.

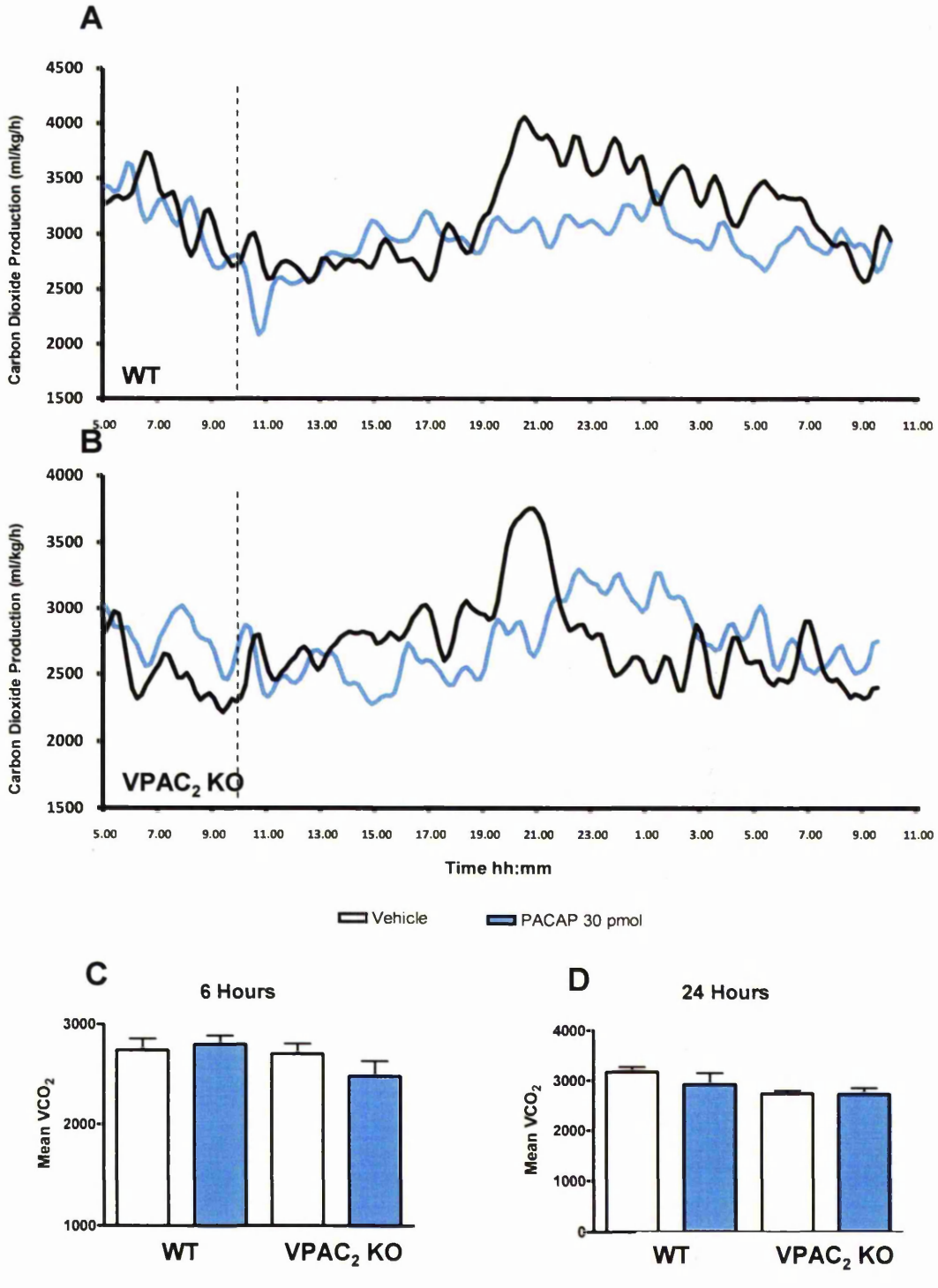


Figure 4.17 PACAP in $VPAC_2$ KO: Carbon dioxide production

Mean VCO_2 5 hours pre and 24 hours post injection in **A.** WT C57BL/6 controls and **B.** $VPAC_2$ KO mice. Error bars are left off for clarity. Injection is indicated by the dotted line.Mean ± SEM VCO_2 during **C.**1-6 hours post injection and **D.** 1-24 hours post injection for each genotype. Data were analysed using a two-way ANOVA with Bonferroni *post hoc* test.

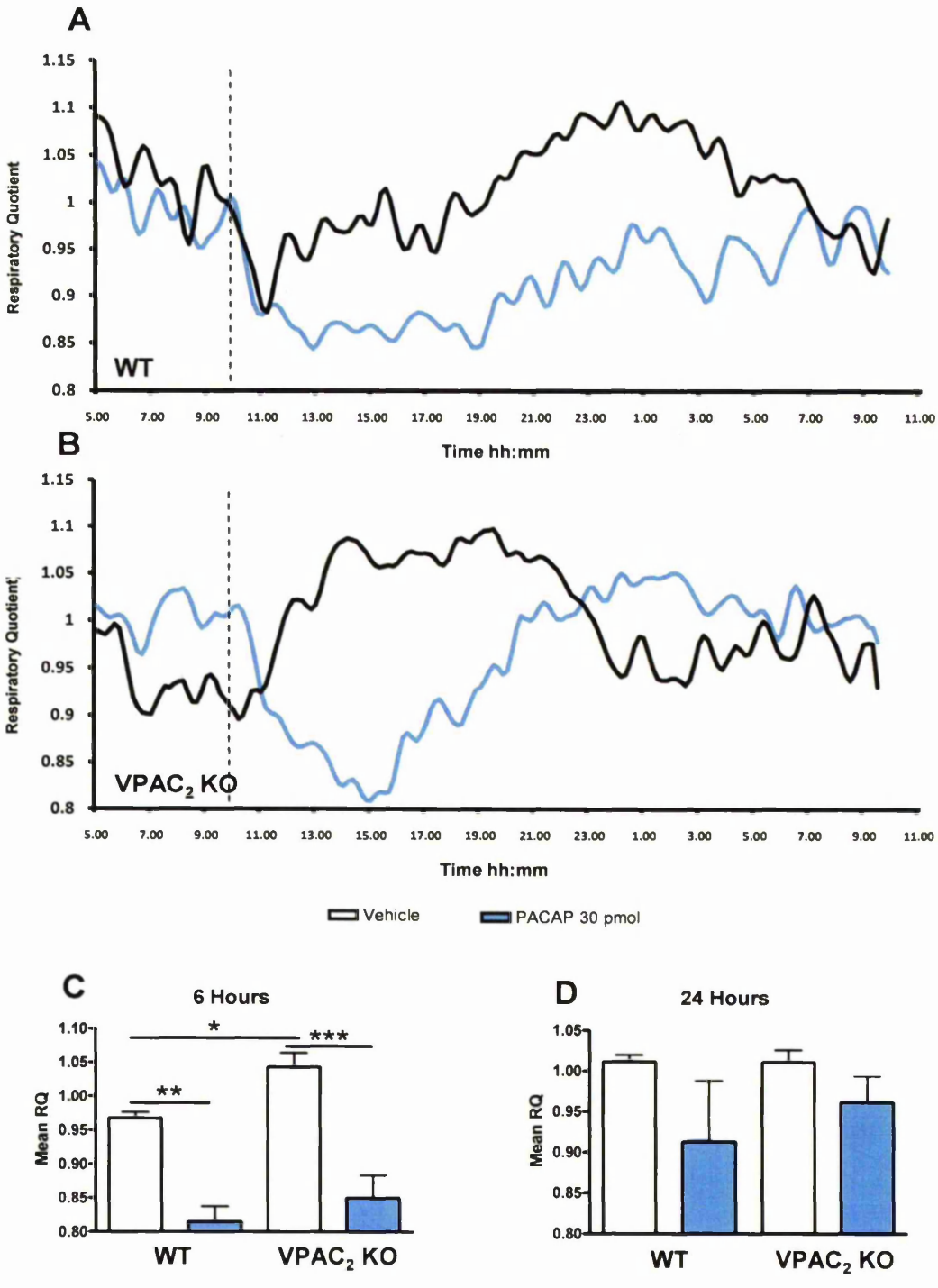


Figure 4.18 PACAP in $VPAC_2$ KO: Respiratory quotient

Mean RQ 5 hours pre and 24 hours post-injection in **A.** WT C57BL/6 controls and **B.** $VPAC_2$ KO mice. Error bars are left off for clarity. Injection is indicated by the dotted line. Mean ± SEM RQ during **C.**1-6 hours post injection and **D.** 1-24 hours post injection for each genotype. *P<0.05, **P<0.01, ***P<0.001. Data were analysed using a two-way ANOVA with Bonferroni *post hoc* test.

4.4 DISCUSSION

Effect of PACAP on feeding and behaviour

The data presented in this chapter confirm that i.c.v. PACAP has a dose-dependent anorexigenic effect in mice, and that doses in the low pmol range are able to elicit this effect both during daytime fast-induced re-feeding, and nocturnal free feeding. However, many exogenous agents can cause cessation of feeding, without inducing satiety *per se*. For example sensations of nausea, drowsiness or hyperthermia can all cause reductions in the amount of food consumed. Even a number of physiologically anorectic peptides including CRH and CCK can have aversive effects when administered at high doses (Deutsch and Hardy, 1977; Heinrichs et al., 1991). We carried out a behavioural analysis following a single central PACAP injection to investigate whether this is also the case for this peptide.

During BSS analysis, great care was taken to ensure all animals were well acclimatised and conditioned to handling and injection. This is important for behavioural analysis, since mice are easily stressed and this could have marked impact on their pattern of behaviour. Furthermore, since behaviour categorisation can be subjective it was important to carry out preliminary monitoring sessions prior to the experiment in order to establish how behaviours would be classed. The interval sampling method used has the advantage of allowing up to eight animals to be observed at once, minimising variation attributed to sampling on different days or at different times of day. However, the main disadvantage of only sampling every 30 seconds is that the observer may underestimate time spent performing each behaviour, or indeed miss behaviours which have a very short duration e.g. drinking. On the other hand, unconscious bias can also be a factor, since an observer frequently will favour a rare behaviour over a more frequent one if an animal is in transition between the two (Altmann, 1974). However, despite these caveats, BSS monitoring was an adequate method of behavioural analysis for this experiment, since overall behaviour was the emphasis, rather than detailed analysis of individual behaviours.

The BSS analysis works on the principal that rodents follow a distinct pattern of predominant behaviours during and immediately after feeding characterised by an early period of food intake intermingled with short bouts of activity, post-prandial grooming, and finally rest (Halford et al., 1998). Substances which cause disruption of this pattern are deemed to act via a non-homeostatic mechanism, whereas true satiety factors cause a leftward shift but do not alter the overall pattern. Our data would imply that at

high doses PACAP does not behave as a true satiety factor when administered into the cerebrospinal fluid (CSF), since the reduction in food intake is largely attributed to an increase in inactivity. During behaviour analysis, 60 pmol PACAP significantly decreased the incidence of feeding over the observation period, but also caused marked disruption of the BSS, most evident by the increase in inactivity at the expense of all other natural behaviours. In contrast, the lower doses of 30 and 15 pmol, did not disrupt the overall sequence of the BSS, while 30 pmol still decreased feeding incidences and food consumed (data not shown). Importantly, activity and grooming which can decrease feeding secondarily by response competition (Hodge et al., 2008) were unaffected, contrary to higher dose studies (Adamik and Telegdy, 2004). An important factor to consider when investigating a novel anorexigen is that centrally-administered peptides can cause aversive effects which are independent of receptor binding but which may manifest as an 'anorexigenic' effect. Nevertheless, the fact that we were able to block PACAP-induced anorexia by co-administration $PACAP_{6-38}$ (also shown by Mounien et al., 2008) shows that this is a receptor-mediated effect.

Our results suggest that like CCK and CRH, high doses of PACAP reduce food intake by mechanisms other than satiety. To further characterise the underlying cause of this anorexic effect, a simple conditioned taste aversion paradigm could be used. This involves exposing animals to a novel and pleasant stimulus; sucrose solution, and immediately following this with administration of the agent to be tested. Forty eight hours later, animals are re-exposed to sucrose solution and the volume consumed is compared with controls. Agents which cause hypophagia by visceral illness significantly reduce the volume of sucrose ingested upon second exposure, presumably by associating the sucrose drink with the unpleasant feelings experienced as a result of the drug. This will help us to clarify whether the increase in inactivity is due to aversive sensations of nausea, hyperthermia or fear, or as a result of motivational or locomotor deficits.

Effect of PACAP on energy expenditure

Energy expenditure is comprised of three elements: that required for normal cell function; that resulting from physical activity; and that which is attributed to non-shivering thermogenesis (Lowell and Spiegelman, 2000), the latter two of which are variable. We initially suspected that PACAP could be affecting energy expenditure following the observation that its central administration decreases body weight three-fold over that accountable by food intake. This indicates that PACAP-injected individuals are using more energy, and since we previously demonstrated that PACAP

does not alter activity, we can deduce that it derives from increased thermogenesis. In rodents, BAT is a major site of non-shivering thermogenesis, and central energy balance networks contribute to drive its activation. We have demonstrated that PACAP expression in the VMN is up regulated in thermogenically active DIO-R mice, and down regulated in *ob/ob* and fasted animals, models of metabo-supression, raising the possibility that it too may promote sympathetic function (see Chapter 3). Indeed, previous studies have shown that exogenous PACAP elicits hyperthermia, and in this study we found a robust transient increase in T_b in response to PACAP, which is slightly preceded by an increase in VO_2. These effects are short-lived, due to the rapid degradation of PACAP *in vivo* (Bourgault et al., 2008), and the resulting negative energy balance is rapidly compensated during the ensuing dark phase by a decrease in energy expenditure.

In addition to increasing thermogenesis, PACAP injection significantly lowered RQ, a useful indicator of the proportion of energy expenditure derived from fat and carbohydrate oxidisation. RQ is negatively correlated with lipolysis and SNS activation, and as such, treatment with catabolic factors that promote fat metabolism, reduce its value. This result indicates that as well as promoting energy expenditure, PACAP also induces lypolysis of body fat stores, perhaps by an altogether independent mechanism. We also observed, although did not quantify, noticeable reductions in WAT fat pad mass following acute PACAP treatment, which was not seen in pair-fed animals. Recently it has been shown that a major stimulator of lipid mobilisation from WAT is via sympathetic innervation of this tissue, similar to that of BAT (Bamshad et al., 1998). PACAP KO mice are characterised by defects in sympathetic activation of BAT, WAT (Gray et al., 2002), kidney (Hatanaka et al., 2008), and respiratory system (Wilson and Cummings, 2008) and we believe that PACAP may be an important contributor to sympathetic outflow to a number of peripheral organs, including those involved in energy balance.

The VMN, where PACAP perikarya are densely located is alleged to be a key regulator of sympathetic outflow, demonstrated by the fact that VMN stimulation increases (Saito et al., 1987; Taniguchi et al., 2003) and VMN lesion decreases SNS activity. Moreover, a series of elegant retrograde tracer studies performed by Bamshad and colleagues (1998; 1999) have revealed other key brain areas which lie upstream of sympathetic outflow to BAT and WAT, many of which express PAC_1. These include the periventricular and dorsomedial nuclei of the hypothalamus, periaqueductal grey (PAG) of the midbrain, and the raphe nuclei of the brainstem. In chapter 6 of this thesis we will

examine more closely whether i.c.v. PACAP causes neuronal activation in these brain regions using c-Fos immunostaining. While the central sites reached by our i.c.v. injections are not known, it is important to bear in mind that sympathetic pre- and postganglionic neurones also express the PAC_1 receptor, and that PACAP potently stimulates sympathetic neurones (Beaudet et al., 1998; Lai et al., 1997). As such, administering PACAP into the CSF may cause increases in sympathetic outflow at least partially by its action on sympathetic neurones of the spinal cord.

Dissection of PACAP receptor pathways

Our results clearly show that PACAP has catabolic effects when administered centrally, and this is in agreement with the literature. *In vitro* PACAP binds with equal affinity at three G-protein coupled receptors, PAC_1, $VPAC_1$ and $VPAC_2$, which are all widely expressed throughout the brain, (Joo et al., 2004) and we sought to establish which are important in mediating these effects. There is evidence that PAC_1 could be responsible for PACAP's metabolic actions, owing to marked similarities between the phenotypes of the two knockout models (Hashimoto et al., 2000; Hashimoto et al., 2001), although this has never been confirmed pharmacologically. Here we have shown, for the first time that the N-terminus truncated analog $PACAP_{6-38}$ can prevent PACAP-induced catabolic effects when the two are co-injected into freely-behaving mice. Based on binding studies, $PACAP_{6-38}$ was discovered as a potent PAC_1 antagonist and has since been referred to as such in many studies; however, there is evidence that its actions could be far more complex. Gourlet et al., (2005) showed that rather than binding exclusively to PAC_1, $PACAP_{6-38}$ also has strong affinity for $VPAC_2$, although not for $VPAC_1$. Furthermore, very recent reports have found that it even retains some agonist properties, behaving in a similar manner to full-length PACAP in certain systems (Reglodi et al., 2008). Being that $PACAP_{6-38}$ fully blocked PACAP-induced hypophagia and hyperthermia in our experimental paradigms, we can assume that it behaves solely as an antagonist under these conditions, although we cannot say for certain which of PAC_1 and $VPAC_2$ is primarily responsible.

In order to clarify this further, we tested whether centrally administered VIP has the same catabolic effects as PACAP when given at equal doses, and whether PACAP could exert its effects in the $VPAC_2$-deficient mouse. VIP belongs to the same peptide superfamily and has 68 % amino-acid homology with full-length PACAP. Like PACAP, VIP recognises both VPAC receptors, but as it has near-zero affinity for PAC_1, pharmacological disparities between VIP and PACAP can be attributed to this receptor. We found that centrally administered equimolar VIP did not elicit the same responses

as PACAP; 30pmol VIP had no significant effect on food intake, body temperature or oxygen consumption, but interestingly did cause an analogous decrease in RQ. These effects are similar to those reported in chicks (Tachibana et al., 2007), and suggest that while VPAC receptors are redundant in the anorexic and thermogenic PACAP pathways, they may partially mediate lipid mobilisation/utilisation. In agreement with this, knockout of the $VPAC_2$ receptor had no significant effect on the magnitude of hypophagic response to PACAP injection, while effects on VO_2 and RQ were also conserved. Corresponding studies will subsequently be carried out in $VPAC_1$ and PAC_1 knockout strains, which are currently being rederived into the Manchester Incubator Building. If our hypothesis is correct, we would expect PACAP effects to be conserved in the $VPAC_1$-deficient strain, but lost in the PAC_1 knockouts. However, a masking effect whereby related receptors compensate for the genetic loss of one another may exist, making interpretation more difficult.

General considerations

A particular difficulty involved in characterising a pleiotropic peptide is that although subpopulations of PACAP cells may mediate homeostatic energy balance pathways, other, quite separate, central responses could impinge on tests such as BSS analysis when PACAP is administered into the lateral ventricle. For example, PACAP signalling in the amygdala and in the SON has been implicated in fear responses (Legradi et al., 2007) and the light-entrainment of the circadian clock respectively (Fahrenkrug et al., 2005). The combination of these discrete functions could underlie the complex phenotype of the PACAP knockout mouse, and the behavioural effects observed when PACAP is administered into the brain. In chapter 5 of this thesis we investigate whether PACAP signalling is required for the acute hypophagic and hyperthermic responses to leptin, which would suggest physiological relevance. Ultimately, however, targeted deletion of specific subsets of PACAP neurones, for example those of the VMN, using genetic engineering is the only way to remove any ambiguity; an approach which is detailed in the appendix of this thesis.

4.5 SUMMARY

- Central PACAP administration decreases food intake in a dose-dependent manner.
- High doses of PACAP causes marked disruption of the BSS, whereas lower doses have minimal non-feeding behavioural effects.
- Central PACAP administration causes body weight loss, which is significantly greater than that of pair-fed counterparts.
- Central PACAP administration increases T_b and VO_2 and decreases RQ.
- Co-administration of $PACAP_{6-38}$ blocks PACAP's effects on food intake and T_b.
- Central administration of VIP does not alter food intake, T_b or VO_2, but decreases RQ.
- Genetic loss of the $VPAC_2$ receptor does not reduce the magnitude of PACAP's effects on food intake, VO_2 or RQ.

4.6 CONCLUSIONS

Centrally administered PACAP causes robust decreases in food intake, body weight and RQ and increases in Tb and VO_2, typical of a catabolic peptide. Behavioural analysis revealed no changes in other natural behaviours, although higher doses did cause increased inactivity, which could be indicative of sickness, or limbic function deficits such as motivation or anxiety. Effects could be blocked with $PACAP_{6-38}$ and were not reproduced by injection of VIP, a related peptide. Furthermore, $VPAC_2$ KO mice showed normal responses to PACAP, leading to the conclusion that PAC_1, or an undiscovered PACAP-specific receptor is responsible for PACAP's hypophagic and hyperthermic properties. In conclusion, PACAP may have a key role in the regulation of energy balance, which is currently relatively unexplored.

CHAPTER 5

PACAP AND LEPTIN

5.1 INTRODUCTION

The adipose-derived hormone, leptin, was discovered over ten years ago, but only now are we unmasking fully the downstream pathways which result in reduced energy intake (feeding) and increased energy expenditure (thermogenesis). Peripheral or central leptin treatment induces cellular activation not only in the Arc, but in neurones of several hypothalamic nuclei, as well as areas of the thalamus and brainstem (Elmquist et al., 1997). Furthermore, recent transgenic models have challenged the long-standing theory that the Arc is responsible for the majority of centrally-mediated responses to leptin, and research focus is now beginning to shift towards extra-arcuate sites (Balthasar et al., 2004; Dhillon et al., 2006).

The VMN in particular expresses large amounts of the signalling form of the leptin receptor (Mercer et al., 1996b), which are readily engaged by peripheral leptin, as evidenced by the induction of c-Fos and pSTAT3 immunoreactivity (Elmquist et al., 1997; Frontini et al., 2008; Hubschle et al., 2001). Furthermore, when these receptors are selectively knocked out in SF1 cells this results in mice which are as obese as the POMC-driven lepr-B deleted mouse. This finding demonstrates that SF1 neurones of the VMN are at least as important as POMC neurones in mediating the effects of leptin (Balthasar et al., 2004; Bingham et al., 2008; Dhillon et al., 2006). However, since SF1 is not a releasable signalling molecule, the identity of the molecular and cellular correlates of this relationship remain unknown. Chapters 3 and 4 of this thesis provide evidence that VMN PACAP expression is regulated by leptin, and that like leptin, exogenous PACAP has centrally-mediated catabolic effects on appetite, thermogenesis and lipolysis. In light of these data, this chapter examines the evidence behind the hypothesis that leptin and PACAP activate common endogenous pathways and that they may interact at the level of the VMN.

Objectives:

To establish whether PACAP cells of the VMN are a direct leptin target

Since VMN PACAP expression appears to be correlated with central leptin signalling, it is conceivable that activation of the lepr-B signalling cascade may cause transcriptional changes in PACAP gene expression as it does with POMC. Our semi-quantitative *in situ* hybridisation histology data are consistent with the notion that leptin signalling alters PACAP expression in the VMN; however the mode of action remains to be clarified. Although cells in the dorsomedial VMN strongly express lepr-B and are likely

to be a direct leptin target (Funahashi et al., 1999; Mercer et al., 1996b), we must also consider that the VMN has dense reciprocal connections with other hypothalamic nuclei that could intercede transcriptional changes in PACAP indirectly.

