UNIVERSITY OF SOUTHAMPTON

Hypothalamic Neuropeptide Y in the Control of Normal and Abnormal Food Intake and Metabolism

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Thesis submitted for the degree of Doctor of Medicine

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March 1994

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Doctor of Medicine

HYPOTHALAMIC NEUROPEPTIDE Y IN THE CONTROL OF NORMAL AND ABNORMAL FOOD INTAKE AND METABOLISM

by John Paul Howard Wilding

I have investigated the role of hypothalamic peptides, with particular emphasis on neuropeptide Y (NPY), in the regulation of food intake and metabolism. NPY is a peptide neurotransmitter found in several hypothalamic regions involved in the control of food intake. It is the most powerful appetite stimulant known, and its concentration and mRNA rise in food deprived animals, suggesting a physiological role.

In rats with acquired obesity, produced by feeding a palatable diet, I found increased hypothalamic NPY concentrations, but unchanged NPY mRNA, suggesting increased storage and reduced release. Reduced NPY release would be expected to increase thermogenesis, a recognised change in this model of obesity. The NPY levels in these animals were not altered by administration of the anorectic drug, dexfenfluramine. In contrast, the ob/ob mouse, which has obesity and insulin resistance inherited as an autosomal recessive trait, was found to have a three-fold increase in hypothalamic NPY mRNA, which may be a factor in the development of the obese phenotype.

Hypothalamic NPY is regulated by several hormones and I have demonstrated a reciprocal relationship between stimulation by glucocorticoids, and suppression by insulin. This may be relevant to genetic obesity, where the combination of generalised insulin resistance and high corticosterone levels may explain the observed changes in NPY mRNA. NPY mRNA is increased in the hyperphagia associated with lactation in rodents, and a further increase was found with food deprivation, suggesting a separate population of neurones may be involved in lactation and food deprivation. Given that NPY suppresses LH secretion, it seems likely that reduced LH secretion observed in lactation and food deprivation is related to increased hypothalamic NPY activity.

I have found that NPY monoclonal antibodies injected into the third cerebral ventricle in rats fasted for 24h reduces their feeding in a dose-dependent manner by up to 30%, demonstrating the importance of endogenous NPY in deprivation-induced feeding. I have found interactions between NPY and catecholaminergic and opioid feeding systems. Diabetic animals have an attenuated food intake response to NPY, perhaps because of alterations in receptors or other appetite regulating neurotransmitters.

Finally, I have investigated the acute effects of centrally administered NPY on glucose metabolism in rats, using a hyperinsulinaemic euglycaemic clamp to assess insulin resistance, and a tracer technique to measure hepatic glucose output. NPY enhances peripheral insulin sensitivity, increases hepatic glucose output, and stimulates the release of insulin and glucagon from the endocrine pancreas, suggesting complex, neurally mediated effects on carbohydrate metabolism.

Thus NPY in the hypothalamus has potent effects on food intake and metabolism; its physiological role may be to coordinate the metabolic and behavioral responses to food deprivation. Altered hypothalamic NPY production and action in obesity and diabetes suggest that inappropriate adaptive responses of the hypothalamic NPY system may play a role in the pathophysiology of these conditions.

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Acknowledgements

I would like to thank all the people in the Francis Fraser Laboratory who helped me in any way whilst I was carrying out this work. I will particularly mention other members of the hypothalamic group, including Stephen Gilbey, Phil Lambert, Michael Ajala and Aziz Al-Dokhayel. Other senior members of the lab gave vital assistance at different stages, including Mohammad Ghatei, Phil Jones and Dave Smith. I am grateful to Kay Davies for her helpful comments after reading the thesis in its entirety. Thanks must also go to various external collaborators, particulularly Cliff Bailey, Roy Batt, Gareth Williams and Yolanta Kruszynska. Thank you Mandy, Marion and John Gregory for looking after the animals. Special thanks must of course go to Steve Bloom, without whom none of this would have been possible.

Chapter 1

Introduction

"The body has a head."

The central nervous system is essential to the normal control of metabolic processes. This was recognised by the French physiologist Claude Bernard (1813-1878), when he produced diabetes in dogs by puncturing the floor of the fourth cerebral ventricle (piqure diabetes) [1]. Maintenance of energy balance is an important part of this regulation, and this involves the control of input (food intake) and output (energy expenditure). Loss of this homeostatic regulation results in either obesity or cachexia.

Understanding the role of the central nervous system in the regulation of body weight has important implications for human disease. It may provide insight into eating disorders such as anorexia and bulimia. Obesity is becoming increasingly prevalent in the Western World and is an important risk factor for the development of non insulin-dependent diabetes and ischaemic heart disease. Knowledge of the CNS mechanisms involved may lead to the development of new therapeutic approaches directed towards the prevention of these conditions.

A. The hypothalamus and its role in the control of food intake and peripheral metabolism.

The hypothalamus is the region of the brain situated at the anterior region of the diencephalon. It is an integrating centre for the control of many homeostatic processes, and influences pituitary hormone secretion, body temperature, sexual behaviour, autonomic responses to changes in emotion, plasma osmolarity and energy balance.

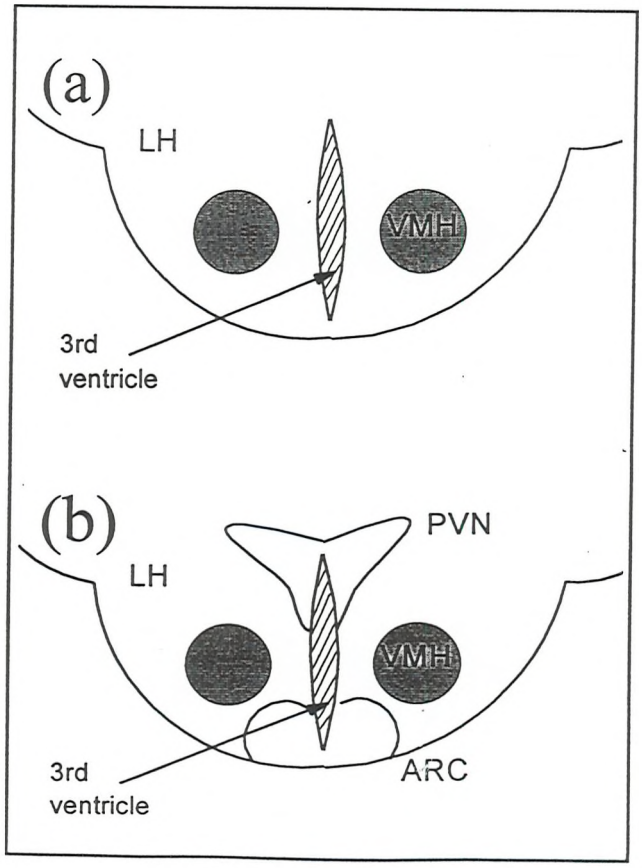
Regulation of body weight is simply a balance between intake and expenditure. Energy may be expended as work or dissipated as excess heat in 'futile' metabolic cycles (thermogenesis). In most animals, this balance is tightly regulated throughout adult life, and body mass remains constant. There is considerable evidence that the hypothalamus plays a critical role in the coordination of food intake and subsequent metabolic responses necessary to maintain energy homeostasis. Control of food intake and peripheral energy expenditure are closely linked, but will be dealt with separately initially for the purposes of this introduction.

Anatomical Pathways

Experiments carried out in the 1940's and 1950's first identified the hypothalamus as an important brain region concerned with the control of food intake. Lesions of the ventromedial hypothalamus were found to produce uncontrolled hyperphagia and obesity [2] and lesions of the lateral hypothalamic area, aphagia and weight loss [3]. This led to the development of the concept of feeding being controlled by two opposing areas in the hypothalamus, a lateral hypothalamic 'feeding centre' and a ventromedial 'satiety centre'(Fig 1.1a). This simplistic model is now outdated for several reasons. It has become clear that several extrahypothalamic brain regions are also important in controlling food intake, for example the nucleus of the tractus solitarius and the area postrema in the brainstem [4]. Diencephalic structures such as the amygdala are also involved [5]. Within the hypothalamus other important structures include the paraventricular nucleus (PVN), which is the site of action of several appetite stimulating neurotransmitters, and the arcuate nucleus (ARC), their site of synthesis [6] (Fig 1.1b).

Figure 1.1

Schematic coronal sections through the rat hypothalamus. Early studies concentrated on the opposing influences of the ventromedial hypothalamic satiety centre, and a lateral hypothalamic feeding centre (a). It is now known that many other regions are involved, including the paraventricular nucleus (PVN), and the dorsomedial nucleus (DMN) which receive fibres from the arcuate nucleus and from the brainstem (b).



The suprachiasmatic nuclei may also be involved, perhaps as part of the diurnal control of feeding. Early lesion experiments may have interfered with fibres passing through the ventromedial nucleus from the brainstem to the PVN. PVN lesions may also produce obesity, but this depends on the extent of the lesion [7]. Further evidence against the theory of two opposing centres is the fact that in animals with ventromedial lesions producing obesity, food intake initially rises and obesity develops, but after some time body weight reaches a plateau, suggesting that there is another level of control [8,9]. Similarly, animals with lateral hypothalamic lesions maintained on tube feeding for some months eventually escape from their initial anorexia, and again eat spontaneously, although at a lower level than previously [10]. This redundancy is important to remember when studying appetite control, as it means that any intervention may be rapidly compensated for by a change in the activity of other components of the system.

Hunger and satiety - opposing influences in the control of food intake

Although the anatomical concept of feeding and satiety centres is now outdated, it is still useful to consider feeding as being controlled by the two opposing influences, hunger and satiety; this has the advantage that it does not presuppose particular pathways. Hunger is only satisfied when sufficient food is eaten, producing satiety. One can envisage a system which is permanently set to stimulate food intake. This is only inhibited following a meal. The degree and duration of inhibition will vary according to the calorific content and macronutrient composition of the meal consumed, and the overall nutritional status of the animal before the meal. This process of satiation probably involves many factors including circulating metabolites, hormones and neural transmission via the autonomic nervous system (Fig 1.2). This has been termed the peripheral satiety system.

Gut Hormones

Following ingestion of a meal, several signals are activated which produce satiety. Gastric distension increases vagal activity. Nutrients promote the release of numerous gastrointestinal hormones and neurotransmitters, notably cholecystokinin (CCK) and bombesin [11]. Both of these peptides have been shown to produce satiety following peripheral injection in experimental animals [12,13]. These act on receptors which convey information to the brain via afferent parasympathetic fibres. In the case of CCK, these fibres have been traced from the GI tract via the vagus to the nucleus tractus solitarius in the brainstem and hence to the PVN [14]. This has been demonstrated by abolishing CCK

induced satiety by truncal vagotomy, and with selective lesions in the brainstem and PVN. More recently specific CCK antagonists have been developed and suggest that a peripheral CCK-A receptor and possibly a central CCK-B receptor may be involved [15,16]. The effect of bombesin is also neurally mediated, but complete spinal and vagal denervation is required to block bombesin induced satiety [17]. Pancreatic hormones may also be important, particularly glucagon, which stimulates receptors in the liver, information again being conveyed via afferent vagal fibres [18].