In order to indicate whether leptin might directly activate PACAP cells, we will first use dual *in situ* hybridisation histology to examine whether VMN SF1 neurones co-express PACAP. SF1 is a transcription factor which is expressed exclusively in VMN cells of the brain, and which is essential for VMN development (Majdic et al., 2002). Currently a role of SF1 in the adult CNS has not been described, although electrophysiological analysis of these cells shows SF1 expression is a useful indicator of leptin sensitivity. Like POMC cells of the Arc, over 80 % of SF1 neurones are excited by leptin, compared with only 12 % of non-SF1 VMN cells. Furthermore, SF1 cells respond uni-directionally to leptin, indicating that they are likely to also express a catabolic signalling molecule(s). In agreement with this data, selective lepr-B knockout in SF1 cells (SF1-Cre $lepr^{flox/flox}$) reduces leptin-mediated VMN pSTAT3 induction by over 80 %, indicating that SF1 is an adequate surrogate marker of leptin-excited VMN cells (Dhillon et al., 2006). Our second approach will be to use semi-quantitative *in situ* hybridisation histology to examine whether PACAP mRNA expression is altered by the selective removal of leptin input in SF1 cells using the SF1-Cre $lepr^{flox/flox}$ strain. This model lacks leptin input onto SF1 cells of the VMN, while other leptin targets such as those in the Arc, PVN and LH respond normally, therefore any abnormalities in PACAP expression can be attributed directly to VMN leptin signalling, or lack thereof.

To test whether $PACAP_{6-38}$ can block leptin-induced hypophagia

Leptin increases firing of first-order catabolic neurones such as POMC expressing cells of the Arc in order to exert acute hypophagic effects. As such, central antagonism of the MC_4R with the potent synthetic antagonist SHU9119 prevents leptin-induced hypophagia (Seeley et al., 1997). This work has formed an important basis in establishing POMC cells as a leptin target and we intend to determine whether the PACAP antagonist $PACAP_{6-38}$ has a similar effect. It was demonstrated in chapter 4 that central co-administration of $PACAP_{6-38}$ completely blocks PACAP-mediated hypophagia, establishing it as an effective tool for eliminating PACAP anorexigenic pathways. We will use a similar approach to investigate whether blocking central PACAP signalling using this non-functional analog significantly affects the hypophagic response to exogenous leptin. The contribution of VMN neuronal populations in this leptin response is relatively unexplored, although direct VMN injection does produce hypophagia, albeit with less potency than Arc injection (Satoh et al., 1997a). Based on

this evidence we might only expect a partial reversal of leptin-induced feeding effects following PACAP antagonist administration.

To test whether $PACAP_{6-38}$ can block leptin-induced hyperthermia

Increased sympathetic outflow to BAT and WAT may be one of the major catabolic actions of PACAP and would explain the striking weight loss which results from a single central injection (see Chapter 4). These effects are also observed following centrally administered leptin (Collins and Surwit, 1996; Haynes et al., 1997) and since both PACAP and lepr-B are highly expressed in the VMN, it is possible that the two interact at this site. Satoh and colleagues (1999) demonstrated that a single direct leptin injection into the VMN, but not the Arc, DMN or PVN significantly increased plasma noradrenaline and adrenaline concentrations, reliable markers of SNS activity. Furthermore, pre-obese VMN-lesioned rats fail to show this response to central administration, suggesting that the VMN is essential for leptin's effects on sympathetic activation (Satoh et al., 1997b). Recent transgenic studies have also shown that SF1-driven deletion of the leptin receptor renders mice incapable of adaptive thermogenesis in response to a high-energy diet, despite extra-VMN leptin targets remaining intact (Dhillon et al., 2006). In light of these observations, and considering PACAP's own thermogenic properties (Pataki et al., 2000), we sought to find out whether $PACAP_{6-38}$ could prevent leptin-induced increases in body temperature.

5.2 METHODS

PACAP and SF1 ribonucleotide probes

Ribonucleotide antisense probes designed to bind PACAP mRNA (bases 506-980; DIG-labelled) and SF1 mRNA (bases 2038-2870; ^{33}P-labelled). cDNA sequences corresponding to these regions were generated using oligonucleotide primers (PACAP: 5'-AAGTACCTGCAGTCGGTCGT-3' and 5'-TGTGCACCCATCTTAATTGC-3'; SF1: 5'-ATTGGGTCCCCAGAGGATAC-3' and 5'-ACTTCTTGTCTGGGGCTGTG-3'), cloned into pGem-T-Easy plasmid (Promega, UK) and linearised using appropriate restriction enzyme (PACAP SpeI; SF1 ApaI). mRNA riboprobes were transcribed from linearised template in nuclease-free conditions at 37 °C for 1 hour using appropriate polymerise enzyme (PACAP T7; SF1 SP6). Reactions contained: 4 µl transcription buffer, 2 µl DTT, 1.5 µg linear template plasmid, 1 µl RNA polymerase (all Promega, UK) and 1 µl Superasin® (Applied Biosciences, UK). DIG transcriptions also contained 2 µl DIG-labelled NTP mix (Roche Diagnostics, Germany), while in ^{33}P transcriptions this was replaced with 1 µl each rATP, rCTP, rGTP (Promega, UK) and 3 µl ^{33}P-UTP (30 µCi; Perkin Elmer, USA). Transcription reactions were carried out in a volume of 20 µl. For transcription of the radioactive probe, excess cold UTP was added 15 minutes prior reaction termination, to ensure all products were full-length. Once transcription was complete, 1 µl DNase-1 (Promega, UK) was added to destroy DNA plasmid template which could contaminate RNA riboprobes. Probes were then precipitated at -20 °C overnight in 75 % ethanol (VWR International, UK), 700 mM ammonium acetate (Sigma Aldrich, UK) and 2.5 µl glycogen (Applied Biosciences, UK) in a total volume of 550 µl. The following day, tubes were centrifuged at 4 °C at high speed for 30 minutes and pellets de-salted in 70 % ethanol and air dried at RT. Probes were re-suspended in 100 µl nuclease-free water and quantified by gel electrophoresis (DIG-labelled) or by scintillation counting (^{33}P-labelled). Both probes were initially tested for binding specificity by DIG-labelling followed by NBT (Nitro blue tetrazolium chloride)-BCIP (5-Bromo-4-chloro-3-indolyl phosphate toluidine salt; Roche Diagnostics, Germany) detection and signal distribution compared with the literature.

Dual *in situ* hybridisation histology

Hypothalamic sections from naïve adult male CD1 mice (n=3) were pre-treated and dehydrated as for oligonucleotide *in situ* hybridisation (see general methods chapter), except that tissue was then de-lipidated in 100 % chloroform (VWR International, UK). Sections were hybridised with SF1-DIG-conjugated (300 ng/ml), and PACAP-^{33}P-conjugated (4 x10^6 counts/ml) probe cocktail in hybridisation buffer (4x SSC; Promega,

UK, 50 % deionised formamide, 1 mM EDTA, 20 µg/ml yeast tRNA, 10 % dextran sulphate, 1x Denhardt's solution; all Sigma Aldrich, UK; HB) at 65 °C overnight. Sections were then rinsed briefly in 2x SSC at RT, followed by 30 minute washes twice in 2x SSC, then once in 2x SSC/50 % formamide and 0.5x SSC, all at 60 °C. Sections were then washed twice in 0.1 M Tris pH7.5, 150 mM NaCl (Tris-buffered saline; TBS) for 10 minutes and blocked using foetal calf serum in TBS, 0.1 % Tween-20 (Sigma Aldrich, UK; TBS-T). Slides were then incubated overnight in anti-DIG-AP (Anti-Digoxigenin-POD, Fab fragments; Roche Diagnostics, Germany; 1:1500) in blocking buffer at 4 °C. The following day, sections were washed again in TBS pH7.5, followed by two further washes in 0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$ (all Sigma Aldrich, UK). DIG-probe was then visualised using NBT-BCIP (Roche Diagnostics, 1:100 in same buffer) at 37 °C overnight. Slides were thoroughly washed and air dried and coated in nuclear emulsion solution (K5; Ilford Imaging, UK) diluted 1:1 with distilled water containing glycerol under red light illumination. Once the emulsion was dry, slides were stored in desiccated light-tight boxes at 4 °C for 6 weeks. Slides were the processed in the dark room for 7 minutes in developing fluid (1:5 Phenisol, Ilford Imaging, UK), rinsed briefly in distilled water and finally incubated for 10 minutes in fixer (1:5 Hypam, Ilford Imaging, UK). Sections were then thoroughly washed under running tap water for 30 minutes, air dried and coverslipped using xylene-based mountant. For analysis, the VMN was subdivided into rostral, dorsomedial, central, ventrolateral and caudal using the Paxinos mouse atlas as a guide (Paxinos and Franklin 2004). Cells were considered positive for signal based on pre-set criteria and expressed as mean % ± SEM for each subdivision (see results section).

SF1-Cre $lepr^{flox/flox}$ and $lepr^{flox/flox}$ mice for semi-quantitative ISHH

The SF1-Cre $lepr^{flox/flox}$ strain was a generous gift from the Lowell Laboratory (Harvard Medical School, Boston, USA) and were re-derived and maintained in heterozygote breeding colonies at the University of Manchester Biological Services Facility. Male mice were group housed prior to sacrifice at 8-weeks of age, at which time body weight was recorded. Cre-negative mice of the same strain ($lepr^{flox/flox}$), in which leptin receptor is expressed normally, were used as controls. Animals used for comparison came from two litters of the same generation to minimise confounding effects of genetic background.

Semi-quantitative *in situ* hybridisation histology

Animals were killed by cervical dislocation between 10:00 and 12:00 and brains quickly removed and frozen on dry ice. *In situ* hybridisation of hypothalamic sections was

carried out using ^{35}S-labelled oligonucleotide probes, as detailed in the general methods chapter using the PACAP probe listed in Table 3.1 of Chapter 3. Statistical analysis of resulting signal was carried out as described in the general methods chapter.

Leptin dose response

A fast-induced re-feeding experiment was performed according to the protocol detailed in the general methods chapter. Animals received a single 1 µl i.c.v. injection of 0, 6 or 60 pmol leptin in sterile isotonic saline using minimal restraint (n=5-6 per group).

Co-injection of leptin with $PACAP_{6-38}$

Fast-induced re-feeding (n=6 per group) and radiotelemetry (n=5-8 per group) experiments were performed as described in the general methods chapter, with mice receiving a single 1 µl i.c.v. injection containing either 60 pmol leptin only, 300 pmol $PACAP_{6-38}$ only or 60 pmol leptin + 300 pmol $PACAP_{6-38}$ in isotonic saline. Controls received saline only.

5.3 RESULTS

Co-localisation of SF1 and PACAP

VMN signal was detected strongly with both probes. While SF1 signal was not found elsewhere in the brain, PACAP signal was also strongly detected in the mHab and MeA, analogous to our oligoprobe binding pattern, and confirming probe specificity (see Figure 5.1).

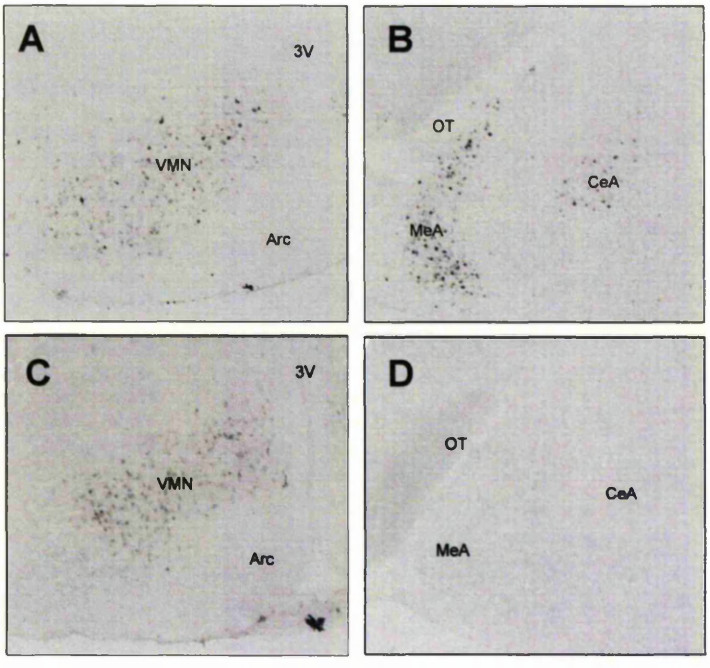
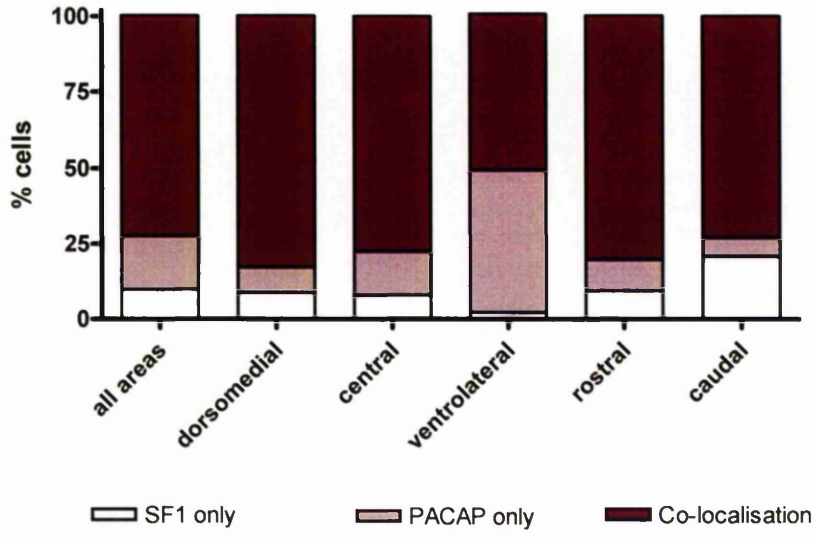


Figure 5.1 Verification of probe specificity
A-B PACAP and **C-D** SF1 DIG-labelled ribroprobe detection in the VMN and Arc (**A, C**) and amygdaloid nuclei (**B, D**). PACAP positive cells were detected densely in the VMN, MeA, CeA and mHab (not shown). SF1 positive cells were found in the VMN only (VMN and amgdala shown only). VMN- ventromedial hypothalamic nucleus; Arc- arcuate nucleus; 3V- third ventricle; OT- optic tract; MeA- medial amygdala; CeA- central amygdala.

In the VMN, PACAP expression extended ventrolaterally beyond SF1 signal as has been reported previously (Dhillon et al., 2006; Maekawa et al., 2006). For dual *in situ* hybridisation histology, a neurone was considered positive for SF1 if the cell body was clearly DIG-labelled (purple stain) and PACAP positive if its cell body was decorated with at least ten silver grains (3x above background). The majority (73±1 %) of total labelled VMN neurones were positive for both SF1 and PACAP, especially in the dorsmedial subdivision where 83±1 % cells were deemed to co-express both mRNA (see Figures 5.2 and 5.3). In the ventrolateral portion, a different pattern was observed; with only 51±2 % co-localisation and 48±5 % positive for PACAP only (see Figure 5.3).

All other subdivisions showed a similar distribution to that found in the dmVMN (see Figure 5.2).

A



B

VMN subdivision	% SF1 only	% PACAP only	% co-localised
All areas	10 ± 2	18 ± 1	73 ± 1
Dorsomedial	9 ± 1	8 ± 1	83 ± 1
Central	8 ± 1	14 ± 0.5	77 ± 3
Ventrolateral	2 ± 1	48 ± 5	51 ± 2
Rostral	9 ± 3	10 ± 0.5	80 ± 2
Caudal	21 ± 2	6 ± 1	73 ± 3

Figure 5.2 Co-localisation of SF1 and PACAP in subdivisions of the VMN

A. Graphical representation and **B**. tabular presentation of the percentage of labelled cells which express: SF1 only, PACAP only or SF1 and PACAP together in subdivisions of the VMN. Data expressed as Mean % (±SEM **B** only).

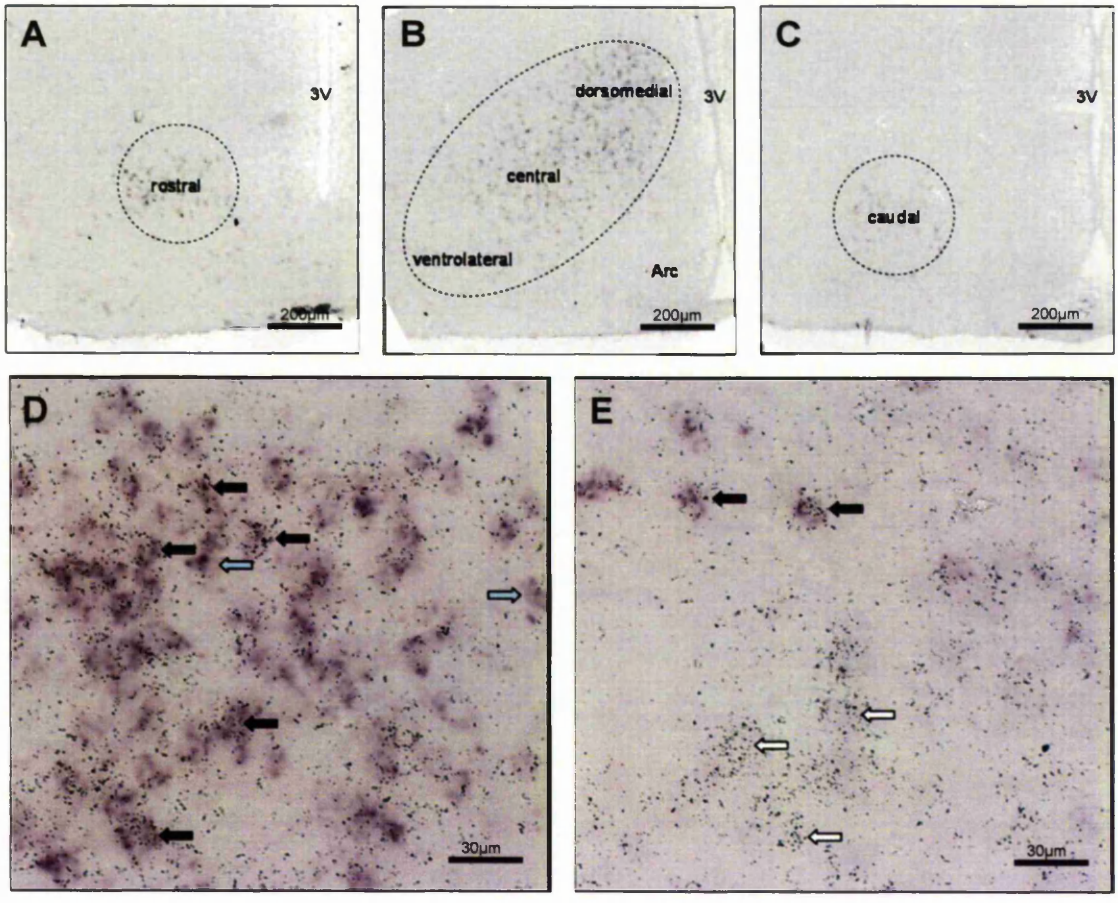


Figure 5.3 Co-localisation in the VMN

A-C Representative sections showing SF1 mRNA detection in the rostral, dorsomedial, central, ventrolateral and caudal subdivisions of the VMN. **D-E** Dual *in situ* hybridisation histology of the dorsomedial (**D**) and ventrolateral (**E**) VMN showing expression of PACAP (silver grains) and SF1 (purple stain). Black arrows denote co-localisation; pale blue arrows, SF1 only; and white arrows, PACAP only.

SF1-Cre lepr$^{flox/flox}$ and lepr$^{flox/flox}$ mice

As expected, 8-week old male SF1-Cre lepr$^{flox/flox}$ mice were marginally heavier than age-matched lepr$^{flox/flox}$ controls, although at this age the difference was not statistically significant (see Figure 5.4). Semi-quantitative *in situ* hybridisation histology revealed significantly lower (-21±2 %) VMN PACAP expression in SF1-Cre lepr$^{flox/flox}$ compared with controls. No difference was observed in the mHab which was used as a comparison region (see Figure 5.5).

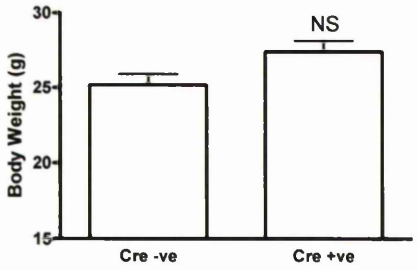


Figure 5.4 Body weight data: SF1-Cre lepr$^{flox/flox}$ and lepr$^{flox/flox}$
Body weights of 8-week old male SF1-Cre lepr$^{flox/flox}$ mice (Cre +ve) and age-matched lepr$^{flox/flox}$ controls (Cre –ve). Data are expressed as mean ± SEM and analysed using the Students' *t*-test.

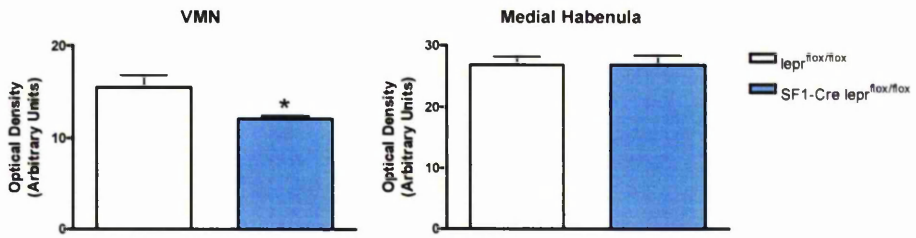


Figure 5.5 PACAP SF1-Cre lepr$^{flox/flox}$ and lepr$^{flox/flox}$
Comparison of PACAP mRNA expression, as measured by optical density, in the VMN and medial amygdala of SF1-Cre and lepr$^{flox/flox}$ mice. Data are expressed as mean ± SEM. *P<0.05; Student's unpaired t-test.

Effect of leptin on food intake

Leptin delivered i.c.v. caused a dose-dependent reduction of food intake in pre-fasted animals. We found that 60 pmol, but not 6 pmol caused a significant reduction in food consumed at 1 and 2 hours post injection, and at 4-hour and 24-hour time points both doses had significant anorexigenic effects (see Figure 5.6).

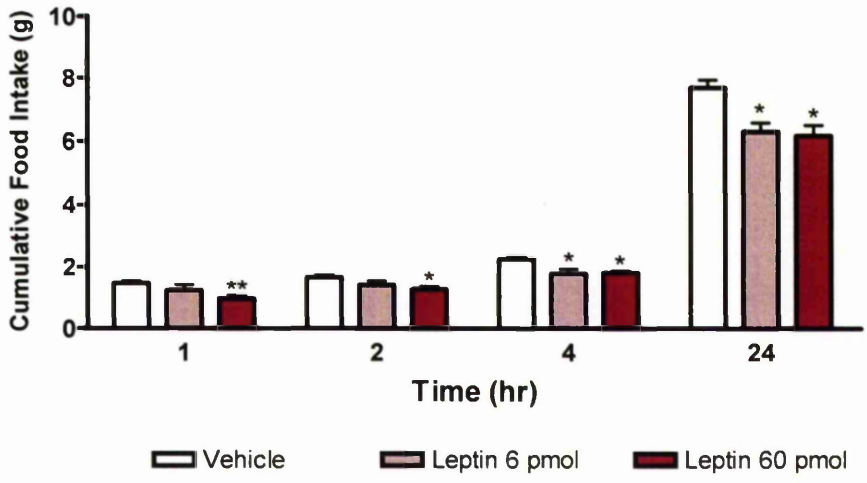


Figure 5.6 Leptin dose response

The effect of different doses of leptin on fast-induced re-feeding at 1, 2, 4, and 24 hours post injection. Groups: vehicle (sterile isotonic saline; n= 6), 6 pmol (n=5), 60 pmol (n=6) leptin. Mean ± SEM. * P<0.05, **P<0.01 all compared with vehicle; one-way ANOVA with Dunnet's *post hoc* test.

Effect of $PACAP_{6-38}$ on leptin-induced hypophagia

We examined the effects of the PACAP antagonist $PACAP_{6-38}$ on the hypophagic responses observed following 60 pmol leptin i.c.v. We found that 300 pmol of $PACAP_{6-38}$ reduced the hypophagic response to 60 pmol leptin by approximately half by 1 hour post injection. This partial attenuation was significant at 1, 2, 4 and 24 hours post injection, when the effects of leptin injection only were still evident. As before, 300 pmol $PACAP_{6-38}$ alone had no effect on food intake at any time point (see Figure 5.7).