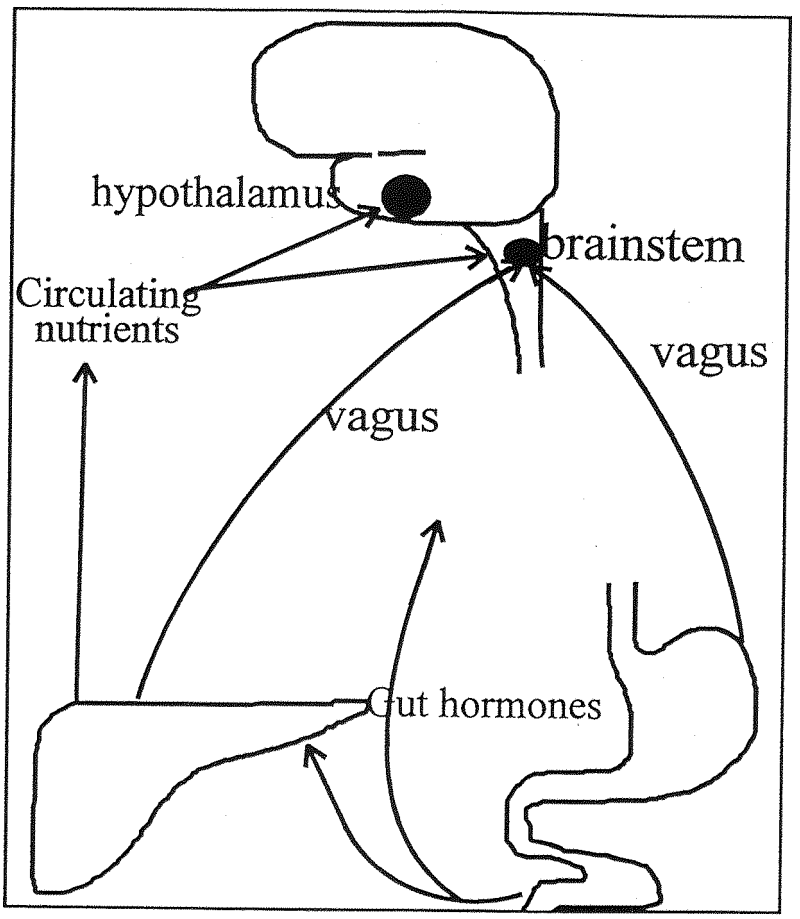


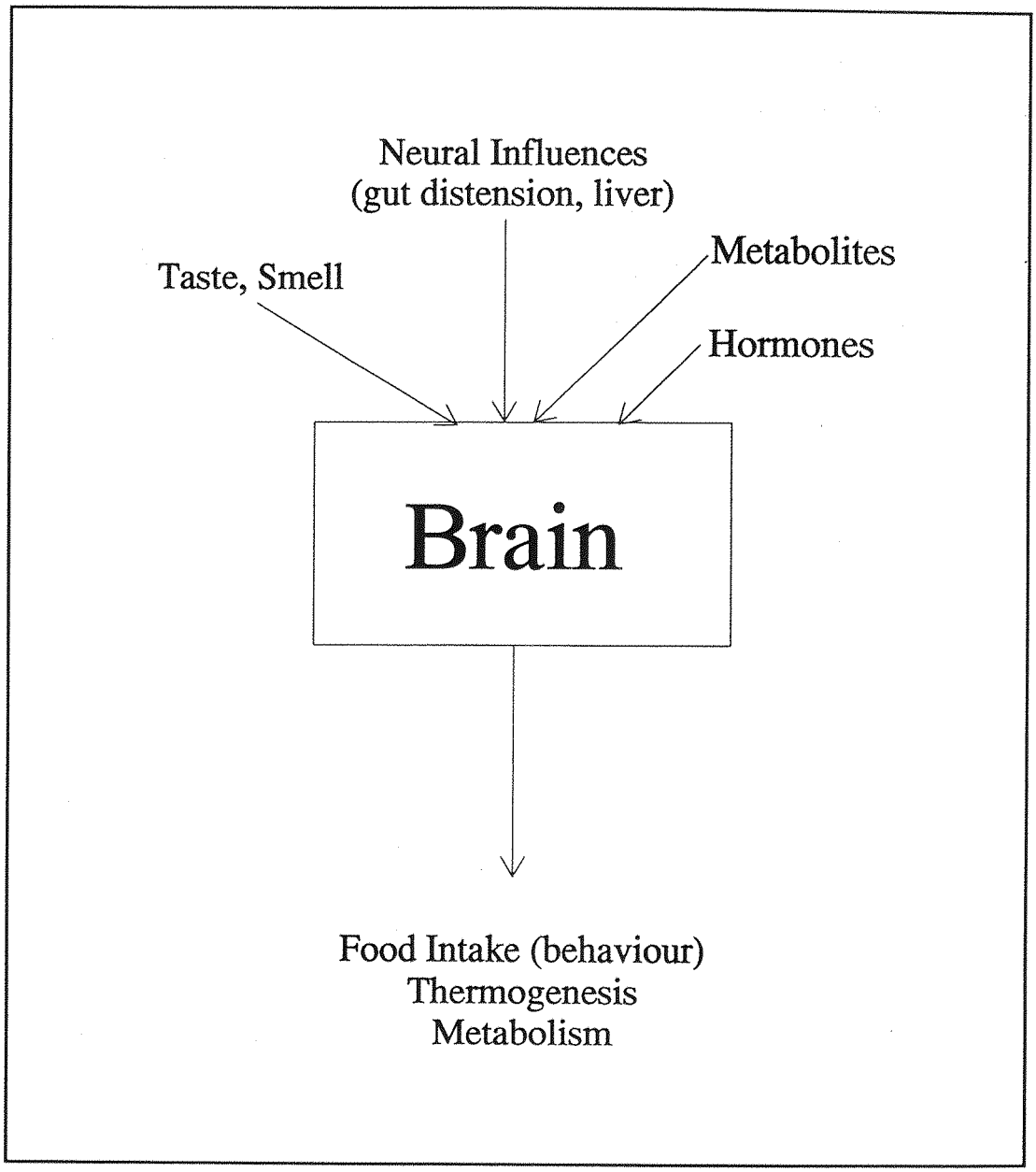
Figure 1.2
The peripheral satiety system. Satiety signals include concentrations of circulating nutrients and hormones, which may act peripherally or directly on the brain, and neural signals from the gut.

Nutrients

Nutrients may themselves serve as a satiety signal, and these effects may be mediated peripherally, from the liver, or centrally. The liver may convey information about the rate of fatty acid oxidation [19]. Examples of central signals are glucose, which may act on specific glucoreceptors in the VMH [19], and the amino acids tryptophan and tyrosine which are precursors for monoamine neurotransmitters known to be involved in appetite control [20]. The sugar acids 3,4-dihydroxybutanoic acid-γ-lactone, 2-buten-4-olide and 2,4,5-trihydroxypentanoic acid-γ-lactone have also been proposed as possible satiety signals [21]. Satietins are circulating glycoproteins which appear to have an anorexic effect, but these have not yet been fully characterised [22].

Figure 1.3

Schematic figure illustrating the various factors influencing hunger and satiety. The various metabolic, hormonal and hedonic signals are integrated in the CNS to determine when and how much food to eat and to control peripheral metabolism in a way appropriate to the nutritional status of the organism.



Insulin

Insulin deserves a special mention here. It is important to distinguish between peripheral and central effects. Peripheral insulin injection produces an increase in food intake, probably secondary to the resultant hypoglycaemia, and promotion of fat synthesis which together increase body weight [23]. Several studies have demonstrated the presence of insulin binding sites and insulin receptor mRNA at various sites in the CNS, notably in the hypothalamic arcuate nucleus [24,25]. Although insulin may be synthesised locally in the brain [26], the majority of brain insulin is probably derived from the circulation, mainly from receptor mediated uptake across the blood brain barrier [27]. Chronic administration of insulin into the CNS of baboons and rats produces a fall in food intake and body weight [28,29]. Whether or not this is physiologically important remains uncertain, but the fact that insulin antibodies increase food intake when injected chronically would support the notion that it is [29]. Brain insulin may therefore be an important signal of medium to long term body energy stores.

Macronutrient selection

The control of food intake does not only relate to quantity or calorific content, other important influences include hedonic aspects such as the sight, smell or taste of food and the proportion of macronutrients (carbohydrate, fat and protein) it contains. These may have modulatory influences on the central pathways involved in appetite control.

Diurnal variation

In both rodents and humans, the time of day is important in determining eating behaviour. Rodents are predominantly nocturnal feeders, and it is interesting that the pattern of food intake in rats given pure macronutrient diets varies according to the time of night. The first meal is typically rich in carbohydrate, with an increase in the proportion of protein and fat as the night progresses [30]. This raises the possibility that specific neurochemical signals may determine which nutrients are selected if a choice is available.

Neurotransmitters that influence food intake

A very large number of neurotransmitters may be involved in the control of food intake. Some may play a purely central role, whereas others are also involved in peripheral satiety signals as discussed above. Most of the experiments that have looked at the effects of various neurotransmitters on feeding involve the injection of the compound into the CSF or localised brain areas via stereotactically implanted cannulae. Effects on feeding in satiated animals may assess stimulatory effects, and effects in food deprived

animals inhibitory effects. Pure macronutrients can be given to study food preference. A large number of substances can be shown to inhibit food intake, whereas relatively few are stimulatory (Tables 1.1 and 1.2).

One of the problems associated with studying the inhibitory transmitters is that the effect may not be behaviour specific. The animal may not eat because it is doing something else, or because it is unwell. Behavioural studies go some way towards controlling for these phenomena, but the true physiological role of a putative appetite inhibiting transmitter cannot be established without the use of specific antagonists. For many of the peptides studied, these antagonists are not available. The known effects of the various transmitters are shown in the tables.