Effect of $PACAP_{6-38}$ on leptin-induced hyperthermia

Mice injected i.c.v. with leptin showed a transient increase in T_b, which persists beyond the initial stress spike and remains elevated over vehicle-treated mice for several hours (see Figure 5.8). Co-administration of $PACAP_{6-38}$ to block endogenous PACAP thermogenic pathways significantly reduces the magnitude of the response to leptin, while antagonist alone has no effect on T_b (data not shown). Analysis of the period between 1 and 6 hours post injection (to eliminate the initial spike) shows a significant elevation in T_b (+1.0 ±0.19°C compared to controls) in the leptin-injected group, which is significantly reduced almost to control levels in the co-injected group. Since the 24

hour mean level of neither parameter was significantly different from controls we can assume that any effects of this dose of leptin on energy expenditure are short term.

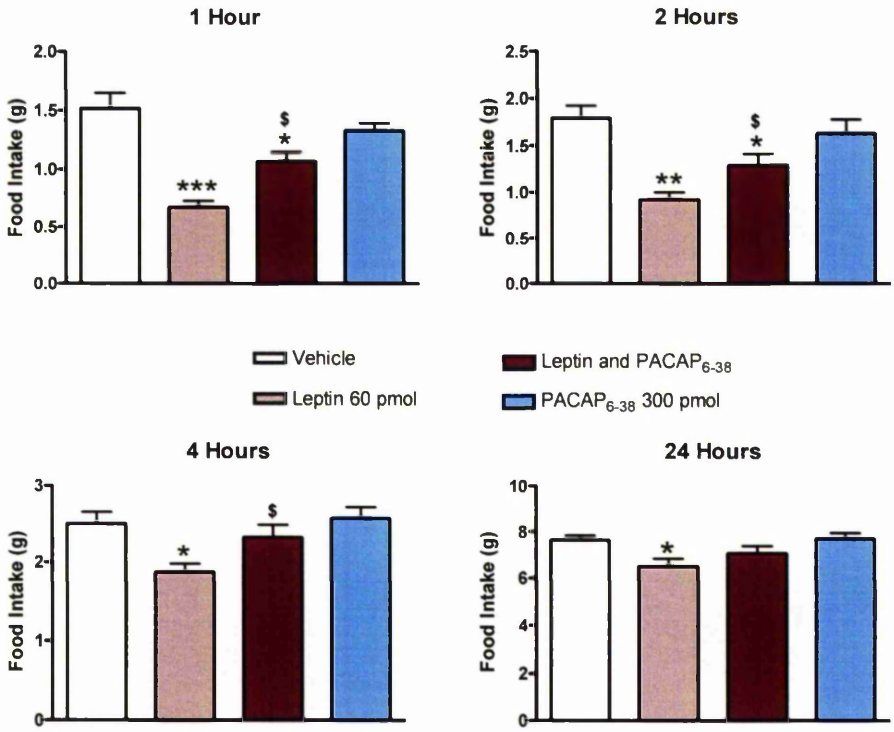


Figure 5.7 Leptin and $PACAP_{6-38}$ co-administration: food intake

The effect of co-administration of $PACAP_{6-38}$ on i.c.v. leptin-induced hypophagia at 1, 2, 4 and 24 hours post injection. Groups: vehicle (sterile isotonic saline; n= 6), 60 pmol leptin (n=6), 60 pmol leptin + 300 pmol $PACAP_{6-38}$ (n=6), 300 pmol $PACAP_{6-38}$ (n=5). Mean ± SEM. * P<0.05, **P<0.01; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with Vehicle, $compared with leptin only.

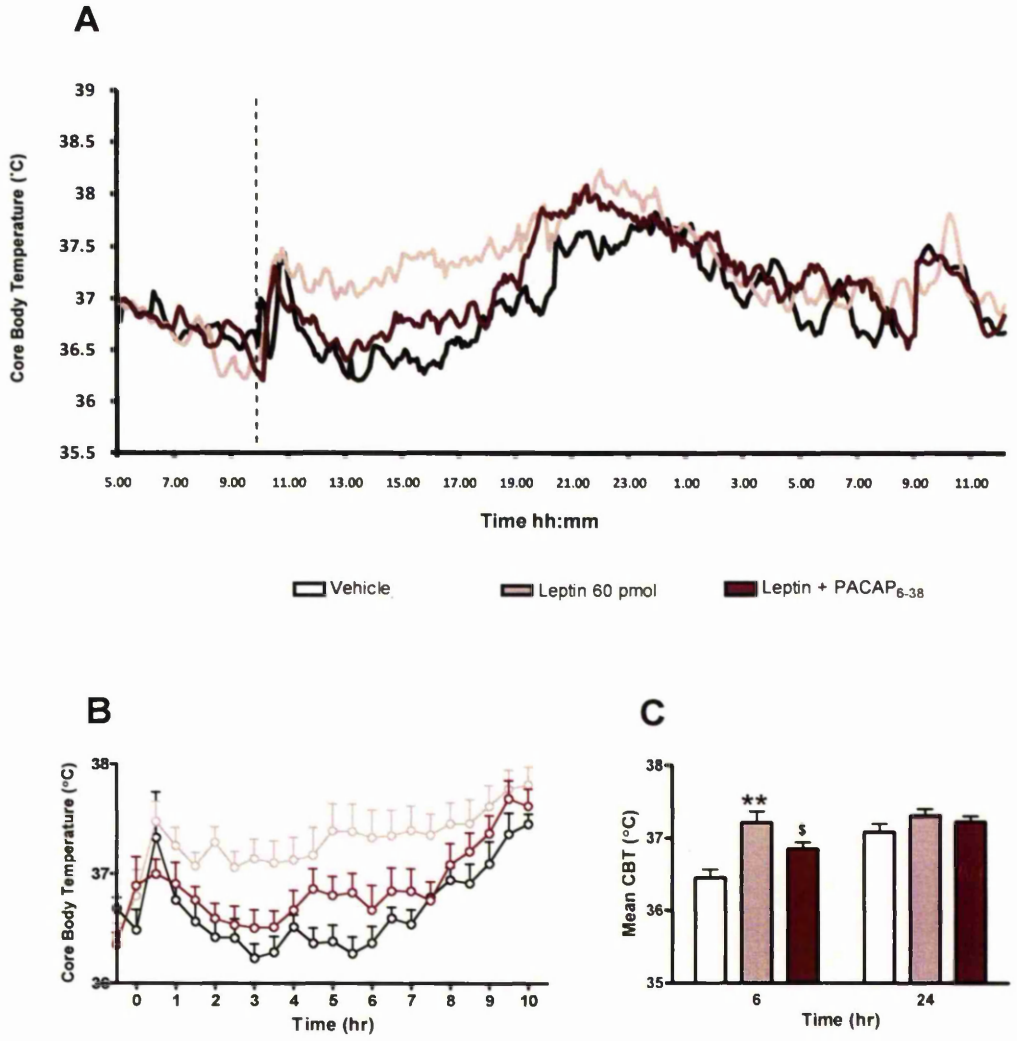


Figure 5.8 PACAP and $PACAP_{6-38}$ co-administration: core-body temperature

Effect of co-administration of $PACAP_{6-38}$ on i.c.v. 60 pmol leptin-induced hyperthermia. Groups: vehicle (sterile isotonic saline; n= 8), 60 pmol leptin (n=8), 60 pmol leptin + 300 pmol $PACAP_{6-38}$ (n=11), 300 pmol $PACAP_{6-38}$ (n=10; not shown). **A.** Mean T_b 5 hours pre and 24 hours post injection. **B.** Mean ± SEM T_b 10 hours post injection. **C.** Mean ± SEM T_b during 1-6 hours post injection and 1-24 hours post injection. **P<0.01; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle control, $compared with leptin-injected group.

5.4 DISCUSSION

Neuro-anatomical studies have led to the belief that the Arc is the main target for processing leptin's anti-obesity effects (Saper et al., 2002; Schwartz and Porte, 2005; Spiegelman and Flier, 2001). Although the data supporting this view are persuasive, they are challenged by the fact that mice lacking lepr-B expression in POMC cells develop only moderate obesity, and mice lacking lepr-B in SF1 cells are equally obese (reviewed in Balthasar, 2006). These observations imply a quantitatively similar contribution of POMC and SF1 cells to the leptin-mediated regulation of body weight. This revelation has re-established the VMN as a key player in energy balance and as a fundamental leptin target. Electrophysiology and immunohistochemistry confirm that SF1 cells in the VMN are a target for leptin (Dhillon et al., 2006), however, since SF1 is not a releasable molecule it cannot itself participate in downstream signalling pathways. Therefore, it is reasonable to assume that leptin alters expression or release of catabolic effectors which are co-expressed with SF1. In chapter 4 we demonstrated that exogenous PACAP has anorexigenic, thermogenic and lipolytic effects, similar to those observed following leptin administration. In this chapter we sought to establish whether PACAP cells of the VMN might be a first-order target for leptin.

VMN PACAP neurones are a direct target for leptin

We found that SF1 and PACAP mRNA are tightly co-localised in the VMN, especially in the lepr-B-rich dorsomedial region, implicating PACAP as a potential leptin target. These histological data support the proposal that PACAP cells in the VMN have the ability to respond to leptin, although whether this is the only mechanism by which leptin impinges on these cells is currently unclear. To shed light on this, we examined PACAP mRNA levels in pre-obese SF1Cre lepr$^{flox/flox}$ mice, which lack lepr-B expression specifically in SF1 neurones of the VMN. These mice go on to exhibit obesity, particularly when challenged with high-energy diet, partly due to deficiencies in adaptive thermogenesis, the compensatory increase in sympathetic drive to BAT (Dhillon et al., 2006). We found that a specific loss of leptin signalling in SF1 cells of the VMN decreases local PACAP expression, despite Arc-mediated leptin signalling being unaffected. The magnitude of this deficiency is around half that observed in *ob/ob* mice which lack universal leptin signalling, and manifest a far more severe metabolic phenotype. This could imply that leptin acts on PACAP cells both directly and indirectly, via other central targets, perhaps even involving non-SF1 VMN cells. Xu and colleagues (2003) recently demonstrated that the MC_4R agonist MTII was able to

partially prevent fasting-induced reductions in BDNF expression, leading to the conclusion that BDNF acts downstream of MC_4R. With this in mind, it might also be valuable to investigate whether manipulating MC_4R tone also affects VMN PACAP expression. Furthermore, although SF1 is a reasonable marker of leptin-responsive neurones, it is not ideal. Preferably we would like to demonstrate that PACAP cells are co-localised with lepr-B and that they express markers such as SOCS-3, pSTAT3 and c-*fos* following peripheral leptin administration. Long term we should also show using electrophysiology on identified PACAP neurones that it is definitely a direct action. This would demonstrate unequivocally that PACAP cells of the VMN are activated by circulating leptin.

PACAP has effects on food intake and body temperature downstream of leptin

Leptin exerts its effects on food intake partly by acting on first order neuropeptidergic cells in the hypothalamus. These include POMC and NPY in the Arc, orexin in the LH and CRH in the PVN (Schwartz et al., 2000). Since we have shown that VMN PACAP cells almost certainly have the ability to respond to leptin, we next sought to establish whether PACAP-induced changes in energy balance are separate, or correlated with leptin-effects. The effects of central PACAP injection are remarkably similar to those observed when mice are given leptin, leading us to speculate that the two are linked. In agreement with this, we found that by blocking central PACAP receptors with $PACAP_{6-38}$ we reduced the potency of leptin-induced anorexia by approximately half. This attenuation was similar in magnitude to that observed when α-helical CRH, a CRHR antagonist is co-applied with leptin (Gardner et al., 1998; Uehara et al., 1998), and provides strong evidence that PACAP receptors form part of leptin's downstream anorectic pathway. By comparison though, the anorectic reversal properties of $PACAP_{6-38}$ were not as powerful as those of SHU9119 (Seeley et al., 1997), in agreement with the notion that the Arc is the primary mediator of leptin-induced hypophagia.

In addition to reducing food intake, we found a single central leptin injection also caused a transient increase in core-body temperature, similar to that seen following central PACAP injection. Leptin is known to increase sympathetic activation of BAT and expression of UCP1 in this tissue (Collins and Surwit, 1996; Haynes et al., 1997; Hwa et al., 1997), however, the downstream players in this function are far less well understood than the effects on food intake. As such there are currently no data showing reversal of the acute hyperthermic response to leptin by specific antagonists. We have shown that blockade of PACAP receptors almost completely prevents leptin-

induced hyperthermia, indicating that PACAP is a key mediator of this response. This suggests that PACAP cells may play a far more relevant role in the increased sympathetic tone in response to leptin, and should be investigated more fully.

Together these data suggest that PACAP is likely to be a novel leptin target which partakes both in acute feeding and thermogenic responses. To confirm that PACAP is involved in the sympathetic activation of BAT, it might be interesting to investigate whether $PACAP_{6-38}$ also blocks leptin-induced increases in UCP1 expression in this tissue. Also, to further confirm a role for PACAP downstream of leptin we plan to investigate whether leptin elicits its effects on food intake and thermogenesis in PACAP receptor knockout strains detailed in chapter 4.

5.5 SUMMARY

- PACAP is co-localised with SF1 in cells of the VMN, particularly in the dorsomedial, central, rostral and caudal portions. Many cells in the ventrolateral subdivision express PACAP only.

- Genetic deletion of Lepr-B in SF1 cells significantly reduces PACAP expression in the VMN, but not the medial habenula.

- $PACAP_{6-38}$ partially blocks leptin-induced hypophagia

- $PACAP_{6-38}$ almost fully blocks leptin-induced hyperthermia

5.6 CONCLUSIONS

The catabolic peptide PACAP represents a strong candidate for the elusive VMN-expressed leptin target. PACAP mRNA expression is tightly co-localised with that of SF1, a proxy indicator of leptin-excited VMN neurones, indicating that that these cells are almost certainly directly activated by leptin. Moreover, we previously showed that PACAP expression in the VMN is correlated with leptin levels and this is reiterated by the observation that specific loss of VMN leptin input causes local suppression of PACAP transcription. Finally, blocking endogenous PACAP receptors using $PACAP_{6-38}$ (previously shown to block PACAP-induced metabolic effects) significantly attenuates leptin's acute effects on both food intake and core-body temperature, indicating that endogenous PACAP pathways are essential mediators of acute leptin responses.

CHAPTER 6

PACAP'S DOWNSTREAM TARGETS

6.1 INTRODUCTION

In Chapters 4 and 5 of this thesis it was demonstrated that a single i.c.v. injection of PACAP causes changes in both energy intake and energy expenditure and that these effects are associated with those of leptin. Furthermore, our *in situ* hybridisation studies (see Chapter 3) show that PACAP mRNA expression, specifically in the VMN, is generally correlated negatively with appetite and positively with metabolic rate, reiterating a physiological role for this peptide. Relatively little is known however about the mechanisms by which PACAP may impinge on energy balance pathways and the identity of its downstream targets. PACAP is a potent stimulator of adenosine 3'5' cyclic monophosphate (cAMP) production (Miyata et al., 1989; Tatsuno et al., 2001; Vaudry et al., 2000) and of cAMP response element binding protein (CREB) phosphorylation, resulting in gene transcription (Schomerus et al., 1994). Genes whose transcription is regulated by CREB include the immediate early gene c-*fos*, and many neuropeptides such as BDNF, somatostatin, and CRH, some of which may modulate PACAP's effects on energy balance.

The results presented in Chapter 4 indicate that PACAP's effects on energy homeostasis are likely to be mediated primarily by the PACAP-specific PAC_1 receptor, as VIP, a co-activator of the VPAC receptors does not share its hypophagic and hyperthermic properties. Expression of PAC_1 is widespread throughout the brain, including regions which have been implicated in energy balance regulation (Joo et al., 2004). Of particular interest are the Arc and PVN, key homeostatic regulatory nuclei which have reciprocal connections with the VMN where the majority of hypothalamic PACAP expressing neurones are found. Also of interest is PAG (also termed mid-central grey) of the midbrain, which has been identified in a number of studies as a regulator of sympathetic function, particularly of BAT thermogenesis. Maekawa and collegues (2005) recently showed that a large proportion of PACAP, but not BDNF-positive neurones of the VMN terminate onto cells in the PAG, suggesting a functional pathway between the two. Furthermore, cells of the PAG express all three PACAP receptors and have been identified by autoradiography as a ^{125}I-PACAP binding site (Masuo et al., 1992). Considering the severely thermogenically-compromised PACAP knockout mouse (Gray et al., 2002), and PACAP's potent effect on body temperature, further investigation of interactions between centrally released PACAP and thermogenic pathways is reasonable.

Objectives:

To identify PACAP-responsive brain areas by c-Fos induction

c-Fos protein is now widely accepted as a marker of neuronal activation in response to receptor binding (Luckman et al., 1994), and has proved an invaluable tool for determining the downstream targets of many signalling molecules. While several groups have individually examined the Arc (Mounien et al., 2006; Mounien et al., 2008), PVN (Agarwal et al., 2005; Legradi et al., 1998) and PAG, amongst others, (Maekawa et al., 2006) as potential PACAP targets, more extensive studies have not yet been described. We aim to carry out c-Fos immunohistochemistry of the mouse forebrain, midbrain and brainstem following a single central PACAP injection at a dose previously shown to affect food intake and core-body temperature (see Chapter 4). Since PACAP is a pleiotropic peptide, and its receptors are extensively expressed in the CNS (Joo et al., 2004), we might expect many regions of the brain to be activated. For the purpose of this study however, only brain regions known to modulate appetite and/or feeding behaviour and thermogenesis will be discussed in detail.

To test whether CRHR or MC_4R antagonists can block PACAP-induced hypophagia

CRH and POMC are two well-characterised anorexigenic neuropeptides which have previously been suggested as potential central mediators of the PACAP-induced hypophagic response. Indirect evidence for interactions between PACAP and CRH neurones of the PVN is strong, and functionally both peptides have been implicated in certain stress responses in addition to effects on food intake (Nussdorfer and Malendowicz, 1998). Neurones of the PVN densely express the PACAP-specific PAC_1 receptor (Nomura et al., 1996), and PACAP immuno-positive terminals directly contact CRH cell bodies of the PVN (Legradi et al., 1998). Furthermore, Agarwal and colleagues (2005) demonstrated that central PACAP injection induces c-Fos expression and CREB phosphorylation in CRH neurones in the rat PVN. In cultured hypothalamic cells PACAP potently stimulates CRH transcription (Kageyama et al., 2007), while *in vivo* PACAP injection rapidly induces, and $PACAP_{6-38}$ reduces CRH mRNA in the rat PVN (Grinevich et al., 1997). Furthermore, pharmacological studies in goldfish (Maruyama et al., 2006) and chick (Tachibana et al., 2004) have also demonstrated that the anorexigenic effects of PACAP are attenuated by pre-treatment with CRH antagonists. Together these data imply that the two peptides converge to form part of the same central pathway.

Evidence for an interaction between PACAP and the hypothalamic melanocortin pathway has also been demonstrated, largely by the work of Vaudry and colleagues. In 2006 this laboratory demonstrated that around 50 % of POMC cells of the Arc express either the PAC_1 or $VPAC_2$ receptor and that PACAP induces POMC expression in hypothalamic explants (Mounien et al., 2006). The latter observation is in line with the earlier work (Aoki et al., 1997; Boutillier et al., 1994) which demonstrated that PACAP is capable of stimulating POMC promoter activity in cultured corticotroph cells via a cAMP-dependent mechanism. Recently, Vaudry's group reported that i.c.v. PACAP induces c-*fos* mRNA expression in POMC neurones, and increases POMC mRNA expression as measured by RT-PCR (Mounien et al., 2006). Furthermore, they were able to partially block effects of PACAP on food intake by pre-treatment with SHU9119, a competitive MC_4R antagonist (Mounien et al., 2008). To determine whether CRH and POMC neurones are downstream components of PACAP feeding pathways, we will use *in vivo* pharmacology to test whether i.c.v. co-administration of the CRH receptor antagonists α-helical CRH and astressin, or the MC_4R antagonists SHU9119 and AgRP can prevent PACAP-induced anorexia.

6.2 METHODS

Animals for c-Fos immunohistochemistry

Adult male CD1 mice were i.c.v. cannulated as detailed in the general methods chapter and allowed to recover for 1 week. Prior to the experiment animals were handled daily to minimise c-Fos induction attributed to stress, which may mask drug effects. During the light phase mice then received a single i.c.v. injection of either 30 pmol PACAP or isotonic saline and were left in quiet conditions for 90 minutes. Animals were then perfused transcardially with heparinised 0.9 % saline followed by 4 % paraformaldehyde as detailed in the general methods section and the brains collected for immunohistochemistry.

c-Fos immunohistochemistry

30 μm coronal sections were cut using a freezing sledge microtome and collected into 0.1M PB. Endogenous peroxidase activity was deactivated by incubating free-floating sections in 1.5 % hydrogen peroxide and 20 % methanol in 0.1 M PB for 20 minutes at RT. Sections were then washed briefly in 0.1 M PB and non-specific staining was blocked by incubation in 5 % normal goat serum (NGS) blocking serum in 0.1 M PB/ 0.3 % Triton X-100 (PBT) for 1 hour at RT. Sections were then incubated overnight at 4 °C in rabbit anti-c-Fos antibody (Oncogene Scientific Incorporated, USA) diluted 1:10,000 in 1 % NGS blocking serum. The following day excess antibody was removed by washing sections in 0.1 M PBT. Sections were incubated for 2 hours in biotinylated anti-rabbit IgG raised in goat (Vector Laboratories Incorporated, USA) diluted 1:500 in 5 % NGS blocking serum at RT. This was followed by 0.1 M PB washes and a further incubation for 1 hour in streptavidin-biotin horseradish peroxidase complex (Amersham Biosciences, UK) diluted 1:500 in 0.1 M PB. The bound antibody-peroxidase complex was visualised using nickel-intensified diaminobenzidine (DAB; Vector Laboratories, UK) following kit instructions. The reaction was followed using a microscope and terminated by repeatedly rinsing in tap water. Sections were mounted from distilled water onto glass microscope slides coated with 1 % gelatine, and coverslipped using xylene-based mountant (Ralmont, BDH UK), left to dry and observed using an Axiovision widefield microscope. c-Fos positive nuclei in selected brain regions were counted by eye and expressed as mean ± SEM for each treatment group.

Dose responses of α-helical CRH, astressin, SHU9119 and AgRP

Daytime free-feeding experiments were performed using dose ranges of each antagonist. Food was removed at 8:00 on the day of the experiment and mice received a single 1 µl i.c.v. injection at 10:00. Food was immediately returned and food intake monitored for 1 hour post injection. Chosen doses were based on previous reports and were as follows: α-helical CRH 0, 0.1, 0.3, 0.6 nmol; astressin 0, 0.3, 0.6, 1.0 nmol; SHU9119 0, 1.0, 3.0, 6.0 nmol; or AgRP 0, 40, 60, 80 pmol all in sterile isotonic saline (n=4-6 per group). The highest dose which did not significantly increase food intake was selected for co-injection with PACAP. These experiments were kindly performed by Irena Reynolds, a technician in the Luckman laboratory.

Co-injection of PACAP and CRH / MC_4R antagonists

Individual fast-induced re-feeding experiments were performed as described in the general methods chapter, with mice receiving a single 1 µl i.c.v. injection containing either 30 pmol PACAP only, antagonist only or 30 pmol PACAP + antagonist in isotonic saline. Controls received saline only (n=5-14 per group). Doses of antagonist were as follows: α-helical CRH 0.3 nmol, astressin 0.6 nmol, SHU9119 0.3 nmol initially (repeated using 0.6nmol), AgRP 60 pmol.