Table 1.1 - *Neurotransmitters that stimulate food intake*

Transmitter	Main Site of Action	Macronutrient Preference	Main Receptor Subtype Involved	Reference
Nor-adrenaline	PVN VMH	Carbohydrate	α_2	[31,32]
GABA	PVN	Carbohydrate	$GABA_A$	[33]
Dynorphin	PVN Amygdala Globus Pallidus	Fat	Kappa	[34]
Neuropeptide Y	PVN	Carbohydrate	Subtype of Y_1	[35]
Galanin	PVN	Fat	Unknown	[36]
Growth Hormone Releasing Hormone	MPO SCN	Unknown	Unknown	[37]

Table 1.2 - *Endogenous substances that inhibit food intake*

Transmitter	Main site of effect	Macronutrient preference	Receptor subtype	Reference
Serotonin	PVN	Carbohydrate	5HT1a	[38]
Dopamine	Lateral hypothalamus	Fat, Protein	D2	[39]
CCK	Peripheral NTS, PVN	Fat, carbohydrate	CCK-A ?CCK-B	[12,16]
Bombesin	Peripheral Various hypothalamic sites, NTS Amygdala		Unknown	[40]
CRF	PVN		Unknown	[41]
Neurotensin	NTS PVN, VMH, DMN		Unknown	[42] [43]
Somatostatin	ICV		Unknown	[44][45]
Calcitonin	PVN, NTS		Unknown	[42][46]
Amylin	ICV		Unknown	[47]
Glucagon	Lateral Hypothalamus		Unknown	[48]
Glucagon-like peptide 1(7-36) amide	ICV		Unknown	[49]
TRH	ICV		Unknown	[33]
Prostaglandins	ICV		Unknown	[50]
Interleukins	ICV		Unknown	[51]
PACAP	ICV		Unknown	[52]

Steroids

Corticosteroids appear to play an important role in the development of obesity in experimental animals and are likely to be important in the control of body weight by the hypothalamus. Adrenalectomy prevents weight gain in rats with lesions of the VMH or PVN [53], and attenuates it in animals with genetic obesity [54,55]. Neurones in the PVN are rich in glucocorticoid receptors, and concentrations of circulating glucocorticoids peak during the dark phase in rats, at the time when food intake is greatest [56]. PVN implants of various steroid hormones have demonstrated a stimulatory effect on carbohydrate intake with corticosterone, and a lesser effect with aldosterone [57]. The PVN is also the site of most CRF synthesis in the hypothalamus, an important inhibitor of feeding [58,59].

Hypothalamic control of peripheral metabolism

As well as the control of food intake, maintenance of energy balance is also dependent on the control of energy expenditure. Many of the transmitters described above have significant effects on the hormonal and autonomic output which influence energy expenditure.

Thermogenesis

As already mentioned, excess energy may be expended in the form of work as useful muscular or metabolic activity, or dissipated as heat. The concept of diet-induced-thermogenesis describes the adaptive phenomenon whereby 'waste' energy can be dissipated as heat in a regulated manner. Studies in laboratory animals fed different diets (particularly cafeteria diets) have demonstrated that as body weight rises there is a compensatory increase in thermogenesis, particularly in brown adipose tissue, which serves to limit the weight gain [60]. Conversely genetically obese animals and those with hypothalamic (VMH or PVN) lesions appear to have a reduction in thermogenesis [61]. Studies of autonomic nervous system activity support the idea that this phenomenon is under nervous control, with the integrating centre of that control being in the appetite regulating areas of the hypothalamus [62]. Stimulation of the ventromedial hypothalamus increases sympathetic activity to brown adipose tissue, whereas its destruction causes the reverse [63,64]. Lesions of the lateral hypothalamus, which cause anorexia and weight loss, are associated with increased sympathetic activity to brown adipose tissue and thus a higher rate of thermogenesis [65].

Glucose metabolism

Since the early experiments of Claude Bernard, it has been recognised that the brain may play a role in the regulation of glucose metabolism [1]. This occurs through a number of mechanisms, including direct effects via the autonomic nervous system on the release of pancreatic hormones, particularly insulin, and the regulation of hepatic glucose metabolism. Effects may also be mediated via alterations in the release of the pituitary hormones ACTH, TSH and GH, which indirectly influence tissue insulin sensitivity or insulin release.

Animal models of obesity and altered food intake

The study of experimental models of obesity is necessary as a means of studying the aetiology of the condition, and to provide insight into the normal control of food intake and metabolism. The models of interest are genetic models of obesity (Zucker rat, ob/ob mouse), acquired obesity (animals fed cafeteria or palatable diets). Models of anorexia due to tumours or endotoxin are also of interest, as is the physiological hyperphagia that occurs during pregnancy and lactation.

B. Neuropeptide Y: Structure, synthesis, tissue distribution, receptors and effects on food intake and metabolism

Structure, synthesis and tissue distribution

Structure and Biosynthesis

Neuropeptide Y (NPY) is a 36 amino-acid peptide first isolated from porcine brain by Tatemoto in 1982 [66]. It is a member of the pancreatic polypeptide family of peptides, which includes pancreatic polypeptide (PP) and peptide YY (PYY), with which it shares up to 50% homology, a common feature is the presence of a carboxy-terminal tyrosine amide group (figure 1.4).

The structural similarities between the various members of the pancreatic polypeptide family of peptides appear to be related to a common genetic ancestry. The genomic organisation of the three genes is very similar, with all having four exons and three introns (figure 1.5). The rat gene for NPY encodes a 98 amino acid precursor which consists of a 29 amino-acid signal peptide, the mature NPY peptide, a Gly-Lys-Arg processing site and a carboxyl-terminal flanking peptide of 30 amino acids. NPY mRNA is detectable in the 16 day old rat foetus, and levels rapidly increase to adult levels. The rat and human NPY peptide sequences are identical, with only one change in the carboxyl

terminal peptide, whereas the porcine NPY has a conservative methionine to leucine amino-acid substitution [67] (figure 1.4). More recent studies show strong evolutionary conservation across a wide variety of species [68].

Figure 1.4

Amino-acid sequences of neuropeptide Y and the related peptides PYY and PP (letters are the international single letter codes for amino-acids).

Porcine NPY

1 10 20 30 36

Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y-NH_2

Rat / Human NPY

Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-M-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y-NH_2

Porcine PYY

Y-P-A-K-P-E-A-P-G-E-D-A-S-P-E-E-L-S-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y-NH_2

Human PYY

Y-P-I-K-P-E-A-P-G-E-D-A-S-P-E-E-L-N-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y-NH_2

Rat PP

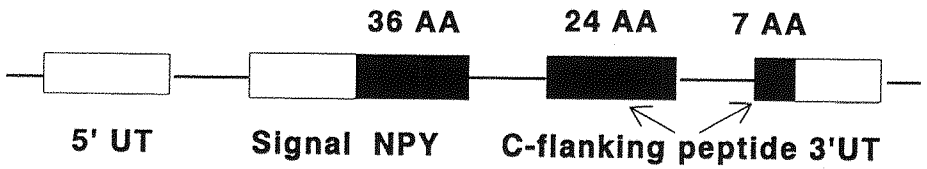
A-P-L-E-P-M-Y-P-G-D-Y-A-T-H-E-Q-R-A-Q-Y-E-T-Q-L-R-R-Y-I-N-T-L-T-R-P-R-Y-NH_2

Human PP

A-P-L-E-P-V-Y-P-G-D-N-A-T-P-E-Q-M-A-Q-Y-A-A-D-L-R-R-Y-I-N-M-L-T-R-P-R-Y-NH_2

Figure 1.5

Gene structure of rat NPY and related peptides. The gene structure for PYY and PP is identical, with 4 exons and 3 introns, suggesting a common ancestral gene.



Tissue distribution

The search which culminated in the discovery of NPY was suggested by the finding of PP-like immunoreactivity in the brain by immunocytochemistry, but little by radioimmunoassay, suggesting the presence of a PP-like molecule in the brain. NPY was found to be widely distributed in the central and peripheral nervous system of mammals, including man [69,70]. Pancreatic polypeptide is not found in the brain, and only small amounts of PYY are present in the brain, with the majority of the latter hormone being

present in endocrine cells in the gastrointestinal tract [71]. In the peripheral nervous system NPY is frequently co-localised with catecholamines, as demonstrated by its depletion following 6-hydroxydopamine lesions [72]. Other sites where there are significant quantities of NPY include the adrenal glands, spleen and pancreatic islets [73,74].