Confirmation of SHU9119 efficacy

A fast-induced re-feeding experiment was performed as described in the general methods chapter, with mice receiving a single 1 µl i.c.v. injection containing either 0.1 nmol MTII only, 0.1 nmol MTII + 0.3nmol SHU9119, 0.1 nmol MTII + 0.6nmol SHU9119 or vehicle (isotonic saline) (n=6 per group).

6.3 RESULTS

PACAP-induced cFos induction in the brain

c-Fos immunoreactivity (IR) was detected in large quantities throughout the hypothalamus and peri-aqueductal region of the midbrain following PACAP administration. Very little induction was identified in the cortex, hippocampus, thalamus or brainstem. In the hypothalamus, quantitative analysis of the number of c-Fos positive nuclei revealed significant increases in the PVN, the periventricular nucleus (PeV), and the DMN, while counts in the Arc, LH and VMN were not significantly different from controls. Significant activation in response to PACAP was also detected in the central, but not medial nucleus of the amygdala. One of the most strongly activated regions was the PAG of the midbrain, where both rostral and, to a greater extent, caudal portions showed significantly higher c-Fos counts in the PACAP-treated mice compared with saline-treated controls. No differences were found in the area postrema (AP) or NTS of the brainstem between the two groups (see Figure 6.1). Representative images of brain regions where statistically significant differences were detected are shown in Figure 6.2.

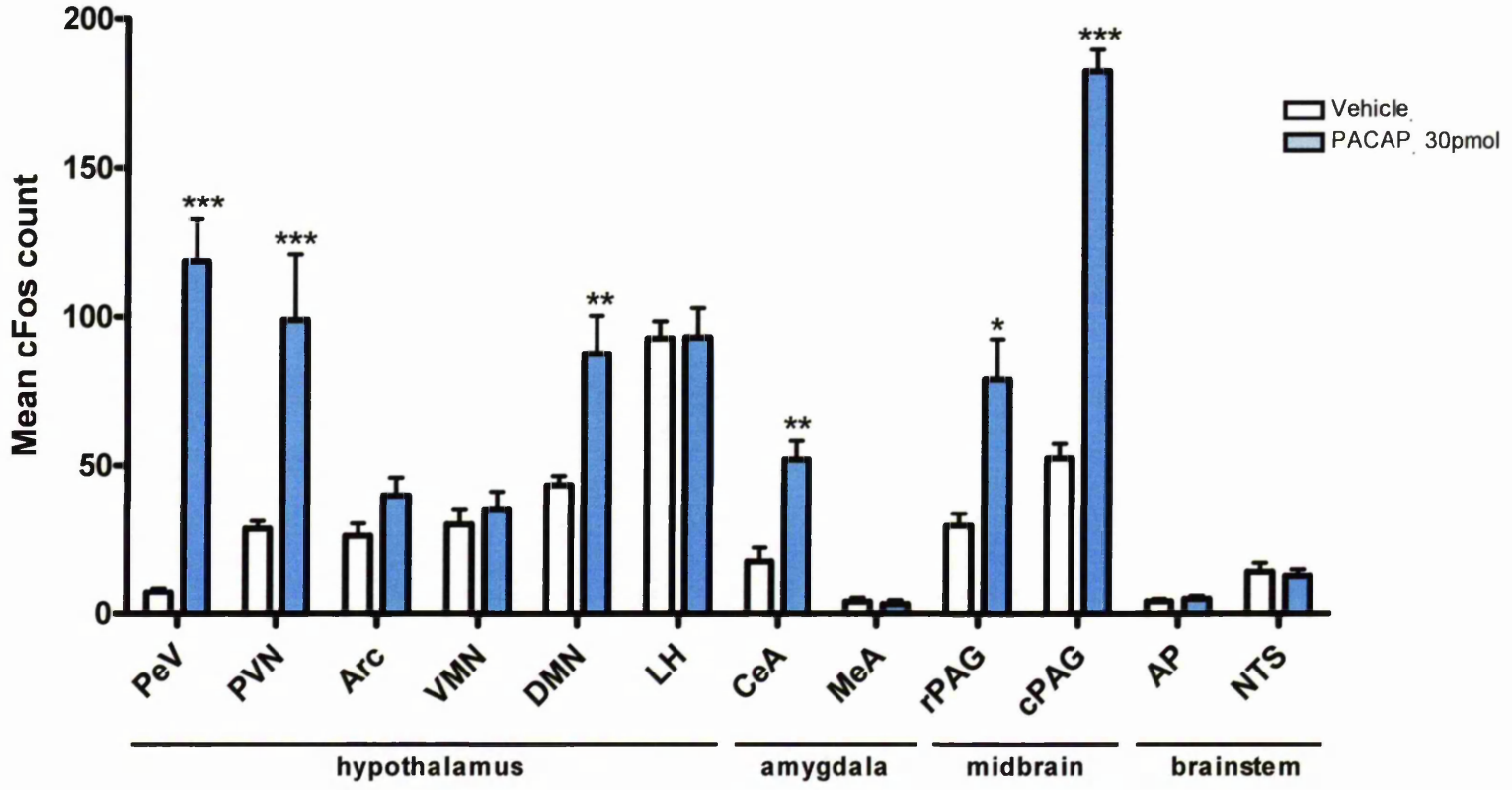


Figure 6.1 c-Fos summary

Analysis of c-Fos induction in areas of the hypothalamus, amygdala, midbrain and brainstem of mice which received saline or 30 pmol PACAP i.c.v. Periventricular nucleus (PeV), paraventricular nucleus (PVN), arcuate nucleus (Arc), ventromedial nucleus (VMN), lateral hypothalamus (LH), central amygdala (CeA), medial amygdala (MeA), rostral peri-aqueductal grey (rPAG), caudal peri-aqueductal grey (cPAG), area postrema (AP) and nucleus of the solitary tract (NTS).Data are expressed as Mean ± SEM. * P<0.05, **P<0.01, ***P<0.001. Data were analysed using the Student's unpaired *t*-test.

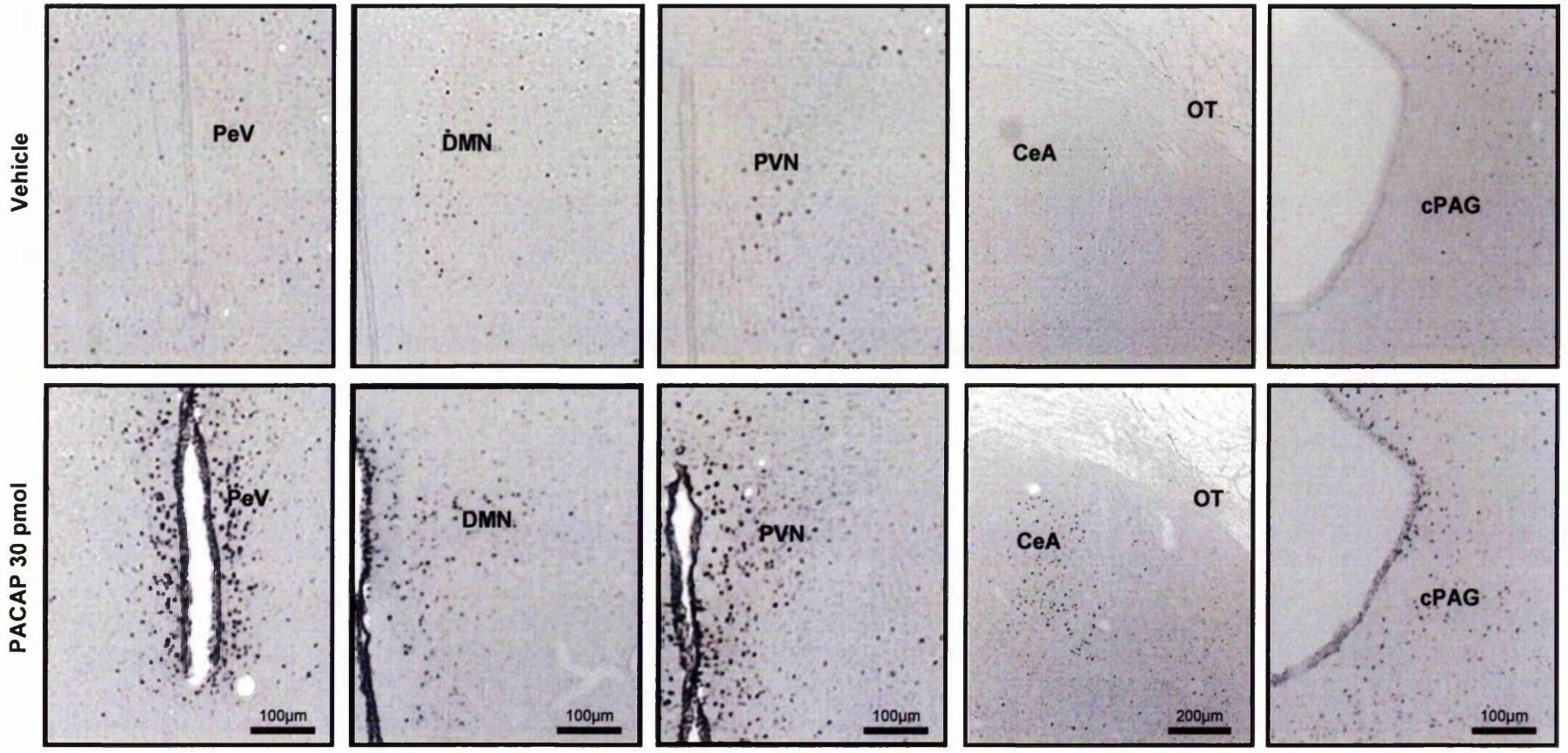


Figure 6.2 Brain areas showing significant c-Fos induction in response to PACAP

Representative images showing c-Fos induction in the periventricular nucleus (PeV), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), central amygdala (CeA), and caudal periaqueductal grey (cPAG) following i.c.v. injection of either saline (upper row) or 30 pmol PACAP (lower row).

Effect of CRH and MC_4 receptor antagonists on food intake

Daytime feeding experiments were performed in non-fasted mice to identify the orexigenic dose-threshold for each of the antagonists. Endogenous tone on anorexigenic receptors under daytime conditions is likely to be high (since mice habitually feed at night) and, as such, administration of antagonists may elicit an independent feeding effect. For the purpose of the co-injection experiments we opted to use the highest sub-threshold dose for an antagonist alone, which should block receptor activation, but not result in false positives caused by independent effects. Using this method, we can deduce that any attenuation of PACAP-induced anorexia observed is due to interruption of a downstream pathway. In all four drugs tested, only the highest dose caused a significant increase in food intake (see Figure 6.3). The dose below this was therefore selected in all cases. Chosen doses were: α-helical CRH 0.3 nmol, astressin 0.6 nmol, SHU9119 0.3 nmol, AgRP 60 pmol.

Effect of CRH and MC_4 receptor antagonists on PACAP-induced hypophagia

We found that the effects of the two CRH antagonists were similar, with 0.6 nmol of astressin (see Figure 6.4) and 0.3 nmol α-helical CRH (see Figure 6.5) significantly reducing the hypophagic response to PACAP. This partial attenuation was significant at 1, 2 and 4 hours post injection, whereas by 24 hours there were no differences between any groups. Neither antagonist significantly affected food intake when administered alone. Unlike astressin and α-helical CRH there were striking differences between the effects of the two MC_4R antagonists in their abilities to block PACAP-induced hypophagia. We found that 60 pmol AgRP completely prevented the reduction in food intake in response to PACAP (see Figure 6.6) while in contrast, 0.3 nmol SHU9119 had no effect on the magnitude of anorexia in response to PACAP (see Figure 6.7). In light of this discrepancy, we felt it necessary to confirm effective doses of SHU9119 by co-administration with an anorexigenic dose of the MC_4R agonist MTII. We found that 0.3 nmol SHU9119 caused a partial reduction, whereas 0.6 nmol completely prevented MTII-induced hypophagia (see Figure 6.8). This observation demonstrates that the original dose of SHU9119 is insufficient for effective MC_4R antagonism. However, even the higher dose of 0.6 nmol SHU9119 was unable to block the anorexic response to PACAP when co-injected centrally (see Figure 6.9).

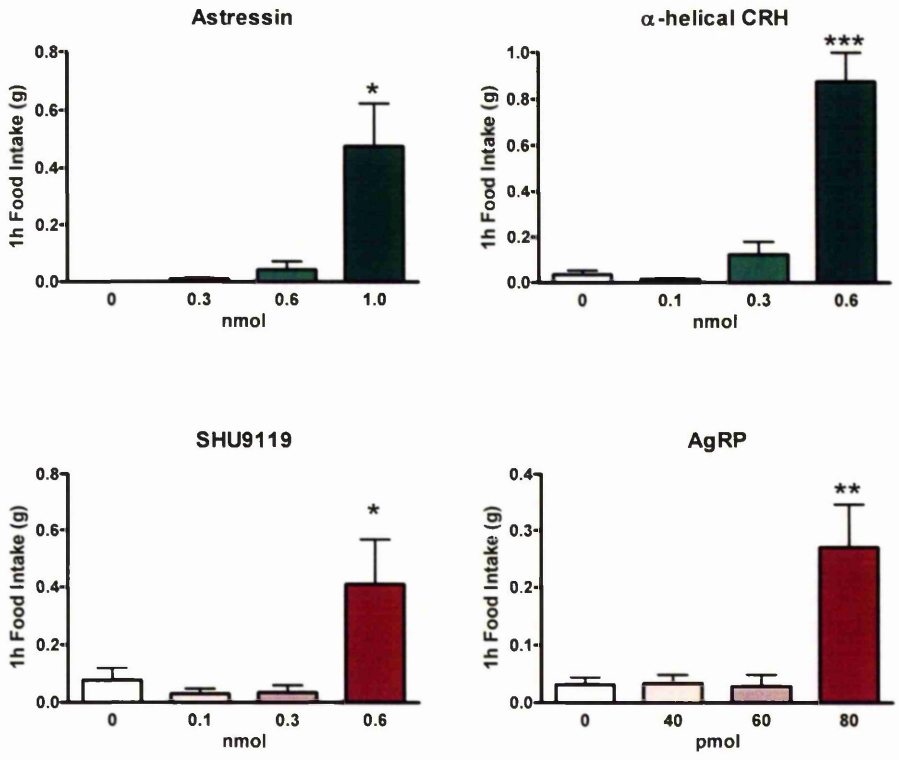


Figure 6.3 Antagonist dose responses

Effect of various doses of antagonists against CRH receptors, astressin and α-helical CRH (green) and MC_4 receptor, SHU9119 and AgRP (pink) on daytime free feeding at 1 hour post injection. Mean ± SEM. * P<0.05, **P<0.01, ***P<0.001 all compared with vehicle; One-way ANOVA with Dunnet's *post hoc* test.

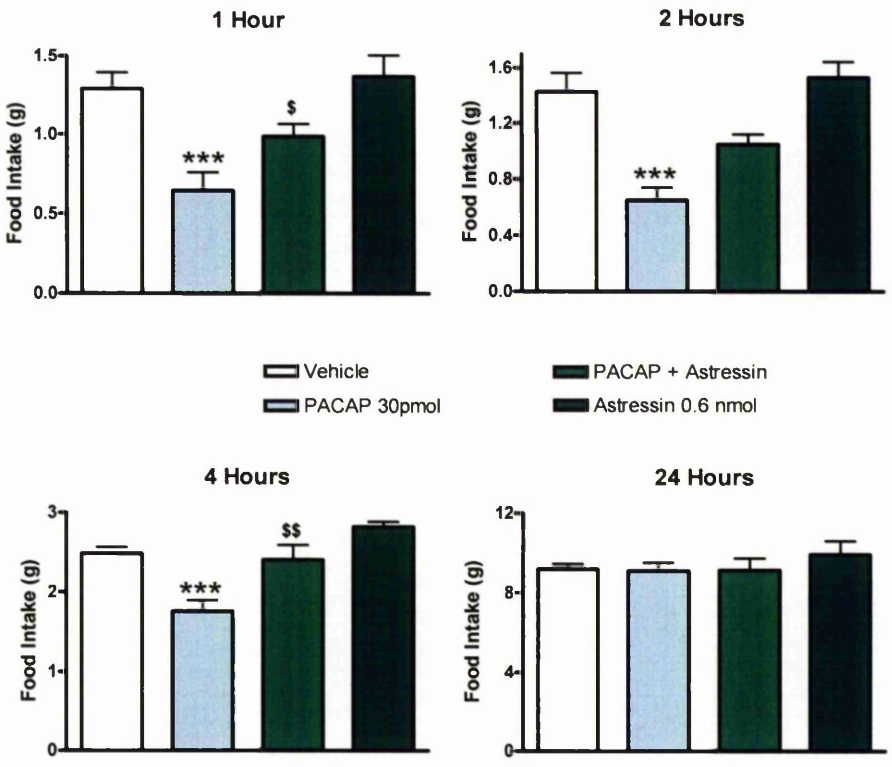


Figure 6.4 Co-administration of PACAP and astressin

The effect of co-administration of astressin on i.c.v. PACAP-induced hypophagia at 1, 2, 4 and 24 hours post injection. Groups: vehicle (sterile isotonic saline; n= 9), 30 pmol PACAP (n=5), 30 pmol PACAP + 0.6 nmol astressin (n=5), 0.6 nmol astressin (n=4). Mean ± SEM. * P<0.05, **P<0.01, ***P<0.001; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle, $compared with PACAP only.

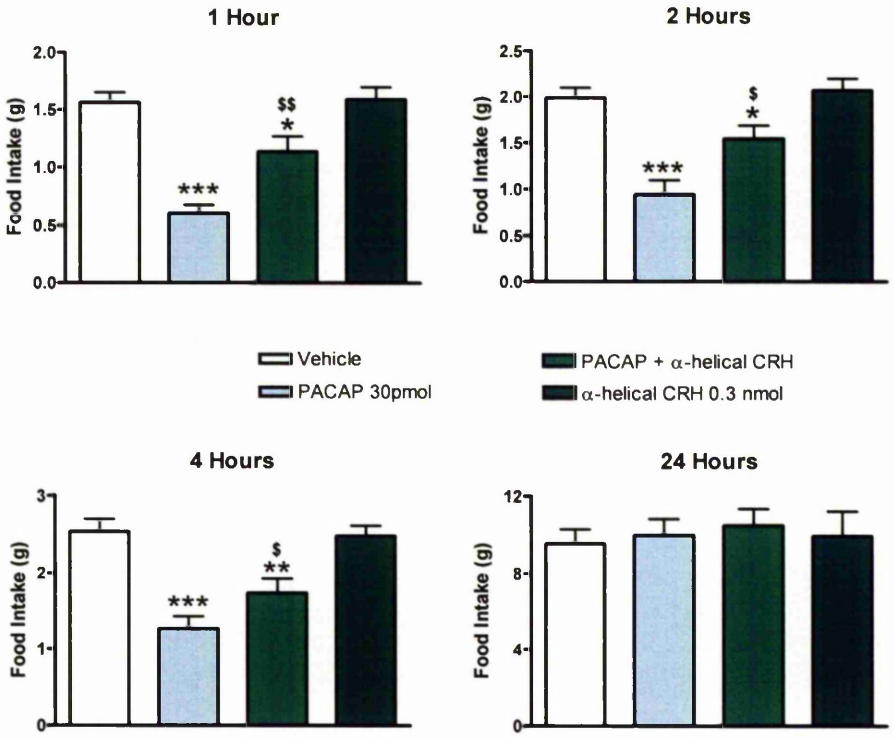


Figure 6.5 Co-administration of PACAP and α-helical CRH

The effect of co-administration of α-helical CRH on i.c.v. PACAP-induced hypophagia at 1, 2, 4 and 24 hours post injection. Groups: vehicle (sterile isotonic saline; n= 16), 30 pmol PACAP (n=9), 30 pmol PACAP + 0.3 nmol α-helical CRH (n=9), 0.3 nmol α-helical CRH (n=6). Mean ± SEM. * P<0.05, **P<0.01, ***P<0.001; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle, $compared with PACAP only.

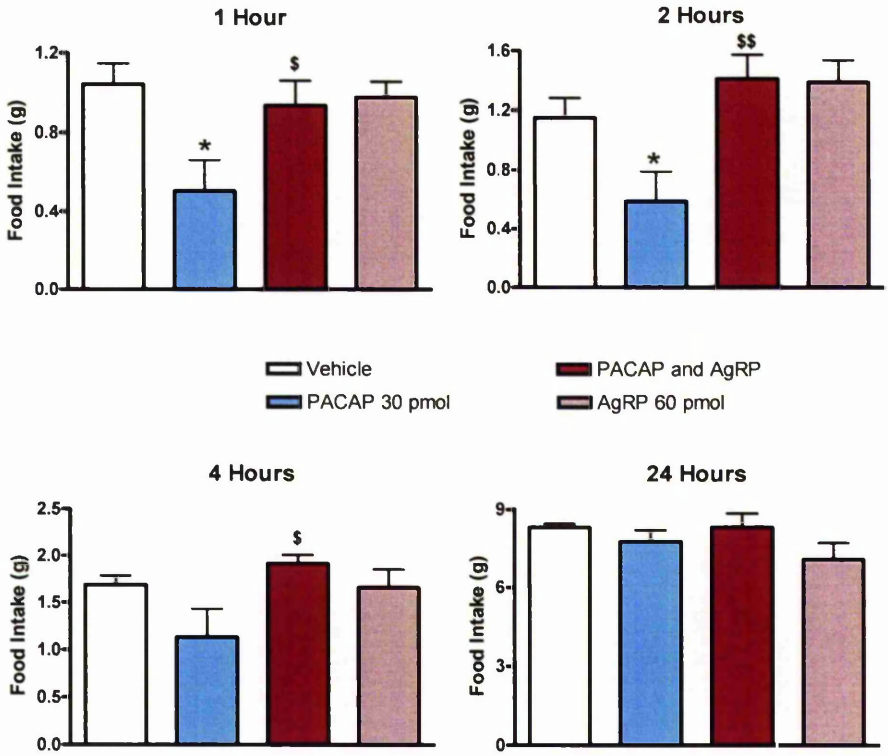


Figure 6.6 Co-administration of PACAP and AgRP

The effect of co-administration of AgRP on i.c.v. PACAP-induced hypophagia at 1, 2, 4 and 24 hours post injection. Groups: vehicle (sterile isotonic saline; n= 6), 30 pmol PACAP (n=5), 30 pmol PACAP + 60 pmol AgRP (n=6), 60 pmol AgRP (n=6). Mean ± SEM. * $P<0.05$; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle, $compared with PACAP only.