NPY immunoreactivity is present in many brain regions, including the cortex, but is particularly abundant in the hypothalamus, which has the greatest concentration of any brain region [69]. Within the hypothalamus NPY perikarya are concentrated in the arcuate nucleus. Fibres are present in the PVN and dorsomedial nucleus (DMN) projecting both from the ARC and from the brainstem, notably from the C1, A1 catecholaminergic cell groups and from the C2 (NTS) [72]. Other projections from the ARC are to the medial preoptic area and to the mesencephalic central grey. An anatomically and functionally distinct NPY system projects from the ventrolateral geniculate nucleus of the thalamus to the suprachiasmatic nucleus of the hypothalamus [75] and has been postulated as being involved in the regulation of circadian rhythms [76]. In the CNS, NPY is not only co-localised with catecholamines, as in the peripheral nervous system, but also with many other neurotransmitters, including somatostatin, enkephalin, GABA, vasopressin and FMRFamide [77-79].

Thus NPY is one of the most abundant brain peptides, and concentrations are particularly high in brain regions known to be important in the regulation of food intake and energy balance.

Peripheral Actions of NPY

NPY has numerous actions in both the peripheral and central nervous systems. The actions of NPY present in peripheral nerves are often similar to those of the frequently co-localised nor-adrenaline. NPY is a powerful vasoconstrictor. Release of NPY from sympathetic nerve terminals appears to occur in similar circumstances to noradrenaline, particularly stress, exercise, and insulin-induced hypoglycaemia in both rodents and humans [80,81]. The effects are mediated via distinct receptors, which appear to act in three main ways: firstly a direct postjunctional vasoconstrictor effect, a post-junctional potentiation of nor-adrenaline induced vasoconstriction, and finally a prejunctional suppression of noradrenaline release [82]. NPY is present in high concentrations in tissue

and plasma from subjects with phaeochromocytomas, and may be important in the development of the associated syndrome [83,84]. NPY inhibits insulin release from the pancreatic islets, and is present both in nerves supplying the islets and in the islets themselves. Within the islets it is regulated by treatments that alter insulin production and secretion and may therefore be both a neurotransmitter and a paracrine or autocrine factor involved in the control of islet hormone secretion [74,85].

Central Nervous System

Effects on blood pressure

NPY is present in many areas of the central nervous system involved in blood pressure regulation. This includes some areas of the hypothalamus, such as the posterior hypothalamic nucleus, certain brainstem regions and the spinal cord. The effects of exogenously administered NPY on blood pressure depend on the site of injection. Intracisternal administration of NPY results in hypotension whereas its administration into the third ventricle increases blood pressure [86,87]. Microinjection studies, injecting NPY into the nucleus tractus solitarius, result in a fall in blood pressure, whereas its vasopressor effects are mediated in the hypothalamus, possibly in the posterior hypothalamic nucleus [86,88]. These effects seem to be related to a general increase in sympathetic nervous system activity [89]. One suggested mechanism is a neuromodulatory effect via inhibition of stimulation evoked nor-adrenaline release [90]. Studies of NPY in the spontaneously hypertensive rat have suggested that central NPY dysregulation may be involved in the pathogenesis of hypertension in this syndrome [91], although it is possible that observed effects are secondary to changes in blood pressure [92].

Effects on behaviour

Apart from the potent effects of NPY on food intake, which are discussed below, central injection of NPY has a number of other behavioural effects. Observations (which have mainly been carried out in rodents) include a general reduction in locomotor activity, although grooming may be increased if food is present. In the absence of food, food seeking behaviour is increased. Muscle tone is increased and there is hypokinesia, suggesting a role in the nigro-striatial system [93]. NPY may also be involved in memory [94]. NPY and its receptors in the brain appear to be altered by the administration of some antidepressant drugs, which led to the suggestion that NPY could be involved in the

development of depression [95,96]. In humans, NPY concentrations have been found to be reduced in the CSF of patients with depressive illness and in patients with depression who committed suicide, however the functional relevance of these findings is not known [97].

Effects on pituitary hormone release

NPY administered icv influences the release of a number of pituitary hormones, notably luteinizing hormone (LH), growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin. The secretion of the latter three are all inhibited by NPY [98]. LH secretion is also inhibited under most circumstances, although secretion is increased in oestrogen primed ovariectomised females [99, 100]

NPY and food intake

One of the most striking effects observed after administration of NPY into the central nervous system of mammals is a dramatic increase in food intake. This was first observed by Clark in 1984, shortly after the demonstration of NPY in the hypothalamus [101], and has since been confirmed by many laboratories, and in many species [102-104]. Water intake is also increased, but to a lesser extent. Cannula mapping studies have found the PVN and the perifornical area to be one of the most sensitive sites for this effect, although injection into many other hypothalamic sites or into the fourth ventricle or frontal cortex also results in increased food intake [35,105]. Repeated injection of NPY into the PVN of the hypothalamus results in a sustained increase in food intake, and ultimately obesity [106]. The magnitude of the feeding response to NPY is greater on a molar basis than that for any other known appetite stimulant. A single injection of NPY of 0.25 nmol, into the PVN causes satiated rats to eat as much as 15g of food in one hour, which is more than 50% of a rat's normal 24 hour food intake [35]. The food intake produced by NPY has been compared to that produced by other appetite stimulating neurotransmitters, notably noradrenaline. The latency to onset of feeding (ie the time from injection to when the animal begins to eat) is notably longer for NPY (10 minutes), as opposed to less than a minute for noradrenaline. The NPY response is longer lasting however, and effects are observed for up to four hours following injection [35]. Like noradrenaline, the feeding response produced by NPY is predominantly for carbohydrate [107,108]. Rats are most

sensitive to the feeding stimulatory effects of NPY at the beginning of the dark phase, a time of day when their food intake is normally at its greatest [109].

Interactions between other appetite stimulating neurotransmitters and NPY

A number of studies have addressed the effects of other known appetite regulating neurotransmitters on feeding stimulated by NPY. These have at times produced conflicting results, but the main findings will be elucidated here. Of the stimulatory neurotransmitters, the most studied in relation to NPY induced feeding is nor-adrenaline. Feeding induced by NPY does not appear to be blocked by the administration of the α-adrenergic blocker phentolamine, although in this study the phentolamine was given after the NPY, which may explain the lack of effect [110]. Phentolamine administered into the PVN does not influence NPY-induced feeding. Yohimbine has been found to inhibit NPY induced feeding by some workers, but this remains to be confirmed [111]. However the effects of noradrenaline and NPY administered together are neither synergistic nor additive, suggesting a common effector pathway [112]. The non-specific opioid antagonist naloxone, administered either peripherally or centrally inhibits NPY-induced feeding, however it is not known whether this is a non-specific effect, as at the doses used, most opioid systems would be inhibited [113].

Effects of known satiety factors on NPY-induced feeding

Centrally administered CCK did not inhibit NPY induced feeding in one study [112], however other workers have found that peripherally administered CCK significantly attenuated NPY induced feeding [114]. The reasons for this discrepancy remain unexplained. Bombesin, calcitonin, corticotropin releasing factor (CRF) and pituitary adenylate cyclase activating peptide (PACAP) have all been shown to decrease NPY induced feeding after intracerebroventricular administration [52,112]. A study demonstrating that the antagonist α-helical CRF enhances NPY-induced feeding suggests that endogenous CRF may be important in modulating the feeding response to NPY [115]. Serotonin is thought to be an important regulator of feeding, and the serotonin agonist fenfluramine decreases feeding induced by NPY [116].