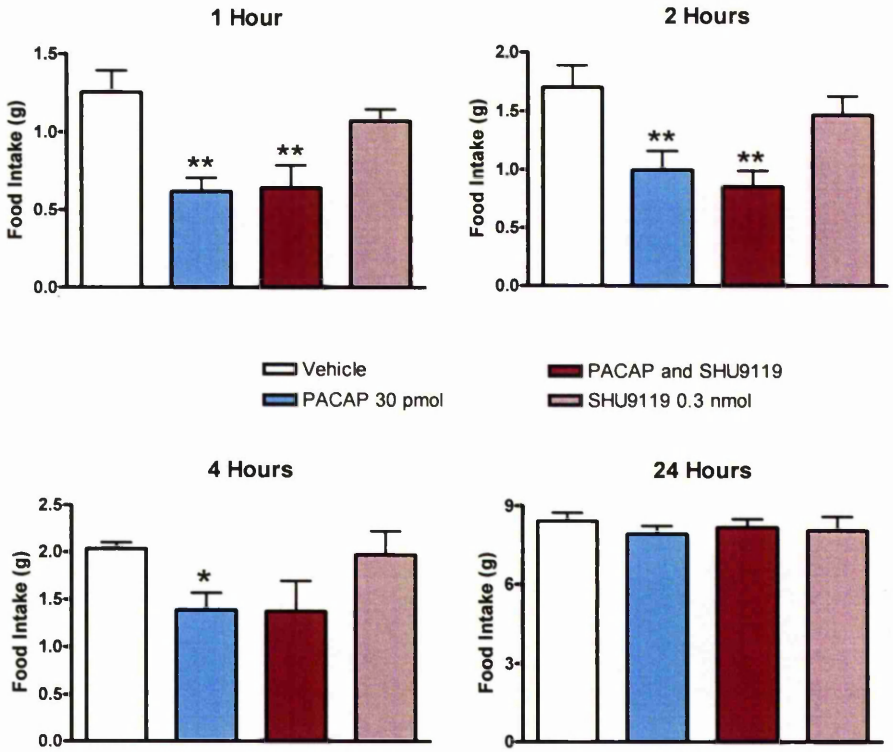


Figure 6.7 Co-administration of PACAP and SHU9119 (0.3 nmol)

The effect of co-administration of SHU9119 on i.c.v. PACAP-induced hypophagia at 1, 2, 4 and 24 hours post injection. Groups: vehicle (sterile isotonic saline; n= 6), 30 pmol PACAP (n=6), 30 pmol PACAP + 0.3 nmol SHU9119 (n=5), 0.3 nmol SHU9119 (n=6). Mean ± SEM. * P<0.05, **P<0.01; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle

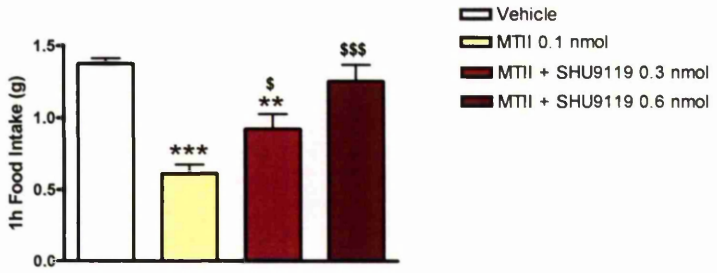


Figure 6.8 Co-administration of MTII and SHU9119

The effect of co-administration of SHU9119 on i.c.v. MTII-induced hypophagia at 1 hour post injection. Groups: vehicle (sterile isotonic saline; n= 6), 0.1 nmol MTII (n=6), 0.1 nmol MTII + 0.3 nmol SHU9119 (n=6), 0.1 nmol MTII + 0.6 nmol SHU9119 (n=6). Mean ± SEM. * P<0.05, **P<0.01, ***P<0.001; One-way ANOVA with Newman-Keul's *post hoc* test to compare all groups. *compared with vehicle, $ compared with MTII alone.

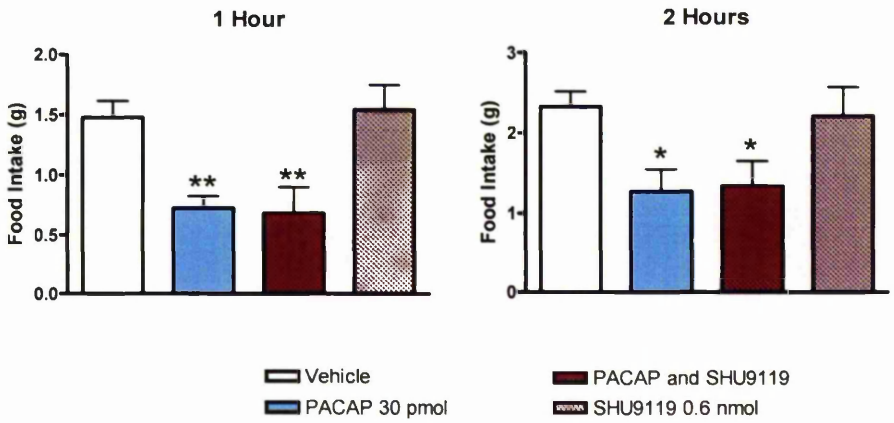


Figure 6.9 Co-administration of PACAP and SHU9119 (0.6 nmol)

The effect of co-administration of SHU9119 on i.c.v. PACAP-induced hypophagia at 1 and 2 hours post injection. Groups: Vehicle (sterile isotonic saline; n= 6), 30 pmol PACAP (n=6), 30 pmol PACAP + 0.6 nmol SHU9119 (n=5), 0.6 nmol SHU9119 (n=6). Mean ± SEM. * P<0.05, **P<0.01; One-way ANOVA with Newman-Keul's *post-hoc* test to compare all groups. *compared with Vehicle

6.4 DISCUSSION

Does PACAP interact with CRH?

We found that central PACAP injection caused the most substantial c-Fos induction in the PVN and CeA, the two major brain areas of CRH expression (Makino et al., 1994). Earlier work showed that cells of the PVN and CeA strongly express the PACAP-specific PAC_1 receptor (Hashimoto et al., 1996; Joo et al., 2004; Nomura et al., 1996) and that PACAP immuno-positive terminals densely, and specifically, innervate these areas (Koves et al., 1991; Legradi et al., 1997b; Piggins et al., 1996). Furthermore, central PACAP injection alters the expression levels of CRH mRNA in the PVN of both rats (Grinevich et al., 1997) and mice (Mounien et al., 2008). This anatomical and histological evidence suggests a physiological connection between the PACAP and CRH systems and in the current study we have confirmed that the anorexigenic action of PACAP in mice involves downstream signalling via CRH receptors, using *in vivo* pharmacology.

The biological actions of CRH are mediated via two receptors, CRH_1 and CRH_2 which exhibit distinct anatomical distribution and pharmacological specificity (Richard et al., 2002). While the well-characterised action of CRH on the HPA stress axis has been proposed to be predominantly CRH_1-mediated, it has been suggested that discrete feeding effects of CRH primarily involve CRH_2 (Steckler and Holsboer, 1999). In our study we used two CRHR antagonists, astressin and α-helical CRH to block endogenous receptor activity centrally *in vivo*. Binding studies have shown that although astressin binds with similar affinity to both receptors, α-helical CRH has preference for CRH_2 over CRH_1 (Perrin and Vale, 1999). Since both antagonists were able to significantly attenuate the degree of anorexia in response to central PACAP injection, this suggests that cessation of feeding by PACAP is not simply a secondary consequence of HPA activation. Furthermore, these results support the hypothesis that CRH cells of the PVN are a target for PACAP and that they are involved in mediating PACAP's effects on food intake (see Figure 6.10). However, because the *in vivo* effects of PACAP were only attenuated and not completely abolished by either CRH receptor antagonist, we cannot exclude the involvement of neural substrates other than CRH in the mediation of PACAP-induced anorexia.

Does PACAP interact with the melanocortin system?

Neuronal populations of the Arc are well characterised as key regulators of energy balance and it was recently shown that i.c.v. PACAP injection (220 pmol), significantly increases c-*fos* mRNA expression in this nucleus (Mounien et al., 2008). In our immunohistological study we found no significant change in c-Fos immuno-positive cells in the Arc following 30 pmol PACAP, although there was a trend to an increase. This discrepancy is likely to be due to a combination of dose effects, and differences in baseline receptor tone, since our animals were fed *ad libitum* throughout and Mounien's were 18-hour pre-fasted. Therefore the detection threshold in our paradigm may be higher and hence we may only be able to detect the strongest-activated brain areas. However, in light of the level of activation elsewhere in the brain, including in the PVN, our c-Fos results do not support the hypothesis put forward by Mounien that Arc neurones are an important target for PACAP-induced anorexia.

Current *in vivo* evidence for a role of POMC cells downstream of PACAP remains ambiguous. Genetic work carried out by Lowell and colleagues demonstrates that the effects of leptin on SF1 (which express PACAP; see Chapter 5) and POMC neurones are non-convergent since knockout of lepr-B in both cell types has an additive effect on the obesity phenotype compared with either single knockout (Dhillon et al., 2006). Moreover, while it was recently published that SHU9119 can block PACAP's anorexigenic effects (Mounien et al., 2008), we were not able to reproduce this result using an almost identical protocol. We found that co-injection of SHU9119 had absolutely no effect on the anorectic response to PACAP; even once we doubled the dose. However, we are confident that our data is sound for several reasons. Firstly, the results discussed above in which we co-injected CRH antagonists with PACAP provide a positive control for our experimental methodology. Secondly, we have confirmed that our PACAP dose does not induce anorexia by non-homeostatic means, whereas the dose used by Vaudry's group is an order of magnitude higher and hence likely to induce aversion. Thirdly, their total injection volume of 10 µl seems excessive when compared with similar studies, and might confound *in vivo* results (Leckstrom et al., 2008; Li et al., 2008; Man and Lawrence, 2008); especially considering mice have a CSF volume of just 35 µl (Pardridge, 1991). Finally, we were very careful to select sub-orexexigenic doses for all our antagonists, so as not to induce independent effects, and further confirmed our SHU9119 dose by antagonism of the MC_4R agonist MTII. The dose of SHU9119 used by Mounien is exceptionally low (18 pmol), and based on our findings would not be expected to block MC_4R activation *in vivo*. The absolute explanation for the inconsistency between the results is unresolved, but we are hesitant

to carry out a direct repeat of the Vaudry study due to the likelihood of adverse effects of increasing our PACAP dose and injection volume. One way to resolve this would be to carry out a dose-response experiment for PACAP to test whether it retains its anorexigenic properties when administered to MC_4R or central POMC-deficient (Huszar et al., 1997; Smart et al., 2007) mouse strains, although these are not currently maintained at University of Manchester.

That said, we also examined the ability of the endogenous MC_4R antagonist AgRP to block PACAP-induced attenuation of fast-induced re-feeding. There is strong evidence that AgRP, in contrast to SHU9119, functions independently as an inverse agonist, causing active down regulation of receptor tone. While anorectic peptides such as CRH, α-MSH and PACAP all stimulate cAMP production in target cells, AgRP causes an active decrease in such intracellular events upon MC_4R binding, rather than purely blocking receptor activation (Chai et al., 2003; Ollmann et al., 1997). Interestingly, and unlike any other antagonist tested, AgRP was able to fully reverse the effects of PACAP on feeding. However, considering our SHU9119 data, we do not judge that a direct relationship between PACAP and POMC should necessarily be inferred from this result. Instead we propose an alternative hypothesis, whereby actions of exogenous AgRP and PACAP converge on common catabolic target cells, resulting in 'cancelling out' of the anorexic effect. We believe these pathways are convergent, rather than independent, since the pre-fast will have reduced the tone on anorexigenic pathways, meaning AgRP would have little further effect if acting on non-PACAP targets. In support of this, AgRP alone under these conditions did not increase food intake. Therefore, we suggest that AgRP decreases the activation of MC_4R-expressing cells, which also respond to PACAP, resulting in a zero net effect on food intake (see Figure 6.11).

The complex networks which control food intake are only just beginning to be elucidated, and it is likely that many neurones express both MC_4 and PAC_1 receptors in the feeding circuits of the brain. One such identified cell type is the CRH expressing population of the PVN, which is becoming the strongest candidate for a downstream anorexigenic mediator of PACAP action. Histological examination using electron microscopy supports this, revealing that CRH cells of the PVN are densely innervated by PACAP (Legradi et al., 1998), α-MSH (Fekete et al., 2000b) and AgRP/NPY (Fuzesi et al., 2007) positive terminals. Furthermore, MTII injection induces CRH expression in the PVN and pre-treatment with α-helical CRH hampers the anorexic function of i.c.v.

MTII (Lu et al., 2003). Our current study has significantly increased our knowledge of how PACAP fits with these recognized hunger/satiety networks of the hypothalamus.

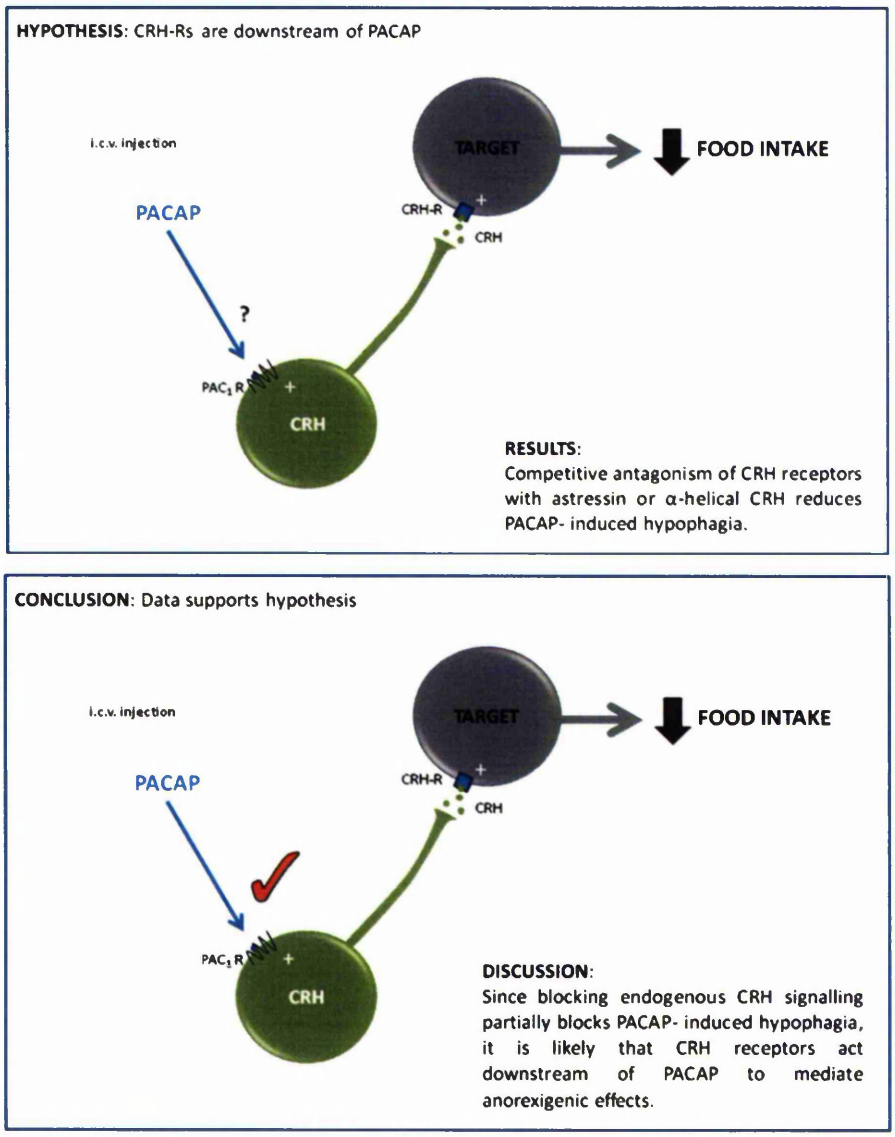


Figure 6.10 PACAP and the CRH pathway

Schematic showing the proposed role of CRH neurones as a downstream mediator of PACAP's feeding effects. Our *in vivo* and immunohistochemical data supports this hypothesis.

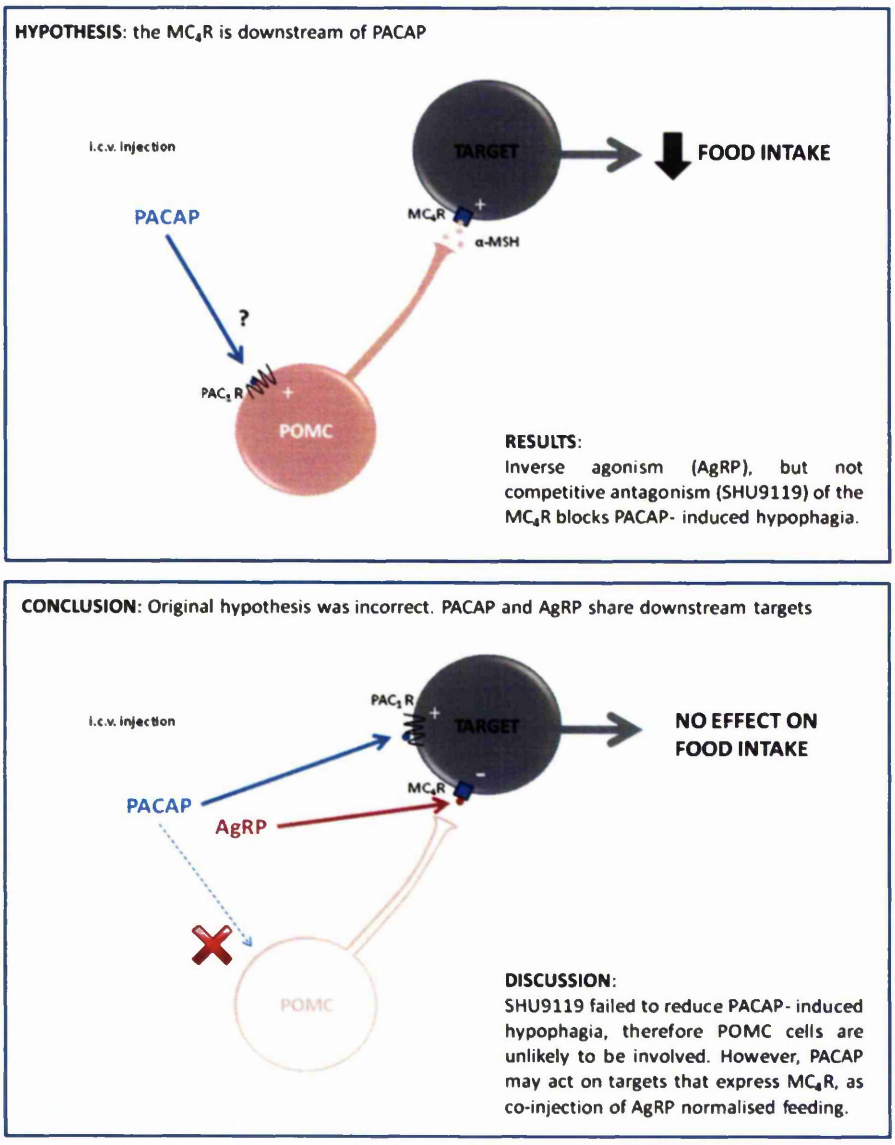


Figure 6.11 PACAP and the melanocortin pathway

Schematic showing the proposed role of POMC neurones as a downstream mediator of PACAP's feeding effects. Our data do not support this, but instead suggest that MC_4R and PAC_1R are co-localised on target neurones.

Does PACAP target brain areas which are involved in thermogenesis?

Pair-feeding and *in vivo* pharmacological studies have shown that PACAP has effects on body weight and energy expenditure which are independent of its feeding effects. In Chapter 4 we showed that PACAP induces thermogenesis and weight loss and hence that one of its major catabolic actions is likely to be promotion of sympathetic drive to peripheral fat depots. However, it is currently uncertain which downstream brain regions could be involved in mediating these effects. In the present study we have examined the induction of c-Fos in the brain following a single PACAP injection, using conditions matched to our calorimetry studies (see Chapter 4) to elucidate potential thermogenic and lypolytic mediators. In the late 1990s the Bartness laboratory produced a series of beautiful trans-synaptic retrograde tracing studies in the Siberian hamster using a modified strain of the pseudorabies virus (PRV) in order to identify the functional neuroanatomical chain from the brain to white and brown adipose tissue (Bamshad et al., 1998; Bamshad et al., 1999). They found that PRV staining was localised to discrete regions in the brain, and that many of these regions innervate both BAT and WAT. Interestingly, in our study, the most strongly activated regions show considerable overlap with those which dominated the tracer studies, suggesting that the sympathetic responses to PACAP could be processed by centres in the brain. These included the PVN and DMN of the hypothalamus, and the PAG region of the midbrain, whose roles in sympathetic drive have been demonstrated by other studies (Arancibia et al., 1996; de Menezes et al., 2006; McAllen, 2007; Nakamura and Morrison, 2007; Yoshida et al., 2005). Regions such as the raphe nuclei which were strongly labelled with PRV tracer and which express PAC_1, did not show c-Fos induction in our study, most likely due to physical limits of the i.c.v. injection. Ideally, we would like to do similar PRV tracer studies and co-localise by ISHH for PAC_1 receptors, as has already been done for the MC_4R (Song et al., 2005; Yasuda et al., 2004).

To summarise, we have shown that PACAP potently activates brain regions which are involved in sympathetic outflow to adipose tissues of the periphery, but as yet direct evidence for a sympathoexcitatory effect of centrally administered PACAP has not been demonstrated. It would be beneficial to test PACAP-injected animals for acute biomarkers of SNS tone, such as plasma levels of noradrenaline and adrenaline, which increase rapidly following central leptin or MTII. Tissue-specific analysis of noradrenaline turnover (Brito et al., 2007) will also reveal whether the increase in energy expenditure is due to a certain tissue type or whether a more general pro-sympathetic effect. In BAT, UCP1 gene expression is regulated by fasting (Champigny

and Ricquier, 1990), dietary overfeeding (Matamala et al., 1996), the *ob/ob* mutation (Ashwell et al., 1985), and exogenous leptin treatment (Zhang et al., 2007); all conditions which modulate PACAP expression. We plan to investigate whether PACAP alters expression of markers of sympathetic activatiation of adipose tissues (already collected), including UCP1 expression (BAT), hormone sensitive lipase (HSL; WAT) and β3-AR (both BAT and WAT) using RT-PCR. We expect that such markers will be upregulated in PACAP-treated animals as a reflection of increased sympathetic drive.

Other considerations

Another brain region which warrants mention due to particularly strong activation following PACAP treatment is the hypothalamic periventricular nucleus (PeV). The PeV is a relatively poorly characterised nucleus, but has been identified as a particularly dense region of somatostatin expression (Elde and Parsons, 1975) and that this is correlated with female reproductive cycle (Van Vugt et al., 2008), a phenomenon thought to involve PACAP (reviewed in Sherwood et al., 2007). Somatostatin, also known as growth hormone-inhibiting hormone, primarily acts on the anterior pituitary, suppressing growth hormone secretion from somatotrope cells. In terms of acute energy balance, central injection of somatostatin in the rat does not affect either food intake or energy expenditure, but does result in decreases in RQ, indicating promotion of fat metabolism (Atrens and Menendez, 1993). It is therefore possible that some of the lipolytic effects of PACAP on WAT may be mediated by this population of neurones.

In terms of methodology of this study, it must be remembered that while c-Fos is a convenient tool for broad neuronal mapping it is not without its limitations (reviewed in Hoffman and Lyo, 2002). c-Fos itself has an extremely short half life, and suppresses its own expression by negative feedback. As such, it is only useful for detection first-order neurones, rather than all the downstream components involved in the response to a stimulus. Another caveat of this technique is that induction of cFos normally indicates neurones which are activated, and not those which are inhibited. However, since PACAP is an excitatory transmitter (Hardwick 2006; Schomerus *et al.,* 1994), this may not be of importance for this study. For the purposes of this investigation, which was to examine the direct targets of PACAP signalling in the brain, c-Fos is a useful experimental approach, although the use of complemetary techniques such as electrophysiology and pharmacological magnetic resonance imaging (phMRI) should be considered for the future.

6.5 SUMMARY

- Central PACAP administration induces c-Fos protein expression in regions known to govern food intake including the PVN and DMN.

- Central PACAP administration induces c-Fos protein expression in brain regions known to govern sympathetic activation of brown and white fat including the PVN, DMN and PAG.

- Co-administration of CRH antagonists α-helical CRH and astressin partially block the hypophagic effect of central PACAP administration.

- Co-administration of the MC_4R antagonist SHU9119 does not block the hypophagic effect of central PACAP administration.

- Co-administration of the MC_4R inverse-agonist AgRP completely blocks the hypophagic effect of central PACAP administration.

6.6 CONCLUSIONS

Central PACAP administration activates regions of the brain known to control energy balance homeostasis. Key regions include the PVN, DMN and PAG which have been previously identified as projecting to both BAT and WAT by retrograde tracing, and which are known to express the PAC_1 receptor. These regions are therefore likely to contribute to the anorexigenic and hypothermic effects discussed in Chapter 4. Interestingly we did not see activation of cells in the Arc following PACAP injection, nor could we block the feeding effect with the MC4R antagonist SHU9119. We can only conclude from this that PACAP and POMC must elicit anorexigenic effects via physiologically distinct pathways. In contrast, the PVN and CeA, areas of dense CRH expression, were strongly activated by PACAP, and furthermore, CRH antagonists reduced PACAP's effects on food intake. It is therefore likely that these pathways interact physiologically.

CHAPTER 7

SYNAPTOLOGY OF THE VMN

7.1 INTRODUCTION

It is established that administration of exogenous leptin to *ob/ob* leptin-deficient or wildtype mice decreases food intake and body weight (Halaas et al., 1995; Pelleymounter et al., 1995; Rentsch et al., 1995), and that it is mediated largely by modulation of hypothalamic feeding pathways including those in the Arc and VMN (Schwartz et al., 2000). In chapter 3 of this thesis we reported that leptin, and stimuli which alter circulating leptin levels have profound effects on gene expression in the VMN. In this chapter, we begin to explore whether another form of hypothalamic plasticity, synaptic re-wiring, is a functional component of leptin responses in the VMN of the hypothalamus. Recently it was found that leptin is able to rapidly alter the GABAergic and glutamatergic inputs of both anabolic and catabolic hypothalamic cell populations (Horvath and Gao, 2005; Pinto et al., 2004), and that this mechanism could represent a significant portion of the hormone's effects. Synaptic remodelling is not a novel concept, in fact it was reported almost twenty years ago that synaptic density in the Arc undergoes rapid changes during the oestrus cycle in rats (Olmos et al., 1989). Since then, short-term rearrangements of synaptic architecture have been described in numerous other regulatory pathways, for example in the magnocellular system during changes in water homeostasis (Miyata et al., 1994) and in the gonadotrophin system during seasonal fluctuations in the ewe (Xiong et al., 1997). However, until recently it had not been imagined that this phenomenon could have a major role in the hypothalamic control of energy homeostasis.

Plasticity in the hypothalamic feeding circuits

In 2004, a fundamental paper published by the laboratories of Horvath and Friedman demonstrated for the first time that energy balance networks are not 'hard wired', but instead are capable of continuous synaptic reorganisation (Pinto et al., 2004). This study demonstrated using electrophysiology, electron microscopy and immunohistochemical stereology, that *ob/ob* mice have severe distortions in the ratio of excitatory to inhibitory afferent inputs terminating on perikarya of the Arc, such that POMC cells are predominantly inhibited and NPY cells excited. Furthermore, treatment of these mice with exogenous leptin normalises synaptic architecture within hours of treatment, a change which precedes the decreases in food intake and body weight. These exciting data demonstrate that leptin has potent and rapid effects on the arrangement of synaptic inputs to key hypothalamic neurones, and raises the possibility that rewiring may be an inherent, obligatory characteristic of energy homeostasis.

Subsequent work has shown that rewiring is not restricted to the *ob/ob* model or to the Arc, and moreover physiological as well as pathophysiological stimuli are capable of inducing plasticity in the hypothalamus. Overnight food deprivation of wildtype mice, which is accompanied by a reduction in circulating leptin levels, promotes the formation of excitatory synapses and resulting post-synaptic currents onto orexin neurones of the LH (Horvath and Gao, 2005). This phenomenon is prevented by concurrent leptin administration, and reversed by re-feeding. Ghrelin too has been shown to induce similar plastic changes in the Arc (Pinto et al., 2004), and also in dopaminergic populations of the ventral tegmental area (VTA) of the midbrain (Abizaid et al., 2006), which have been linked to motivational aspects of feeding (Hommel et al., 2006; Nishino et al., 1987). These data strongly suggest that synaptic restructuring of feeding circuitry could be a widespread and continuous process, which fluctuates according to the daily metabolic environment. We are interested to see whether synaptic arrangement in response to metabolic cues might also be an important function of the VMN, the mechanisms of which remain relatively poorly characterised. In this chapter, we will use immunohistochemistry to assess the excitatory and inhibitory inputs of SF1-GFP neurones of the VMN in control and SF1-lepr-B knockout animals. We anticipate that genetic loss of leptin input onto these cells may cause similar abnormalities in synapse connectivity as are observed in the Arc of the *ob/ob* mouse.

The SF1-Cre lepr$^{flox/flox}$ mouse model

The VMN is an ideal site in which to examine synaptic plasticity, as it expresses markedly high levels of the neurotrophic factor BDNF, which may be a key mediator of synaptic remodelling. To examine whether VMN synaptic architecture is distorted by removal of leptin input to SF1 cells we will use the SF1-Cre loxGFP lepr$^{flox/flox}$ and SF1-Cre loxGFP lepr$^{wt/wt}$ strains developed by Dhillon and colleagues (2006). These strains provide a convenient model for examining synaptic architecture, since they express GFP under the control of the VMN specific promoter, SF1. Furthermore, mice with VMN-specific knockout of Lepr-B present with adult-onset obesity, which could be partly due to abnormalities in local synaptic architecture. In electrophysiological recordings, 82 % of SF1 neurones tested were excited by leptin, similar to POMC cells, with the remaining 18 % showing no response (Dhillon et al., 2006). These data suggest that SF1 cells, or at least subpopulations thereof, have a catabolic, rather than anabolic phenotype. A potential caveat of using this model is that SF1 cells are unlikely to represent a homogenous population; however, since the response to leptin is unidirectional, the degree of heterogeneity is unlikely to be as extreme as the situation found in the Arc.

Objectives:

To determine whether SF1-driven lepr-B deletion affects the overall synaptic architecture of the VMN

We will perform fluorescence immunohistochemistry against VGlut1 and VGAT, vesicular transporter proteins which distinguish excitatory and inhibitory synaptic boutons, respectively. Although both VGlut1 and VGlut2 are expressed abundantly in the hypothalamus, our preliminary studies revealed more robust staining of VGlut1 puncta over VGlut2. VGlut1 was therefore selected as a marker of glutamatergic terminals. Sequential confocal images will be taken of the dorsomedial subdivision of the VMN (dmVMN) of both strains, and analysed using Sigma Stat software (Macro designed in-house by Dr David Bechtold) to calculate synaptic density, expressed as mean number of synaptic puncta per 100 μm^2. This will give a good indication of whether synaptic architecture is distorted in pre-obese SF1-Cre loxGFP $lepr^{flox/flox}$ mice, and whether this may partially underlie the development of increased adiposity.

To determine whether SF1-driven lepr-B deletion alters synaptic inputs onto SF1 perikarya

The next stage will be to use dual immunohistochemistry to examine synaptic inputs onto individual GFP-positive cells. This technique has been used successfully to investigate a number of central systems and its reliability has been confirmed with electron microscopy and electrophysiology (Abizaid et al., 2006; Melone et al., 2005; Pinto et al., 2004). Due to the relatively weak GFP intensity in these strains, it is necessary to perform dual immunohistochemistry, first for GFP, and then either VGlut1 or VGAT on alternating brain sections. A series of high-magnification confocal images, which span the entire cell body will then be analysed visually for contacting VGlut1 and VGAT puncta according to pre-determined strict criteria. The counting will be performed blind by Eliza Kalk, a summer project student in the Luckman laboratory.

7.2 METHODS

Collection of tissue for fluorescence immunohistochemistry

Leptin receptor mutant SF1-Cre loxGFP $lepr^{flox/flox}$ and control SF1-Cre loxGFP $lepr^{wt/wt}$ transgenic strains were bred from mice which were a kind gift from Professor Lowell (Harvard Medical School, Boston, USA). 8-week old male mice (n=8 per strain) were perfused transcardially with heparinised 0.9% saline followed by 4% PFA as detailed in the general methods chapter, and the brains collected for immunohistochemistry. Animals used were of the same generation and littermates where possible, to minimise confounding effects of their mixed-strain background.

GFP and VGlut1 / VGAT dual immunohistochemistry

Four equivalent 30 μm hypothalamic sections were collected rostrally to caudally from each brain using a freezing sledge microtome and stored in 0.1 M PB. Sections spanned the medial portion of the VMN where leptin receptor is most abundantly expressed and were standardised against a counterstained littermate reference brain. Sections were double stained for GFP (green; denoting SF1 positive cells) and for one of either VGlut1 (red; glutamate terminals) or VGAT (red; GABA/glycine terminals) using fluorescence-label immunohistochemistry.

Briefly, sections were transferred to 5 % NGS as a blocking agent in 0.1 M PBT for 1 hour at RT to block non-specific staining. Alternate sections were then incubated overnight at 4 °C in primary antibody cocktails at as shown in Table 7.1 below diluted in 1 % NGS blocking serum. The following day excess antibody was removed by repeated washing in 0.1M PBT and sections were incubated at RT for 2 hours in fluorescent secondary antibody cocktails in 5 % NGS blocking serum as shown below. From this stage onwards sections were kept in the dark to avoid photo-bleaching of the fluorescent signal. Unbound antibody was removed by washing in 0.1 M PB and sections mounted onto polylysine coated glass microscope slides (Mendel and Glazer, UK) according to genotype and section number, and coverslipped using Fluorosave Reagent™ (Calbiochem, USA). Slides were then left to dry in the dark and presence of signal was confirmed using a wide-field fluorescence microscope.

Section	Primary antibody	Dilution	Secondary antibody	Dilution
1, 2, 3, 4	Chick anti-GFP	1:2000	Alexa 488 anti-chicken	1:1000
1, 3	G.-Pig anti-VGlut1	1:10,000	Alexa 594 anti-g.-pig	1:1000
2, 4	Rabbit anti-VGAT	1:5000	Alexa 594 anti-rabbit	1:1000

Table 7.1 Fluorescent antibody cocktails

Details of antibody dilutions used for detection of GFP, VGlut1 and VGAT. Primary antibodies were purchased from Chemicon, UK and fluorescence-conjugated secondary antibodies from Invitrogen (Molecular Probes), UK.

Confocal microscopy and synapse quantification

Images for analysis were collected using a Nikon inverted confocal microscope and EZC1 computer software. The analysis area was standardised across sections using the third ventricle as a guide so that the dorsomedial VMN was centred under 60 x widefield objective. To first assess whether overall bouton count was distorted in the VMN of SF1-Cre loxGFP $lepr^{flox/flox}$ mice, 10 µm thick z-stacks were collected using the red-channel only at 1 µm intervals (10 images per VMN). Puncta count per image (120 µm x 110 µm) was then calculated according to preset size and intensity parameters using Sigma Stat software. For analysis, data for each brain were expressed as mean counts per 100 μm^2 and groups compared using the Student's unpaired *t*-test.

To assess synaptic input onto SF1 neurones selectively, two GFP-positive cells per VMN were chosen randomly for individual analysis. Sequential images using both green and red detection channels were taken at 1 µm intervals spanning the entire cell body, and contacting terminals were quantified visually. Quantification was carried out *post hoc* according to the following criteria: only boutons which were directly in contact with a green cell body were included; any which were close but not touching, or that made axonal/dendritic contact >2 µm distance from the cell body were disregarded (see Figure 7.1). Furthermore, careful comparison of consecutive slices was performed to avoid multiple tally of a single bouton spanning more than one image. For analysis, counts from the same brain were averaged and group data, expressed as mean contacts per cell were analysed using the Student's unpaired *t*-test.

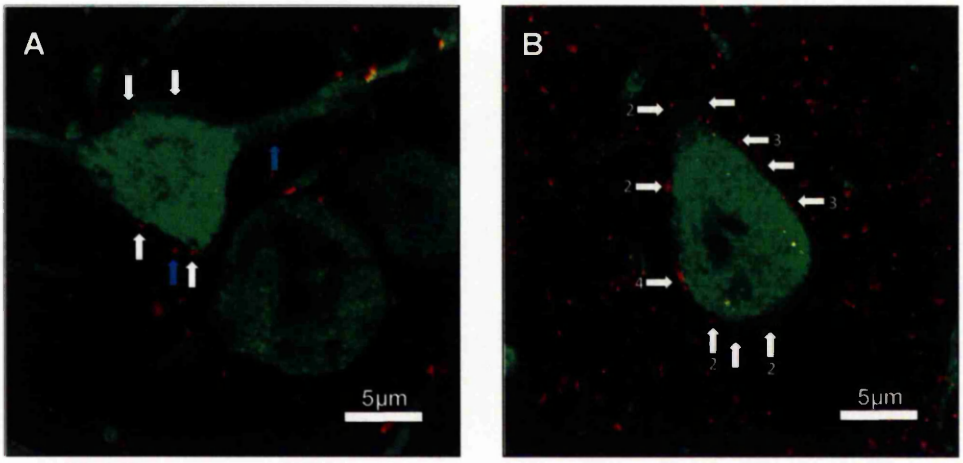


Figure 7.1 Visual evaluation of VGlut1 and VGAT synapses on single GFP positive neurones
Respresentative confocal images taken from a typical z-stack showing a selected GFP cell and **A.** VGlut1 and **B.** VGAT puncta. White arrows indicate axosomatic contacts, while blue arrows denote puncta which do not meet the pre-set criteria and would hence be excluded.

7.3 RESULTS

Dual-fluorescence immunohistochemistry

Expression of GFP, VGlut1 and VGAT in the mouse VMN

SF1 neurones (identified by GFP expression) were detected throughout the dorsomedial and central VMN with no signal elsewhere in the brain, consistent with our SF1 riboprobe binding pattern. In contrast, both VGlut1 and VGAT punctuate staining was widespread throughout the brain, although these expression patterns were not uniform. Of interest to this study, in the dmVMN VGAT signal was more abundant than VGlut1, which is markedly lower compared to surrounding regions (see Figure 7.2), consistent with the understanding that VMN neurones predominantly receive GABAergic inputs (McClellan et al., 2006).

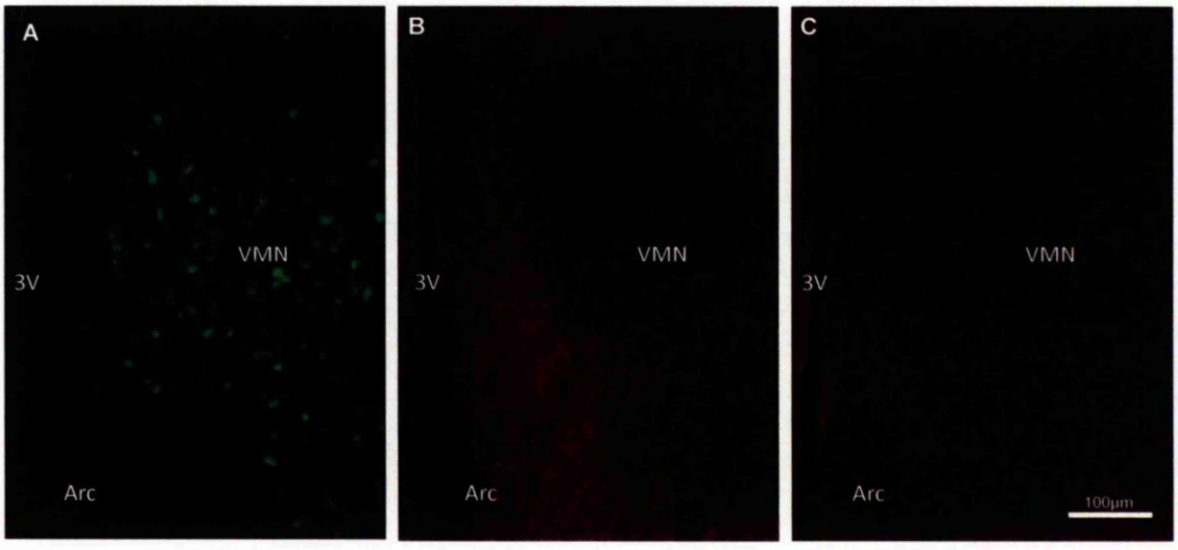


Figure 7.2 GFP, VGlut1 and VGAT expression in the VMN

Signal verification using a widefield fluorescence microscope (10 x objective) showing basomedial hypothalamic immunostaining for **A.** GFP, **B.** VGlut1 and **C.** VGAT in control mouse sections.

Effect of conditional Lepr-B deletion on VMN synaptology

SF1-Cre loxGFP $lepr^{flox/flox}$ mice had significantly lower VGlut1 punta counts per 100 μm^2 in the dorsomedial VMN (-44±5 %) compared with SF1-Cre loxGFP $lepr^{wt/wt}$ controls. Conversely, VGAT punta counts per 100 μm^2 were higher (+26±10 %) in the floxed strain compared with controls (see Figure 7.3).

Individual SF1 neurones selected for analysis were of similar perikaryal dimensions (diameter 8<10μm) and it was therefore not considered necessary to scale data according to membrane size. The mean ratio of VGlut1 versus VGAT putative contacts per cell was approximately 1:3 in the wildtype strain, whereas the floxed strain showed a marked shift in this ratio to around 1:5. Direct strain comparison revealed that the numbers of both excitatory and inhibitory axosomatic SF1 inputs were distorted, with a 29±9 % decrease in VGlut1 ($P<0.01$), and 25±8 % increase in VGAT ($P<0.05$) puncta in SF1-Cre loxGFP $lepr^{flox/flox}$ brains compared with SF1-Cre loxGFP $lepr^{wt/wt}$ controls (see Figure 7.4).

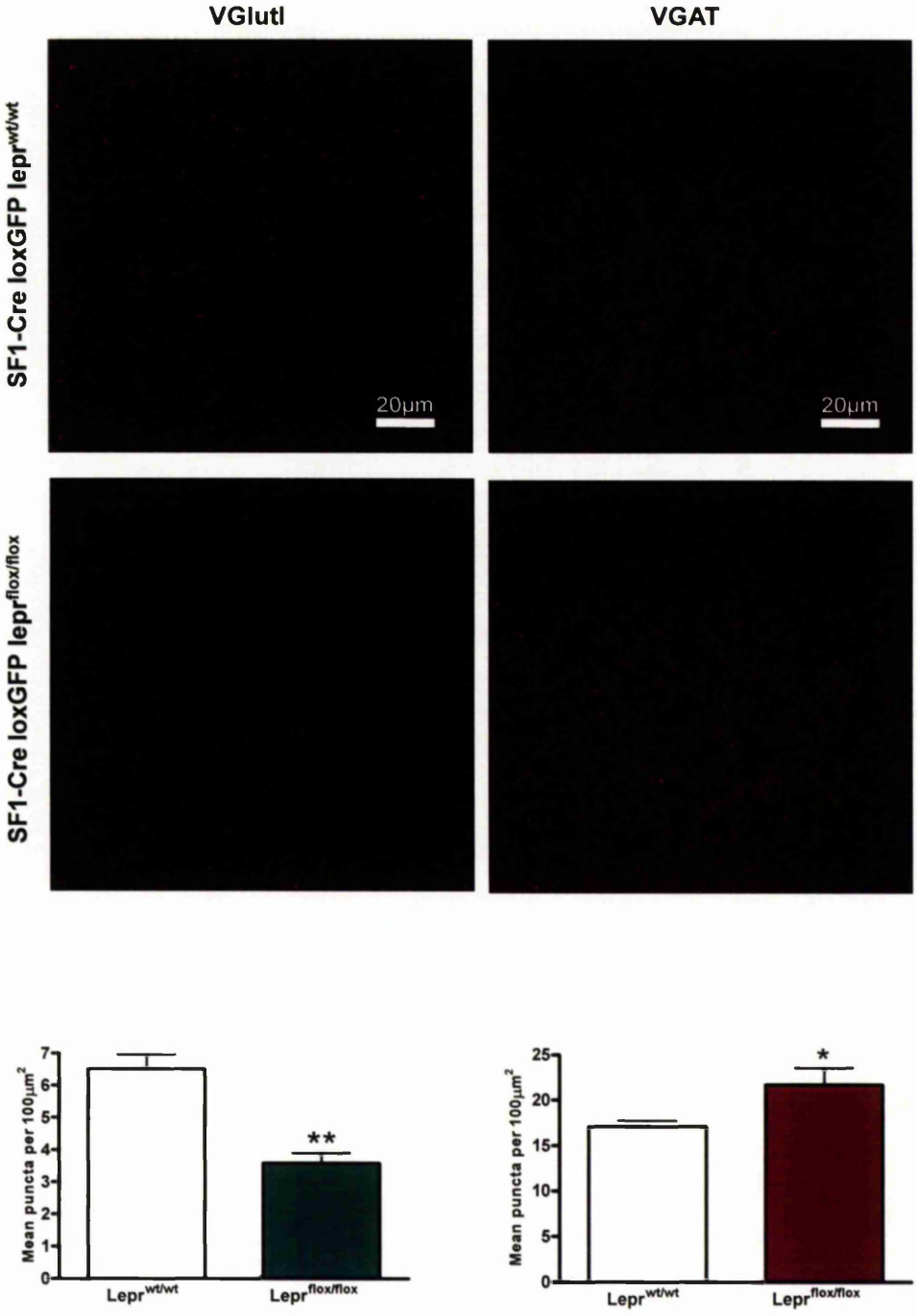


Figure 7.3 Puncta density in the dmVMN

VGlut1 and VGAT puncta density in the dmVMN of SF1-Cre loxGFP lepr$^{wt/wt}$ and SF1-Cre loxGFP lepr$^{flox/flox}$ strains (n=7). Data are expressed as mean puncta per 100µm^2 ± SEM. *P<0.05, **P<0.01; Unpaired Student's *t*-test. Representative images are shown above.

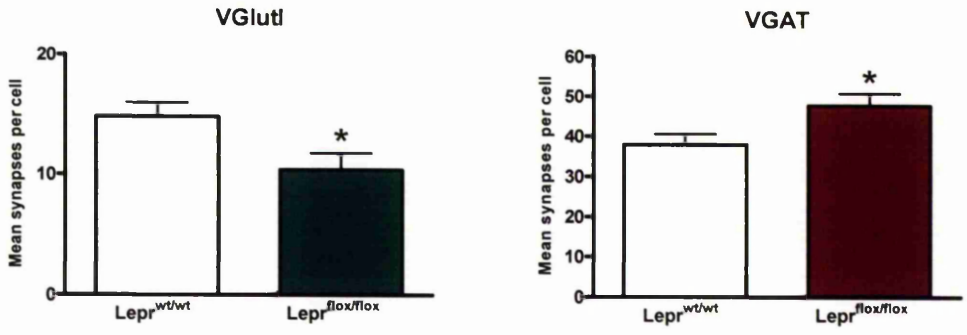


Figure 7.4 VGlut1 and VGAT SF1 inputs

Number of VGlut1 and VGAT axosomatic SF1 contacts in the dmVMN of SF1-Cre loxGFP lepr$^{wt/wt}$ and SF1-Cre loxGFP lepr$^{flox/flox}$ strains (n=7). Data expressed as mean synpases per cell ± SEM. *P<0.05; Unpaired Student's t-test.

7.4 DISCUSSION

Differences in synaptic inputs in the Arc and LH of the hypothalamus have been described in leptin mutant *ob/ob* mice and in wildtype mice in response to metabolic stimuli such as fasting. Here we show, for the first time, that site-specific lepr-B deletion in SF1 neurones of the VMN significantly alters the local synaptology such that VMN inhibitory afferents are more dominant than in the wildtype. Bearing in mind that the SF1-Cre $lepr^{flox/flox}$ mice go on to develop obesity, it is conceivable that these abnormalities directly contribute to decreased energy expenditure and increased food intake. The shifts observed are directionally appropriate for a catabolic cell phenotype, showing a greater intensity of inhibition in the obesity-developing mutant. This is consistent with previous work which shows that SF1 cells are predominantly activated by leptin in a similar manner to POMC cells of the Arc.

Horvath and Friedman's groups (Pinto et al., 2005) were able to show that changes in synaptic architecture were reflected in the frequency of miniature excitatory, and inhibitory postsynaptic currents (mEPSCs and mIPSCs) in single-cell electrophysiological recordings of Arc neurones. mEPSCs are the result of synaptic activation by glutamate, and mIPSCs by GABA/glycine, and the sum of these currents determines the likelihood of an action potential and transmitter release occurring. If the neurone in question is involved in energy homeostasis, as is the case with SF1 cells of the VMN, alteration of electrical inputs could have considerable downstream effects on body weight. We aim to further our current study by carrying out patch-clamp electrophysiology of GFP-positive SF1 cells to quantitatively assess whether neurones deficient in Lepr-B (SF1-Cre loxGFP $lepr^{flox/flox}$) show differences in the frequency of mPSCs compared to SF1-Cre loxGFP $lepr^{wt/wt}$ wildtype cells. This will confirm whether the organisational changes we have detected by immunofluorescence convert into electrical effects as they do in the Arc.