Effects of other interventions on NPY-induced feeding

A number of other local and systemic treatments appear to alter the feeding response to centrally administered NPY. Adrenalectomy results in a loss of NPY-induced feeding, and this effect can be reversed by corticosteroid replacement [117]. Bilateral mesencephalic lesions, which interrupt the pathways from midbrain nuclei to the PVN,

result in decreases in NPY concentration in the PVN, and an increase in the feeding response to NPY, with a reduction in feeding latency [118]. Neonatal administration of monosodium glutamate results in destruction of NPY producing cells in the arcuate nucleus with the expected reduction in NPY content in the arcuate nucleus, the PVN and medial preoptic area, and produces hypophagia [119,120]. NPY induced feeding is attenuated by intra-arterial infusion of glucose, but not fructose, suggesting inhibition via hypothalamic glucoregulatory centres [114].

Effects on pancreatic hormone release and glucose metabolism

The effects of central NPY injection on peripheral metabolism have also been investigated. NPY increases CRF immunoreactivity in the PVN, release of CRF from hypothalamic slices, and increases circulating corticosterone concentrations [121-124]. This is not associated with any change in circulating glucose, but there is an increase in insulin, which may be due to a change in hepatic glucose production, or evidence of regulation of peripheral insulin sensitivity by hypothalamic NPY [125]. Other important metabolic effects observed after central administration of NPY include a reduction in activity in sympathetic nerves innervating brown adipose tissue [126] and alterations in the respiratory quotient favouring metabolism of carbohydrate [127].

NPY Receptors

Receptor heterogeneity

The effects of NPY described above are mediated by specific receptors of which there appear to be at least three pharmacologically distinct subtypes. This classification is based on the different affinities of NPY, PYY and C-terminal fragments of NPY for binding in various tissues, together with different pharmacological specificity. The Y_1 receptor subtype requires the entire NPY molecule for bioactivity and is responsible for NPY-mediated vasoconstriction; selective analogues for this receptor type include {Pro^{34}}NPY and {Leu^{31},Pro^{34}}NPY. In contrast the Y_2 receptor binds the long C-terminal fragments of NPY, such as NPY_{13-36} and NPY_{16-36} and appears to be a presynaptic receptor. This receptor was first postulated following the demonstration that electrically stimulated contraction of rat vas deferens could be inhibited by NPY_{13-36} with equal

potency to the intact molecule, whereas this fragment was inactive in other preparations [82]. Both these receptor subtypes can be classified as PYY preferring, as receptor binding studies suggest a higher affinity for PYY than for NPY. A third, NPY preferring NPY receptor has been postulated to be present in rabbit kidney, and has recently been cloned [128], although the specificity of this receptor for NPY has been questioned [129].

NPY receptors in the CNS

NPY binding sites are present throughout the CNS. Using selective ligands the Y_1 and Y_2 receptors appear to be differentially distributed. The highest concentrations of NPY binding sites are found in the cortex, hippocampus, olfactory bulb and lateral septum. Moderate levels of binding are found in the thalamus, hypothalamus and brainstem [130]. In the cortex binding sites are predominantly of the Y_1 type, with higher amounts of Y_2 binding found in the hippocampus. In the hypothalamus the majority of NPY-binding sites are of the Y_2 type. However studies of the various NPY analogues and fragments on feeding behaviour support the hypothesis that feeding is mediated by a receptor of the Y_1 class, which may be atypical, as feeding is stimulated by NPY_{2-36}, which is inactive in most other Y_1 -like systems [131]. This receptor is unlikely to be NPY-preferring (Y_3), as PYY is equipotent at stimulating food intake [107].

Second Messengers

NPY Y_1 receptors appear to belong to the class of G-protein coupled receptors as the biological effects of NPY, such as vasoconstriction and food intake, can be blocked with pertussis toxin [132]. They appear to be linked to both a pertussis toxin sensitive inhibitory G-protein and to the elevation of intracellular calcium [133].

How is hypothalamic NPY regulated?

The presence of NPY and its receptors in the hypothalamus, and the demonstration of its potent effects on food intake and metabolism is suggestive of an important role in the regulation of energy homeostasis. This can only be fully understood when the factors regulating its expression and release in the hypothalamus are known, and its physiological role established using specific antagonists. At the time of starting work on this thesis, two major observations regarding the regulation of hypothalamic NPY had been made. The first, made in Professor Bloom's laboratory, was that of a wide range of neuropeptides studied, NPY content was specifically increased in the hypothalamus in animals with

diabetes [134,135], and that these changes were mainly confined to known appetite regulating areas, including the arcuate nucleus and the PVN [136]. This increase in NPY content was accompanied by increased NPY mRNA in the whole hypothalamus, and in the arcuate nucleus, suggesting that increased synthesis, rather than increased storage of NPY was occurring in this situation [137]. The second observation was that similar changes were observed following food deprivation, and that the increase in NPY was rapidly reversed by refeeding, suggesting that NPY synthesis was determined by nutritional state [138,139].

Aims

The aims of this research were to identify dietary and metabolic factors involved in the regulation of hypothalamic NPY in the rat and to investigate the importance of NPY in controlling food intake and metabolism under different physiological and pathological conditions. These objectives were achieved by performing various dietary, endocrine and pharmacological manipulations and measuring NPY concentration by radioimmunoassay in microdissected hypothalamic nuclei along with total hypothalamic NPY mRNA (which largely reflects synthesis in the arcuate nucleus). The physiological importance of NPY in the control of food intake was investigated using immunoneutralization of endogenous NPY, with a monoclonal antibody administered into the third cerebral ventricle. Interactions with other appetite regulating neurotransmitters were investigated using specific antagonists. Finally, the role of NPY in the regulation of glucose metabolism was explored using the hyperinsulinaemic euglycaemic clamp and tracer infusions after central injection of NPY.

Chapter 2

Materials and Methods

Introduction

This chapter describes in detail the methodology used for each of the major techniques employed in this thesis. Where variations on technique are used, these will be described in the chapters concerned, as will any techniques unique to a particular series of experiments.

Use of experimental animals

All animal work undertaken was carried out in accordance with procedures described in the Animals (Scientific Procedures) Act 1978, and for which I currently hold a Home Office Personal Licence and, together with Professor Bloom, a Project Licence, for the investigation of the role of regulatory peptides in the control of food intake and metabolism.

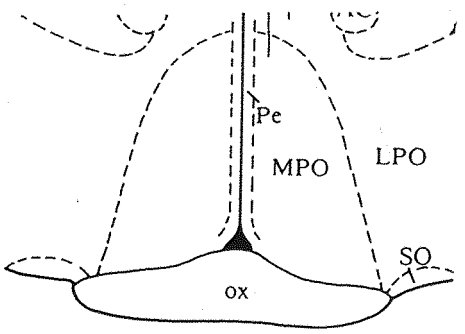
Brain Dissection

Hypothalamic Dissection

Where preparations of whole hypothalamus are required, such as for the preparation of messenger ribonucleic acid (mRNA), the dissection technique is relatively straightforward. Rats were killed by carbon dioxide inhalation, mice by cervical dislocation. Where blood samples were required, these were taken by direct cardiac puncture immediately after the animal was killed. The animal was then decapitated and the brain carefully removed and placed on its ventral surface, the optic nerves and any blood vessels were dissected away. The hypothalamus was dissected out under a binocular dissecting microscope, using coronal incisions at the optic chiasm anteriorly and the mammillary bodies posteriorly, followed by incisions along the perihypothalamic sulci and the anterior commissure. In some experiments the hypothalamus was then divided into two halves by a further vertical cut; one half was kept at -70°C for later measurement of NPY mRNA, the other was divided into medial and lateral parts using vertical cuts through the fornix and the mammillohypothalamic bundle. This separates the hypothalamus into a nucleus rich medial part (including the paraventricular nucleus, arcuate nucleus and ventromedial nucleus) and a nucleus poor lateral part.