It is very probable that plasticity in VMN synaptic architecture is not restricted to the pathophysiological SF1-Cre loxGFP $lepr^{flox/flox}$ model, but that a plethora of stimuli could induce these changes. The VMN expresses a large range of receptors which are activated by peripheral and central mediators of energy balance, any of which could induce subtle changes in synaptology in a continuous and reactive fashion. Since loss of leptin input has such profound effects on VMN wiring, we would predict that in wildtype animals, physiological metabolic stimuli could induce similar changes. Cells of the VMN express both leptin and ghrelin receptors, but whether such signalling could

induce synaptic rewiring in this area has yet to be addressed. Even brain glucose, which fluctuates rapidly to reflect levels in the blood, could potentially initiate changes in VMN synaptic architecture by its effects on glucose-sensing cells. By applying a variety of metabolic stimuli to the SF1-Cre loxGFP $lepr^{wt/wt}$ mice, we will be able to build a thorough profile of the dynamic synpatological reponses of SF1 neurones of the VMN.

Uncovering the mechanism behind VMN synaptic rewiring

In order to gain further insight into the role of synaptic plasticity in the regulation of energy balance, it is critical that we identify the regulatory elements and intracellular correlates of metabolic-state triggered synaptic rewiring. A key question is: how can factors like leptin and ghrelin, which activate very different intracellular pathways, cause similar changes in synpaptology? One possibility is that common targets, such as BDNF or other hypothalamic neurotrophins could mediate these types of responses, downstream of these hormones. Although a link between BDNF and synaptic rewiring has not been shown, BDNF has been associated with forms of plasticity in the hippocampus and in the development of neuronal connectivity networks during embryogenesis (reviewed in Hennigan et al., 2007). As such, BDNF knockout produces a null phenotype, whereas adult onset knockout of BDNF or its receptor Trk-B (Chan et al., 2006; Minichiello et al., 1999) markedly reduces memory and learning capacity in addition to perturbing energy homeostasis. BDNF mRNA is detected at high levels throughout the hypothalamus, particularly in the VMN (see Chapter 3) where its expression levels correlate with energy state. Hence, it is a strong candidate mediator of synaptic plasticity in the hypothalamus. We aim to examine the effects of centrally administered BDNF on synaptic architecture, and subsequently whether BDNF can prevent predicted alterations in VMN synaptic arrangement in response to fasting, downstream of leptin and ghrelin.

Other considerations

All of the hypothalamic rewiring studies to date have focussed on changes in the synaptic contacts onto the cell bodies of identified neurones (Abizaid et al., 2006; Horvath and Gao, 2005; Pinto et al., 2004). While this approach can serve as a useful indicator of re-wiring, it is insufficient when used alone. A major caveat is that axo-dendritic inputs, which contribute strongly to cell activity, are not taken into account. In the current study, we have examined changes in both somatic inputs onto SF1 cells, but also overall puncta density of the dmVMN, which will also include axo-dendritic and non-SF1 inputs. Interestingly, we found more convincing inter-genotype differences in

the overall puncta counts than in the SF1 perikaryal counts, particularly with regards to excitatory inputs. This may suggest that the most significant changes in rewiring do not occur on the cell bodies themselves. While building evidence suggests that synaptic re-organisation is a physiologically relevant phenomenon, current methodology is relatively limited. Axo-dendritic synaptology is impractical to quantify in non-cultured neurones, since cellular projections rarely follow the cutting plane and hence are difficult to track. Furthermore, until useful markers for VMN cell types are established, re-wiring data collected from this area have limited impact in terms of their relevance to physiological function. We are currently endeavouring to generate a PACAP-Cre transgenic strain which will be used to drive GFP expression, allowing analysis of inputs specifically on PACAP cells.

7.5 SUMMARY

- SF1-Cre loxGFP $lepr^{flox/flox}$ mice have a significantly lower density of VGlut1 puncta in the dorsomedial subdivision of the VMN compared with SF1-Cre loxGFP $lepr^{wt/wt}$ controls.

- SF1-Cre loxGFP $lepr^{flox/flox}$ mice have a significantly higher density of VGAT puncta in the dorsomedial subdivision of the VMN compared with SF1-Cre loxGFP $lepr^{wt/wt}$ controls.

- SF1-Cre loxGFP $lepr^{flox/flox}$ mice have significantly fewer VGlut1 inputs contacting SF1 cells of the dmVMN compared with SF1-Cre loxGFP $lepr^{wt/wt}$ controls.

- SF1-Cre loxGFP $lepr^{flox/flox}$ mice have significantly more VGAT inputs contacting SF1 cells of the dmVMN compared with SF1-Cre loxGFP $lepr^{wt/wt}$ controls.

7.6 CONCLUSIONS

Synaptic rewiring is emerging as a fundamental regulatory mechanism in the hypothalamic energy balance circuits. We have shown that SF1-Cre loxGFP $lepr^{flox/flox}$ mice, which lack leptin signalling to SF1 cells of the VMN, have abnormal VMN synaptology, both overall, and specifically onto SF1 cells. This suggests that leptin input is essential for normal VMN wiring and that abnormal synaptic architecture could partially underlie the development of obesity in this strain.

CHAPTER 8

GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

Aim of this thesis

The hypothalamus is a key regulator of homeostatic feeding drive and sympathetic drive to fat stores, which together determine body weight. Much of the research into energy balance pathways puts massive emphasis on the role of the Arc, yet recent transgenic models have demonstrated that cells of the VMN may be equally important, particularly with regards to leptin signalling (Balthasar et al., 2004; Dhillon et al., 2006). Since the VMN remains poorly characterised (Segal et al., 2005), the VMN cell types which mediate leptin responses are currently unknown. The aim of this thesis was to better characterise the role of VMN cell types in energy balance and the effects of leptin in this nucleus.

8.2 VMN NEURONAL MARKERS

Stimuli such as fasting or leptin administration, which acutely alter energy balance cause regulatory changes in the expression of key genes in the hypothalamus (Schwartz et al., 2000). While the VMN has been strongly implicated in glucose handling and (Borg et al., 1997; Borg et al., 1995) leptin sensing (Elmquist et al., 1997; Irani et al., 2008), to date there have been relatively few studies which have examined the effects of different energy states on gene regulation in the VMN. Fasting-induced down regulation of PACAP and BDNF mRNA have been reported previously (Mounien et al., 2008; Xu et al., 2003), and we expanded this to look at gene expression in HED-fed, leptin-deficient and leptin-injected animals. Genes were selected based on a number of factors: firstly, whether they had been implicated in energy balance previously, and secondly, the relative expression in the VMN compared with other parts of the brain. Our selection was not limited to neuropeptides, which have come to form the emphasis of energy balance research, but also included the potassium-calcium co-transporter KCC2 and the retrograde neurotransmitter nNOS. It was found that all the genes examined undergo expression changes following metabolic stimuli, and that two of these (BDNF and PACAP) were directionally comparable to changes in catabolic genes in other nuclei. Furthermore, PACAP, but not BDNF, expression appears to be regulated by leptin, since *ob/ob* mice displayed markedly lower levels (similar to fasted animals) which were subsequently rescued by leptin treatment. We would also speculate that leptin replacement would prevent fasting-induced down regulation of PACAP.

The role of VMN cells in diet-induced obesity

It is known that rodents possess differing susceptibility to excess weight gain when exposed to obesogenic diets, though it is debated whether this results in a normal or bimodal distribution of body weights (Archer et al., 2003; Enriori et al., 2007; Koza et al., 2006; Levin et al., 1997). Studies which have looked at differences between naturally DIO-prone and DIO-resistant individuals in cohorts of outbred rodents reveal that defects may exist at the level of the hypothalamus. For example, DIO-prone animals fail to undergo adaptive thermogenesis on HED (Koza et al., 2006), and due to their obesity, responses to glucose challenge and leptin treatment (Enriori et al., 2007) are impaired. Hence, there appear to be two main questions regarding DIO. Firstly, what are the early divergences that contribute to the initiation of increased fat deposition? And secondly, what is different about the brains of obese mice that prevents them from responding normally to energy challenge? Other studies have suggested that expression of some of the major players in energy balance including NPY, Agrp, POMC and CRH are unchanged by HED-feeding in rats (Archer et al., 2003; Lauterio et al., 1999), yet sympathetic drive is increased and, following initial hyperphagia, calorie intake is decreased. As part of our investigation we used the HED-feeding paradigm in order to assess whether VMN genes could be involved in the counter-regulation against obesity. Interestingly, expression of all four genes was altered by this treatment, suggesting that plastic expression changes occur in the VMN in response to positive energy balance. Moreover, there were clear differences between animals that were susceptible and those which were resistant to obesity, particularly in the expression of PACAP, but whether these changes precede the change in body weight is currently unclear. Koza et al., (2006) were able to predict DIO based on an animal's body weight prior to the HED-feeding period and hence identify potential predeterminants of DIO. To better interpret our current data it will be necessary to examine VMN gene expression, plasma leptin levels and responses to peripheral leptin treatment at various timepoints following HED exposure, using initial body weight as a guide. Using this approach we will be able to build a temporal profile for the divergence of VMN gene expression and how this relates to diet, body weight and leptin resistance.

8.3 FOCUSSING ON PACAP

in vivo effects of central PACAP

Since we found that PACAP expression in the VMN correlates negatively with hunger and positively with sympathetic drive, we wanted to ascertain whether increasing brain PACAP impinged upon these processes *in vivo*. To clarify how PACAP acts on energy balance, we looked not only at food intake and body weight, but also core-body temperature, oxygen consumption and respiratory quotient, key indicators of energy expenditure. The processes that control energy expenditure are far less well characterised than those that regulate appetite, although these parameters are markedly affected by central administration of other neuropeptides. In the current study, we showed that as well as the well-documented potent anorexigenic effect, PACAP markedly increased indicators of sympathetic tone, and produced weight loss above that accounted for by reduced food intake. Hence, it appears PACAP acts as a classical catabolic factor, affecting both 'arms' of energy homeostasis in order to promote weight loss. While we cannot be certain that these effects are not due to non-homeostatic actions, our behavioural and antagonist data suggests that the effects at least partially reflect a physiological role. While other groups have reported that high doses of PACAP can cause changes in grooming, locomotor and anxiety behaviours in rats (Adamik and Telegdy, 2004; Masuo et al., 1995), our BSS analysis study revealed that during fast-induced re-feeding, effects of low doses of PACAP on other behaviours are mild. Of course, PACAP is a pleiotropic signalling molecule, and injection into the lateral ventricle is likely to activate other central PACAP pathways, including those which mediate fear, circadian entrainment and visual pathways, and these may explain the BSS disruption that occurs at higher doses.

PACAP's downstream targets

We believe that the effects of PACAP on energy balance are mediated by the PACAP specific PAC_1 receptor, as opposed to the VPAC receptors, which also have affinity for VIP (Gourlet et al., 1995). In order to examine this *in vivo*, we showed that central co-administration of $PACAP_{6-38}$ results in complete blockade of the effects observed when PACAP is given alone. This suggests that PACAP-induced attenuation of appetite and increase in body temperature are a result of action at the PAC_1 receptor, as opposed to a non-specific response. Evidence from knockout animals also suggests that PAC_1R is dominant in mediating PACAP's effects, since the phenotypes of PAC_1 and PACAP knockout mice are strikingly similar (Hashimoto 2000; 2001). In agreement with this, we

have shown that PACAP retains its anorexigenic and thermogenic effects in the $VPAC_2R$ knockout mouse, suggesting that $VPAC_2$ does not mediate these functions. Furthermore, central injection of VIP at equimolar doses to PACAP has no discernable effect on energy balance. Preliminary work is currently underway in order to confirm that the *in vivo* effects of PACAP are conserved in the $VPAC_1$, but lost in the PAC_1 deficient mouse strain.

PACAP receptors are widespread throughout the brain (Joo et al., 2004), and we aimed to identify which brain areas, and neuronal cell types, could be mediating PACAP effects on energy balance. We found PACAP-mediated activation of cells in regions known to be involved in the control of food intake and sympathetic activation of adipose tissues, including the DMN, PVN and PAG. Evidence suggests that a major PACAP target is the PVN. Neurones of the PVN densely express the PACAP-specific PAC_1 receptor (Nomura et al., 1996), and PACAP-immunopositive terminals directly contact CRH cell bodies of the PVN (Legradi et al., 1998). In agreement with this, we were able to attenuate the feeding effects of PACAP using CRH receptor antagonists, as has been shown in other species (Maruyama et al., 2006; Tachibana et al., 2004). In contrast, the MC_4R antagonist SHU9119 had no effect on the potency of PACAP-induced anorexia, and Arc activation was minimal following PACAP treatment. In contrast, the Vaudry group recently suggested an association between PACAP-mediated hypophagia and the melanocortin pathway of the Arc, since PACAP receptors are co-expressed with POMC, and in their hands SHU9119 attenuates PACAP-induced anorexia (Mounien et al., 2006; Mounien et al., 2008). As yet however, it is not clear whether there is substantial physiological interaction between the two systems, especially since Lepr-B deletion on SF1 and POMC cells appears to cause obesity by distinct pathways (Dhillon et al., 2006). It may be that the PAC_1R and MC_4R modulate different components of leptin signalling, and that administration of both antagonists would more potently block leptin effects than either does alone.

8.4 FOCUSSING ON LEPTIN

Leptin acts via PACAP cells

Recently, the role of the Arc in leptin signalling was called into question, since mice lacking Lepr-B expression in POMC cells develop only moderate obesity, and mice lacking Lepr-B in the SF1 cells are equally obese (reviewed in Balthasar, 2006). Electrophysiology and immunohistochemistry confirm that SF1 cells in the VMN are a

target for leptin (Dhillon et al., 2006), however, since SF1 is not a releasable molecule it cannot itself participate in downstream signalling pathways. Therefore, it is reasonable to assume that leptin alters expression or release of effectors which are co-expressed with SF1. A major finding of my thesis was that SF1-expressing cells in the VMN also express PACAP, and that PACAP expression relies on intact leptin pathways. *ob/ob*, SF1-Cre lepr$^{flox/flox}$ and DIO animals all have impaired leptin signalling and reduced PACAP expression in the VMN compared with their counterparts. Although we are currently unable to show that PACAP is co-localised with lepr-B due to difficulties with signal strength, demonstrated that PACAP comprises part of leptin's downstream energy balance pathways using *in vivo* pharmacology. Leptin exerts its effects on food intake and metabolic rate by acting on 'first order' neurones in the hypothalamus. These include POMC and NPY cells in the ARC, orexin cells in the LH and CRH cells in the PVN (Schwartz et al., 1999). As has been shown previously with MC_4R and CRHR antagonists (Gardner et al., 1998; Seeley et al., 1997), we found that by blocking central PACAP receptors with $PACAP_{6-38}$ we reduced the efficacy of leptin-induced anorexia by approximately half. This provides strong evidence that PACAP receptors form part of leptin's downstream anorectic pathway.

In addition to its effects on food intake, leptin also impinges on energy expenditure by increasing brown-adipose tissue (BAT)-mediated thermogenesis. Leptin injection either centrally or peripherally increases sympathetic nerve activity to brown adipose tissue (BAT) (Collins et al., 1996; Haynes et al., 1997), and expression of uncoupling protein (UCP1) by action at central targets. We found that leptin and PACAP-induced thermogenic responses form part of the same endogenous pathway, since blockade of PACAP receptors also blocked leptin-induced hyperthermia. This was a more potent reversal than we observed for the feeding response, suggesting that PACAP may be more important in mediating the 'energy expenditure' component of leptin signalling. Together, these data highlight PACAP as a novel effector of leptin signalling, both in feeding and in thermogenesis.

Leptin and synaptology

Leptin can rapidly alter the connectivity of glutamatergic and GABAergic afferents onto POMC and NPY cells of the Arc, orexin cells of the LH and dopaminergic neurones of the midbrain (Abizaid et al., 2006; Horvath and Gao, 2005; Pinto et al., 2004). However, a surprising discovery was the extent to which wiring of SF1 cells of the VMN depends on leptin input. We found that animals that are VMN-deficient in lepr-B, but which have normal leptin signalling elsewhere have significantly diminished excitatory

input and enhanced inhibitory input, suggesting that VMN tone may be inhibited in these animals. The VMN is classically considered a 'satiety' centre, and contains catabolic cell types including those which express BDNF, and PACAP (Maekawa et al., 2006). It is reasonable to postulate that hyper-inhibition of these pathways may contribute to the development of obesity and the abnormal responses to HED that characterises this mouse strain.

8.5 SUMMARY AND FUTURE DIRECTIONS

In this thesis, we have demonstrated that exogenous PACAP is able to evoke centrally-mediated effects both on energy intake and expenditure. However, due to the numerous PACAP pathways present in the brain, it is currently unclear which neuroanatomical chain(s) are responsible for these effects, and whether they represent a true functional role, or simply a pharmacological phenomenon. We initially became interested in PACAP following the observation that its expression level in the VMN, a known regulator of energy balance, underwent significant adaptations during metabolic challenges such as fasting or high-energy diet. This response was more robust than for any other VMN gene we tested, and moreover, was absent in other PACAP populations, suggesting that this subset of PACAP cells are functionally distinct from those found elsewhere. Subsequently, we demonstrated that these neurones co-express SF1, a surrogate indicator of leptin sensitivity, and that genetic loss of Lepr-B in these cells markedly reduces local PACAP expression. Hence, although we strongly suspect that VMN cells have a physiologically relevant role in the control of energy metabolism and appetite, this remains to be proven conclusively. The next stage of this project, which is currently ongoing, will investigate this by testing whether VMN-specific knockout of the PACAP gene produces an abnormal metabolic phenotype.

Over the past decade the prolific expansion of transgenic technology has led to rapid advances in our understanding of physiological processes, including those which maintain metabolic equilibrium. Genetic mutation of components of central energy balance networks often (but not always) results in a metabolic phenotype, some of which are quite severe. POMC and MC_4R knockout mice (Huszar et al., 1997; Smart et al., 2007), for example, both exhibit obese phenotypes, as does the AgRP over-expressing strain (Ollmann et al., 1997). However, knockout of NPY, the most potent endogenous orexigen known, has remarkably little effect on appetite or body weight, highlighting that while knockouts are a useful tool, they are not incontrovertible

(Thorsell and Heilig, 2002). Global knockout of the PACAP gene does not result in the expected obese phenotype, but does bring about various metabolic abnormalities. The most striking of these are severe impairment of sympathetically-mediated BAT thermogenesis, and inefficient glucose handling, (Adams et al., 2008; Gray et al., 2001; Gray et al., 2002) both of which may involve the VMN. The lack of obesity and in fact slightly lean phenotype of these mice is intriguing, and could be attributed to a number of factors. Firstly, redundancy is always an issue when gene knockout occurs before embryogenesis i.e. other anorexigenic factors such as melanocortins or BDNF may be up-regulated to compensate for the lack of PACAP. More likely, loss of peripheral PACAP function may result in detrimental effects which secondarily impinge on body weight, masking specific central effects. The high embryonic mortality and a complex adult phenotype of PACAP knockout reiterates the multiple roles of this peptide, and makes phenotypic characterisation of specific manifestations difficult. The way we plan to circumvent these complications is by deleting PACAP in a tissue-specific manner, using Cre-*loxP* transgenic technology (see Appendix).

8.6 SUMMARY OF KEY FINDINGS

- Expression of genes encoding neuropeptides (BDNF and PACAP), neurotransmitters (nNOS) and ion transporters (KCC2) in the VMN are responsive to alterations in energy status including fasting, diet-induced obesity and modulation of leptin signalling.

- Central PACAP injection decreases food intake in mice in a dose-dependent manner, with higher doses also causing aversive effects as assessed by analysis of the behavioural satiety sequence.

- Central PACAP injection increases core-body temperature, oxygen consumption and fat metabolism.

- PACAP is colocalised with SF1 in the VMN and genetic deletion of lepr on SF1 cells decreases VMN PACAP expression.

- Antagonism of endogenous PACAP signalling using $PACAP_{6-38}$ attenuates leptin-mediated hypophagia and hyperthermia.

- The anorexigenic effects of PACAP can be blocked using the CRHR antagonists astressin and α-helical CRH and the MC_4R inverse agonist AgRP, but not the MC_4R antagonist SHU9119.

- PACAP causes c-Fos induction in areas known to control food intake and thermogenesis including the paraventricular nucleus of the hypothalamus and the periaqueductal grey of the midbrain.

- Genetic deletion of the leptin receptor in SF1 cells causes abnormal synaptic architecture in the VMN.

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A.1 INTRODUCTION

The aim of this section is to describe the design stages involved in creating a PACAP$^{flox/flox}$ mouse that will be used to generate conditional PACAP knockout strains. This part of the project is an ongoing collaborative study being carried out with Dr. Tina Ivanov (Simon Luckman laboratory), who is working concurrently to generate a PACAP-Cre expressing line. We will be using the recently developed *Escerichia coli* (*E.coli*)-based 'recombineering' method (recombination-driven genetic engineering) (Copeland et al., 2001; Lee et al., 2001) for construction of DNA targeting vectors. Since this is a novel technique for our laboratory, literature review and close correspondence with experienced advisors Dr. Harveen Dhillon (Harvard Medical School) and Dr. Nina Balthasar (University of Bristol), have been essential for us to produce a strategy which is both cost and time effective, and which will result in successful production of the desired transgenic animals.

Tissue-specific gene deletion

The most common method to achieve tissue-specific gene deletion is using the recombinase systems Cre-*loxP* and Flippase (Flp)-*frt* (Metzger and Feil, 1999; Morris et al., 1991; Palazzolo et al., 1990). These rely on the ability of the recombinase enzymes Cre and Flp to bind specific 34bp DNA sequences (*loxP* and *frt* respectively) and catalyse recombination between two sites of the same type. DNA that lies between the two sites is excised and a single *loxP* (or *frt*) site remains in its place following recombination. We will utilise the Cre-*loxP* system to conditionally target the PACAP gene, although the Flp-*frt* system will also be required for part of a targeting vector construction step.

In order to conditionally knock-out a gene, *loxP* sites are inserted into the DNA so that they flank essential coding regions of the target gene, termed 'floxing'. Conditional knockouts can then the generated by mating the 'floxed' animal with one that transgenically expresses the Cre gene under the control of a tissue-specific promoter (Balthasar et al., 2004; Dhillon et al., 2006; Gu et al., 1994). Hence, the targeted gene will function normally in cells where Cre is not present, but be deleted in cells where Cre is expressed (see Figure A.1). This appendix describes the stages required to make a floxed PACAP mouse (PACAP$^{flox/flox}$) which can subsequently be bred with an SF1-Cre strain (Bingham et al., 2006; Dhillon et al., 2006) to delete PACAP selectively in the VMN. Based on our co-localisation study (see Chapter 5), we would expect a significant knock-down of PACAP expression in the whole VMN, with near-complete

depletion in the leptin-responsive dorsomedial subdivision. The phenotype of this strain will provide invaluable insight into the specific role, metabolic or otherwise, of VMN PACAP cells.

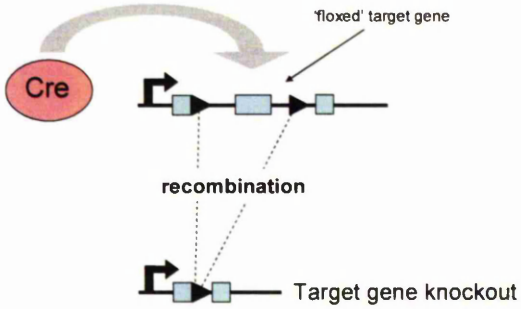

Figure A.1 Cre-driven deletion of a floxed gene
LoxP sites (black triangles) are positioned in non-coding regions such that they flank essential exons (blue rectangles) of the target gene. The Cre enzyme catalyses recombination at *loxP*, excising intervening sequences and removing target gene function.