Figure 2.1

Areas of hypothalamus removed in microdissection of individual nuclei. Figures are taken from the atlas of Paxinos and Watson [140]



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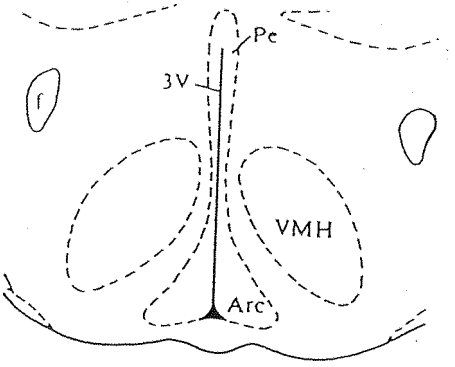

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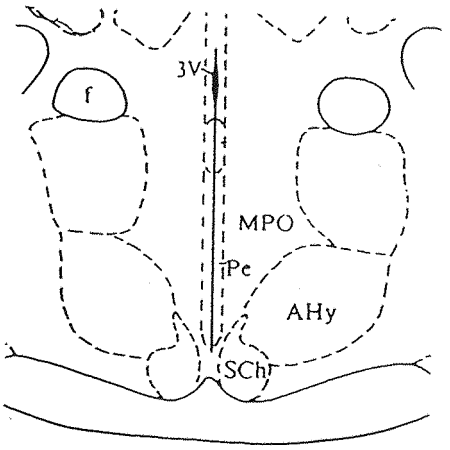

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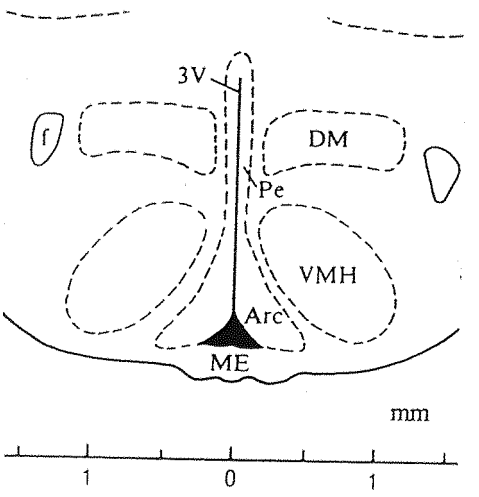

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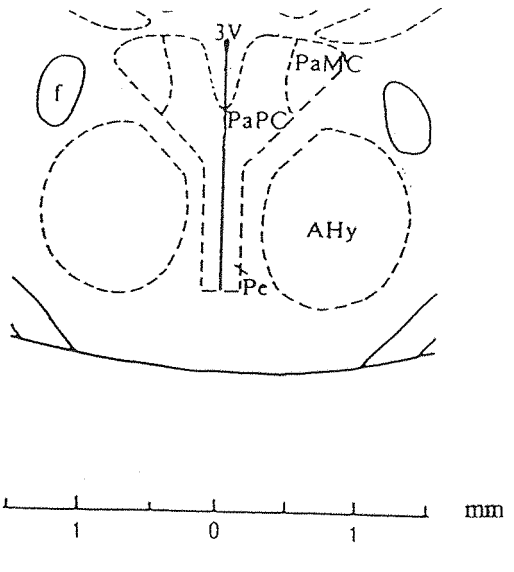


Bregma - 1.8mm

3V = Third Ventricle
f = fornix
MPO = Medial Preoptic Area
LPO = Lateral Preoptic Area
SO = Supraoptic nucleus
OX = Optic Chiasm
Pe = Periventricular nucleus
SCh = Suprachiasmatic nucleus
AHy = Anterior Hypothalamus
PaMC = Magnocellular paraventricular nucleus
PaPC = Parvocellular paraventricular nucleus
VMH = ventromedial nucleus
DM = Dorsomedial nucleus
ARC = Arcuate nucleus
ME = Median Eminence

Hypothalamic microdissection

For most of the studies employing radioimmunoassay, individual hypothalamic nuclei and regions were dissected out using a modification of the method described by Jacobowitz [141]. The brain was rapidly removed, as described above, and the frontal cortex and cerebellum were separated by vertical slices in front of the optic chiasm and behind the mammillary bodies, aided by the use of a special slotted perspex box to ensure consistency in the plane of slicing. The resulting brain slice was attached to a cold-stage using cyanoacrylate adhesive (Superglue 3, Loctite, Welwyn Garden City, Hertfordshire, UK), with the anterior surface uppermost and placed in a vibrating microtome containing ice-cold saline. Cuts of 100μm were then made until the anterior commissure was clearly visible at an equivalent level to the illustration in a rat brain atlas [140]. From this reference point serial slices were obtained through the brain at 500μm intervals. These were examined under a binocular dissecting microscope and hypothalamic nuclei were punched, using a blunt 19g needle attached to a 1ml syringe, using a rat brain atlas [140] as a reference. The medial preoptic area was carefully dissected using a small scalpel. Samples were expelled into acetic acid (500μl of 0.5mol/l) by gentle pressure on the syringe plunger and boiled for 10 minutes to extract the peptides. All samples were then frozen at -20°C until assay. The nuclei sampled were the medial preoptic area (MPO), anterior hypothalamic area (AHA), lateral hypothalamic area (LHA), suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), ventromedial nucleus (VMH), arcuate nucleus (ARC) and the dorsomedial nucleus (DMN) (Fig 2.1).

Radioimmunoassay of NPY and other peptides

Introduction

Radioimmunoassay is an invaluable technique for measuring the content of biological substances in tissue and plasma. It has gained widespread use since its first description by Yalow and Berson in 1958 [142]. Radioimmunoassay is based upon the binding of antigen (Ag) to a specific antibody (Ab). At equilibrium, the amount of antibody bound to antigen and the amount of free antigen and antibody are constant:

$$K_a = \frac{[AgAb]}{[Ag][Ab]}$$

K_a is the association constant, and describes the avidity of the antibody for the antigen and is in the order of 10^7 to 10^{12} M^{-1} for a typical radioimmunoassay antibody. Radioimmunoassay depends upon the incubation of a constant amount of radiolabelled antigen, a constant (and limiting) amount of antibody with a sample containing an unknown amount of antigen. At equilibrium, the proportion of antibody-bound to free labelled antigen is proportional to the amount of unlabelled antigen present. A standard curve is constructed using known quantities of unlabelled antigen, and unknown samples can then be compared to the standard curve.

The requirements for a radioimmunoassay are therefore an appropriate specific antibody with a high binding avidity (which will be a determinant of assay sensitivity), a quantity of radiolabelled antigen (^{125}I is most commonly used, but ^{3}H is an alternative), and known quantities of antigen from which a standard curve can be constructed. Other conditions such as pH and temperature should also be controlled, and ideal conditions may vary for each assay. Most antibodies are obtained from rabbit immunization with peptide + adjuvant, although monoclonal antibodies can also be used.