Practical gene targeting

All gene-targeting experiments comprise the following steps:

1. Construction of a mutant 'targeting vector' (see Figure A.2B) which will recombine with the endogenous gene and which consists of: the modified target gene (containing *loxP* sites); an antibiotic resistance gene for selection of targeted embryonic stem (ES) cells (flanked by *frt* sites for later removal); substantial DNA 'arms' upstream and downstream of the target sites that are homologous with the wildtype allelle.

2. Introduction of the linearised targeting vector into mouse ES cells and selection and screening for correctly targeted clones.

3. Microinjection of targeted ES cells into blastocysts to generate chimeric mice which will form the founders of the PACAP$^{flox/flox}$ line.

A.2 PACAP TARGETING STRATEGY

Development of a transgenic targeting strategy is a lengthy process which requires careful consideration of all the downstream stages of mouse generation. In order to successfully target the PACAP gene we must consider the following (Liu et al., 2003):

- Inclusion of >5 kilobases (kb) of wildtype genomic DNA homology arms to maximise successful genomic incorporation of the final targeting vector.
- Positioning mutant *loxP* sites in non-coding regions so they do not disrupt gene function in the absence of Cre.
- Inclusion of antibiotic resistance genes (*Neo*) for selection of targeted *E.coli* and ES cells. *Neo* genes must be flanked by *loxP* or *frt* sites to allow them to be removed by recombinase enzymes (Cre or Flp) once they are no longer required.
- Introduction of a unique restriction enzyme site (in this case, SphI) in the inserts for subsequent genotyping of ES cells (and mice) by Southern blotting.

The mouse PACAP gene contains five short exons, separated by larger non-coding introns (NCBI GeneID:11516; see Figure A.2.A). Exon 1 and the distal portion of exon 5 are not translated, whereas all other exons contribute to the protein. To be sure that gene disruption occurs in a Cre-conditional manner, and not globally, we have selected non-coding target sites: insert1 100bp distal to exon 2; and insert 2 immediately after the stop codon in exon 5 (see Figure A.2.B and C). Using this approach, Cre will delete the coding exons 3, 4 and 5 (see Figure A.2.D) and almost certainly result in a PACAP null outcome in these cells.

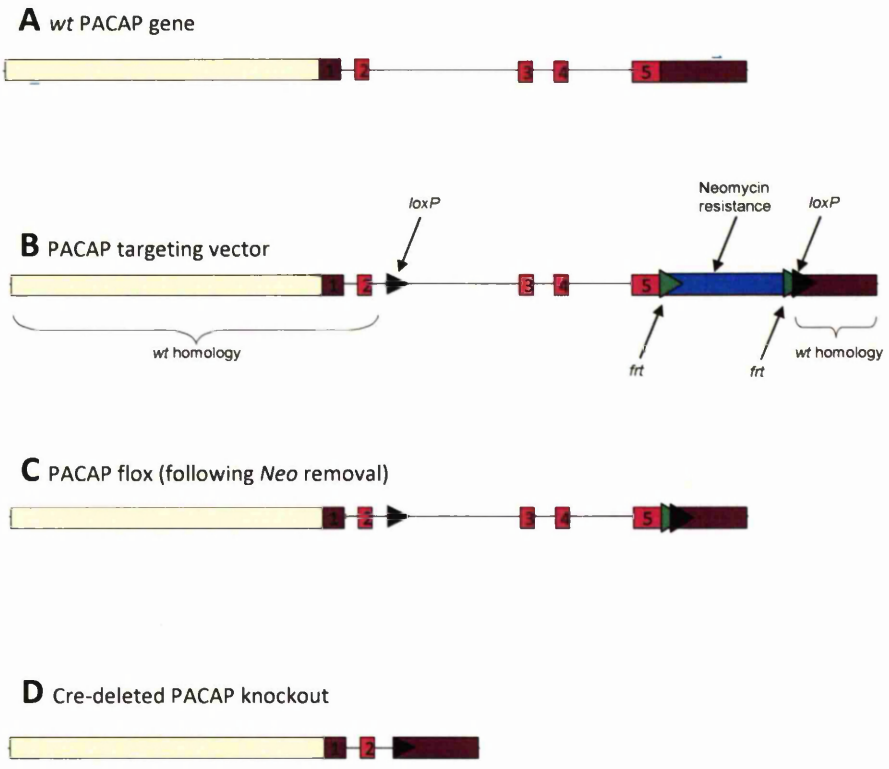

Figure A.2 Targeting the PACAP gene

Schematic showing **A.** the wildtype PACAP gene (plus 3kb upstream sequence) **B.** the 'targeted' PACAP gene that will be electroporated in ES cells (*frt-Neo-frt* is used for ES cell selection) **C.** the 'floxed gene' following *Neo* removal using Flp. N.B. This will function normally in the PACAP$^{flox/flox}$ mouse. **D.** the Cre- deleted, non-functional PACAP gene.

A.3 CONSTRUCTING THE TARGETING VECTOR

The PACAP gene (~7kb) plus 3 kb upstream sequence will be subcloned from a bacterial artificial chromosome (BAC) into a 'retrieval' plasmid, PL253 (Liu et al., 2003), using the recombinogenic properties of specially modified *E.-coli*, SW106 (Warming et al., 2005) using a technique known as 'gap-repair' (Copeland et al., 2001; Lee et al., 2001; Liu et al., 2003). PL253 contains an *Amp* (ampicillin resistance) cassette, which can then be used to select for PACAP-positive cells. Mutant *loxP* sites and the *Neo* selection marker (encodes kanamycin resistance in bacteria and neomycin resistance in ES cells) will then be introduced by two further rounds of recombineering, one for each *loxP* insert using the protocol developed by Lui and colleagues (Liu et al., 2003).

The first targeting step (single *loxP*)

The first targeting step initially involves introducing insert 1 (*loxP-Neo-loxP*) into the subcloned PACAP gene via homologous recombination, selecting for '*Neo*'-positive *E.coli*, then subsequently removing the *Neo* gene by inducing Cre expression, leaving a single *loxP* (see Figure A.3). Insert 1 contains the *EM7* (prokaryotic) promoter which will transcribe *Neo* in correctly-targeted bacteria, allowing for selection by kanamycin. The insert is generated by PCR using 100 nucleotide chimeric primers, consisting of 45 bases of homology with the PACAP target site; 34 bases of *loxP* sequence and 20 bases of homology with the *EM7-Neo* plasmid template, PL451 (Liu et al., 2003). A SphI restriction enzyme site is also included in the antisense primer for later genotyping by Southern blotting. Following insertion and *E.coli* selection, *Neo* gene excision is achieved using the arabinose-inducible Cre gene present in the SW106 *E.coli* genome according to the protocol used by Warming and colleagues (Warming et al., 2005).

The second targeting step (*frt-Neo-frt-loxP*)

The second targeting step (see Figure A.4) is identical to the first, except that the *Neo* gene must persist for selection of targeted ES clones (Liu et al., 2003). For this reason, a *PGK* (eukaryotic) promoter and downstream poly-A region are also amplified from the PL451 template plasmid with *Neo,* as are flanking *frt* sites and a single *loxP*. Following recombination and selection, the plasmid is linearised to give the finished targeting vector that will be electroporated into mouse ES cells. The *frt* sites allow the *Neo* to be removed after ES cell selection. Although this step is not essential, most investigators choose to remove *Neo* as it can sometimes interfere with gene expression in the mouse (Kaul et al., 2000). Excision is achieved either by transient transfection of ES

cells with an Flp-containing plasmid (Schaft et al., 2001), or later by crossing mice with a ubiquitous Flp-deleter strain (Kanki et al., 2006; Rodriguez et al., 2000).

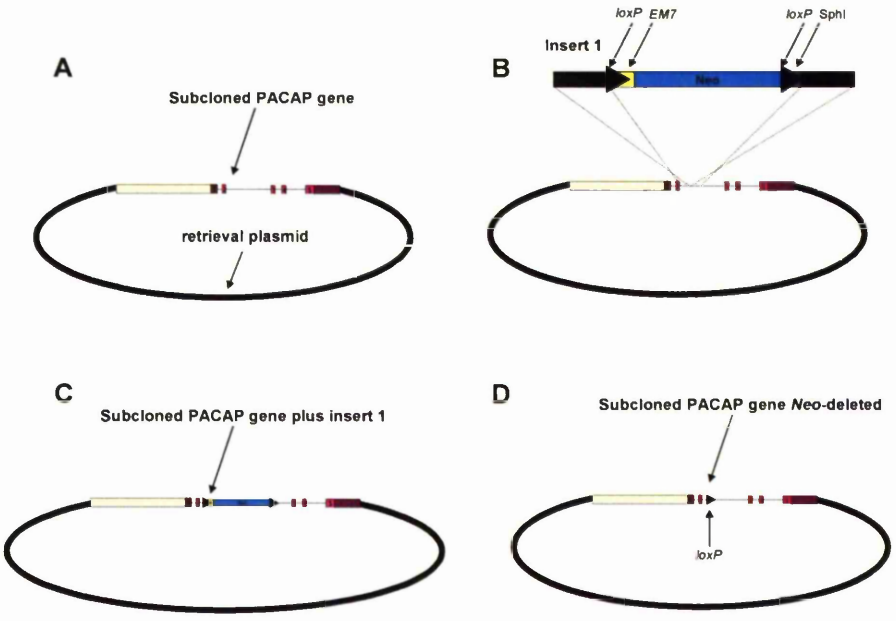

Figure A.3 The first targeting step

The PACAP gene is subcloned into the retrieval plasmid (**A.**) and undergoes homologous recombination with insert 1 (*LoxP-Neo-loxP*) at the target site 100bp downstream of exon 2 (**B.**), resulting in insert 1-incorporation (**C.**). Following kanamycin selection, the *Neo* cassette is removed by Cre leaving a single *loxP* site (**D.**).

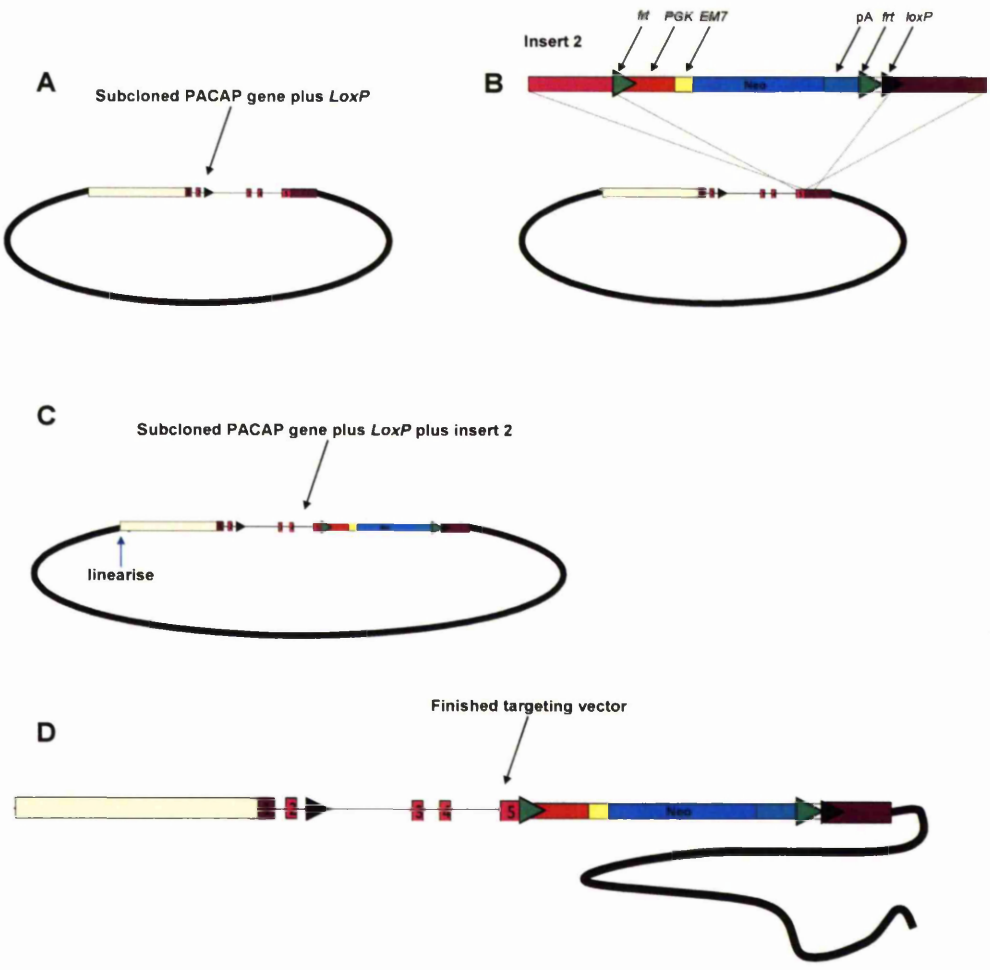

Figure A.4 The second targeting step

The *loxP*-containing PACAP gene (**A.**) undergoes homologous recombination with insert 2 (*frt-Neo-frt-loxP*) immediately after the 'stop' codon in exon 5 (**B.**), resulting in insert 2-incorporation (**D.**). Following kanamycin selection, the plasmid is linearised to give the finished targeting vector that will be electroporated into mouse ES cells (**D.**).

A.4 BEYOND THE TARGETING VECTOR

ES cells, blastocyst injection, chimeric mice and breeding

The ES cells maintained for transgenic work at the University of Manchester are derived from male mouse embryos of the 129/SvJ strain, and produce a white coat colour. For ES cell targeting, the purified targeting vector is electroporated into pleuripotent ES cells (Ramirez-Solis et al., 1993) and homologous recombination occurs between the homology arms and the endogenous PACAP gene on chromosome 17 (see Figure A.5). G418 (neomycin) resistant cells can then be screened by Southern blotting to test for correct incorporation of the transgene (Ramirez-Solis et al., 1993). Unfortunately, the frequency of successful recombination is extremely low, as it relies on the cellular machinery which drives random genetic recombination between parent alleles. As such, it is not uncommon for hundreds of ES clones to be tested before a correctly targeted one is found.

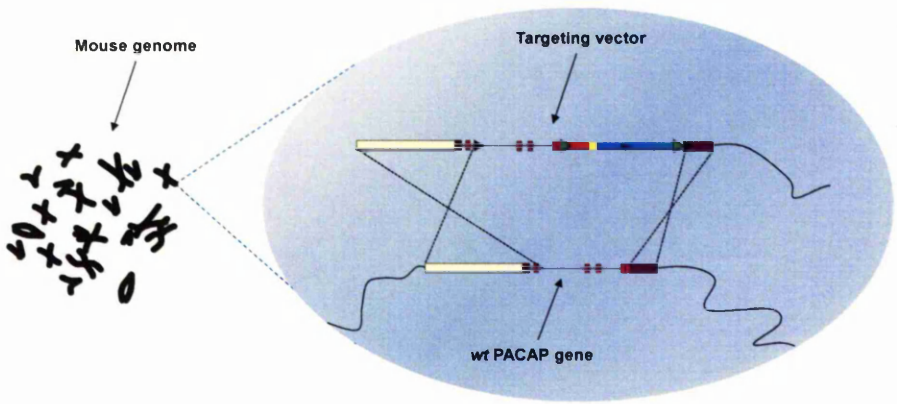

Figure A.5 Targeting vector incorporation

Schematic showing homologous recombination between the targeting vector and the wildtype PACAP gene on chromosome 17 of the ES cell genome. Targeted clones will be identified by neomycin resistance encoded in the targeting vector and screened using Southern blotting.

Once a successfully targeted ES line has been identified and expanded, this can be used to create the targeted mouse line (Stewart, 1993). This involves inducing superovulation in C57/BL6 females (black coat) with hormone treatment, while at the same time mating a CD1 female with a vasectomised male to induce pseudopregnancy. C57BL/6 blasocysts are then harvested and ES cells immediately injected into the blastocoelic cavity under a cooling microscope. Injected embryos are then implanted into the uterine horns of the CD1 surrogate female and allowed to go to term (Joyner 2000; see Figure A.6A).

Chimeras are the result of both wildtype host blastocyst, and heterozygous ES cells contributing to the soma (Stewart, 1993), and are most readily identified by their patchy coat colour. Generally, the contribution of ES clone to coat colour is a reliable indicator of its transmission to germline and thus the decision of whether or not to breed a chimera depends on the proportion of white fur. If germ-line transmission is 50 % for example, a cross with a C57BL/6 female will generate 50 % agouti and 50 % pure C57BL/6 offspring (Joyner 2000). These F_1 agouti offspring can now be tested by Southern blot and PCR genotyping for presence of the targeted gene. Because the targeted ES cells were heterozygous, half will be expected to carry one copy of the transgene (PACAP$^{flox/wt}$) half will be wildtype. (see Figure A.6B). Finally, F_2 PACAP$^{flox/flox}$ mice are achieved breeding F_1 heterozygous agouti pairs (see Figure A.6C) and can then be used to generate a stable line (Joyner 2000).

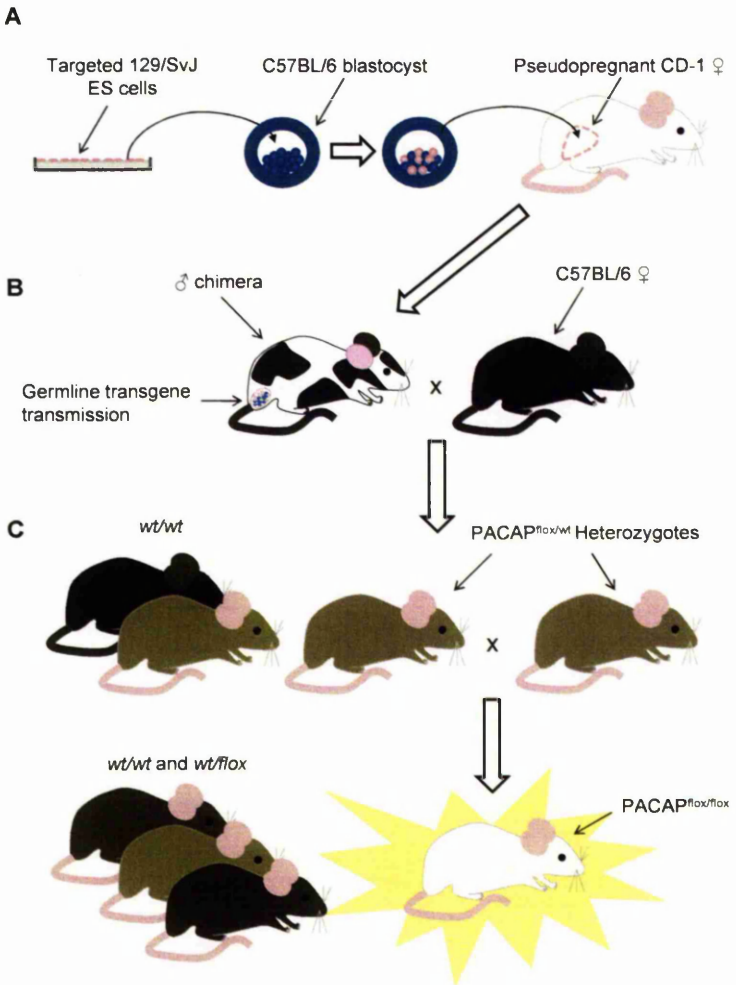

Figure A.6 Generation of PACAP$^{flox/flox}$ mice

A. Targeted ES cells are microinjected into C57BL/6 blastocysts which are then implanted in a CD1 surrogate female. **B.** Chimeric male offspring are mated to C57BL/6 females to generate heterozygote agouti, wildtype agouti and wildtype C57BL/6 offspring. **C.** Heterozygous agoutis are mated to produce homozygotes (PACAP$^{flox/flox}$). N.B. Coat colour is no longer an indicator of trangene presence as it sorts independently from the PACAP gene (Joyner 2000).

Southern blotting strategy

Our Southern blotting strategy relies on pre-incubation with the restriction enzyme SphI which naturally cuts either side of the original subcloned DNA sequence. Denatured DNA Southern blots will then be probed using ^{32}P-labelled oligonucleotide primers which recognise the DNA sequence immediately inside each SphI site. In the wildtype animal both probes will bind to identical 17.5 kb DNA fragments (see Figure A.7A). In contrast, if homologous recombination has occurred and an ES cell contains the targeted gene, Southern blotting with probe 1 will produce a 10 kb band, while probe 2 will produce a 3.5 kb band (see Figure A.7B). This is a result of the SphI site which was co-introduced with the single *loxP*, and the natural sites occurring within the *Neo* cassette of insert 2.

This strategy can also be used to review the success of the later Flp-driven *frt-Neo-frt* excision step. The floxed gene resulting from *Neo* removal produces a 7.5 kb band with primer 2 which is easily distinguished from either the wildtype (17.5 kb) or targeted gene (3.5 kb; see Figure A.7C). Once a PACAP$^{flox/flox}$ line is established it is sensible to initially cross with a ubiquitous Cre-deleter strain to confirm that the Cre-driven gene knockout works effectively. This will essentially produce PACAP knockout offspring, and the removal of the 4 kb portion of the PACAP gene can be detected by Southern blotting as a 13.5 kb band using either primer (see Figure A.7D).

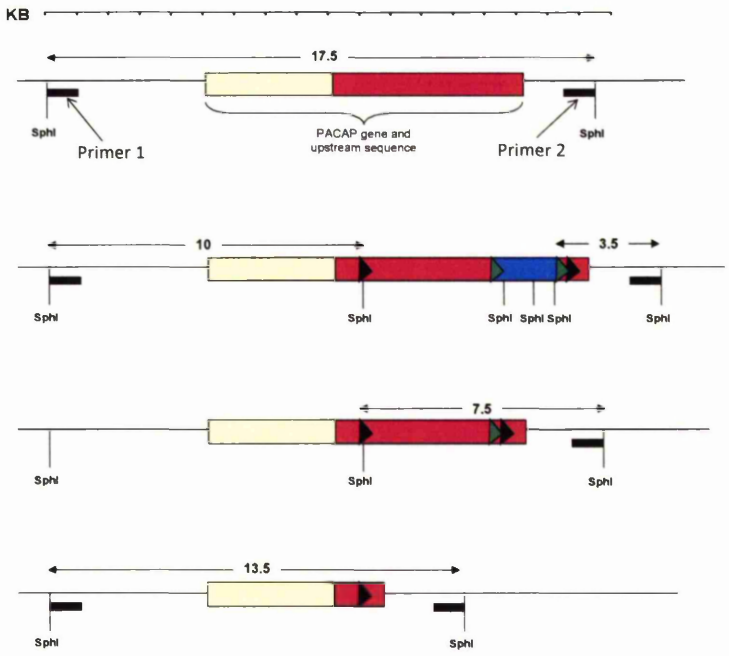

Figure A.7 Southern blotting strategy

Southern blot DNA predicted band sizes (kb) detected using oligoprobe primers 1 and 2. **A.** wildtype gene, **B.** PACAP targeting vector, **C.** floxed PACAP gene (following *Neo* deletion), **D.** deleted gene. SphI restriction sites (natural and inserted) are indicated.

A.5 SUMMARY

The development of conditional knockout technology has allowed the manipulation of genes in specific subsets of cells in order to determine their physiological relevance. This approach has been used successfully in recent years to grade the relative importance of neurones such as POMC and SF1 in the control of energy balance (Balthasar, 2006). While we and others (Gray et al., 2002; Morley et al., 1992; Mounien et al., 2008; Pataki et al., 2002) have provided histological, transgenic and pharmacological evidence for a role of PACAP in food intake and energy expenditure, this data does not distinguish between PACAP populations. As part of my PhD I am fortunate enough to have been involved in the design process of the PACAP$^{flox/flox}$ mouse which will provide invaluable information about the physiological importance of specific populations of PACAP-expressing cells.