Separation of free and bound fractions is necessary after incubation. This may be achieved in several ways. Immunoprecipitation involves the addition of a second antibody which reacts with the first (eg a goat anti-rabbit antibody). Chemical separation involves the use of alcohol, polyethylene glycol or ammonium sulphate. The method used in these studies was physical separation using dextran-coated charcoal.

Once the free and bound fractions have been separated, radioactivity is counted in all the fractions. Although it is possible to then calculate the results by hand (ie ratio of free and bound antibody for each tube), this is quickly and conveniently carried out by a microcomputer linked to the gamma counter.

Quality Control

Quality control is an essential aspect of any assay system. Throughout these studies all assays for a particular experiment were carried out in a single assay to remove any possibility of interassay variation. Systematic errors due to intraassay variation do occur, and one of the commonest is 'assay drift', which may occur due to systematic errors, particularly in the timing of assay separation. These errors were avoided by 1) placing samples where direct comparisons were to be made (eg all samples from a particular nucleus) close together in the assay, and 2) Alternating tubes from different limbs of the study. Intraassay variation was also checked for by the use of zero tubes at

regular intervals during the assay. Counting error is kept to a minimum by counting for at least 4000 counts, giving a counting error of about 1.3%.

Radioimmunoassay of neuropeptide Y

I will describe the assay procedure used for the measurement of NPY in detail, and then note briefly any variations in procedure used in the measurement of other peptides in these studies.

All assay measurements were carried out in duplicate. Numbered plastic tubes were filled with 600µl of 60mM phosphate buffer pH 7.4 (containing EDTA and 0.3% bovine serum albumin). For microdissected hypothalamic nuclei, the optimum sample volume addition was 20µl, for whole hypothalami 0.2µl additions were needed, which were obtained by dilution of a 10µl aliquot. As the samples were extracted into 0.5M acetic acid, 20µl of this was added to all non-sample tubes to maintain constant pH. Two standard curves were constructed for each assay, along with blank and excess antibody tubes. Zero tubes were inserted every 20-30 assay tubes as internal quality controls. Standard curves used porcine NPY (Bachem), calculated as being 80% pure, made up to a nominal concentration of 0.5pmol/ml in assay buffer and added in duplicate in volumes of 1,2,3,5,10,15,20,30,50 and 100µl using glass micropipettes to ensure accuracy. Volumes of 20µl and above were corrected by removal of buffer from the appropriate tubes. The antibody used (NPY YN10) was developed by rabbit immunisation [143], and used at a final titre of 1:120000. There was a preincubation period of 24hrs at 4° C before the addition of Bolton-Hunter labelled porcine NPY at approximately 300 counts per assay tube. The assay was then incubated for a further 4 days before separation using 250µl of dextran-charcoal slurry (0.6mg:6mg w/w) suspended in 60mM phosphate buffer containing EDTA and 0.3% gelatine. Tubes were then centrifuged at 4°C at 2500 rpm. The supernatant was removed to fresh tubes by hand using glass pipettes. The radioactivity was counted in the free (pellet) and bound (supernatant) fractions using four 16 well gamma counters (NE1600, Nuclear Enterprises, Scotland) linked to a microcomputer. The computer calculated free and bound fractions and was then able to produce a standard curve and compute sample concentrations for the unknowns. This data could then be imported into a spreadsheet for further analysis.

The detection limit for this assay, calculated as the smallest standard curve value measured, which is 2 SD from the zero standard (ie at the 95% confidence interval) was 0.6 fmol / assay tube.

Measurement of protein content of tissue samples

Measurement of peptide content alone may give an incorrect or variable answer if the original amount of tissue added is not taken into consideration. For large samples this can easily be accomplished by weighing the tissue, but this is impractical for small samples such as microdissected hypothalamic nuclei. The amount of protein or DNA present in a sample is usually proportional to the amount of tissue present, and for these studies I chose to measure protein content as there is a sensitive and rapid assay available.

Protein content was measured in a 50µl aliquot of the sample, diluted in 60mM phosphate buffer to a volume of 500µl, using the Coomasie Blue Micromethod (Pierce, Chester, UK). Standard curves were constructed using bovine serum albumin supplied in the kit, diluted with phosphate buffer to a volume of 500µl. The addition of 500µl of the Coomassie Blue reagent produces a colour change proportional to the amount of protein present. The absorbance at 595nM was then read in a spectrophotometer (Shimadzu, Kyoto, Japan), which automatically calculates a standard curve. The final results for the unknowns were then calculated from this curve by the machine. These were then combined with the results produced from the radioimmunoassay on a spreadsheet, and the final results calculated and expressed as fmol/µg protein.

Extraction of RNA and Northern Blotting

For the semiquantitative measurement of NPY mRNA in hypothalamus, total hypothalamic RNA was extracted, size separated on agarose-formaldehyde gels, transferred to nylon membranes by capillary blotting, and then probed using a specific cDNA probe. The amount of radioactivity present was determined using a surface β-counter and compared to the total amount of poly A+ RNA present by repeating the probing procedure using an oligo dT probe.

RNA Extraction

Total RNA was extracted from individual hypothalami using acid guanidinium thiocyanate-phenol-chloroform extraction [144]. This method relies on guanidinium thiocyanate to lyse cells and inactivate RNAse enzymes. Each hypothalamus was homogenised in 2mls of Solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7;0.5% sarcosyl, 0.1M 2-mercaptoethanol), and transferred to a sterile glass tube to which 0.2mls sodium acetate (pH5.2), 2mls phenol and 1ml chloroform in IAA were added. This was mixed well and left on ice for 15 minutes. Following this the lysate was

centrifuged at 10000g for 20 minutes, with the brake off to avoid disturbing the interface. The supernatant was then transferred to a fresh siliconised tube and the RNA precipitated by the addition of 2mls isopropanol, and left for one hour. Sedimentation was then carried out at 15000g for 20 minutes, and the resulting pellet resuspended in 0.4ml solution D, precipitated again with 0.4ml isopropanol for 1hour, centrifuged again, the supernatant removed, and washed in 1ml 70% ethanol to remove any traces of phenol or salt contamination, centrifuged and the resulting pellet dissolved in autoclaved, glass distilled water. The amount of RNA was determined by reading the absorbance at 260nM in a spectrophotometer. This yielded approximately 50µg RNA per hypothalamus. The RNA was again precipitated, by the addition of 2.5ml absolute ethanol, left for 1 hour, centrifuged and the pellet vacuum dried for 15 minutes. The pellet was then resuspended in autoclaved, distilled water at a final concentration of 5mg/ml. Integrity of the RNA was confirmed by separating 5µg of RNA on a 1% agarose formaldehyde gel, staining with ethidium bromide (stained for 20 minutes in 100mls of 1X TE, to which 10µl ethidium bromide had been added, and destained for 16 hours in 1X TE, then visualised under UV light.

Northern Blotting

40-50µg of RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel and transferred to a Hybond nylon membrane (Amersham International, Amersham, Bucks., UK). The filter was baked for 2 h at 80C before being probed for NPY mRNA. A marker track was run on the same gel, and stained with ethidium bromide as described above.

Probe synthesis and hybridisation

Prehybridization was for 2 h at 42C in 50 mmol sodium phosphate buffer/l (pH 6.8)/50% formamide/5x SSC (1x SSC = 0.15mol NaCl/l/0.015mol sodium citrate/l, pH 7.0)/1x Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/100 µg denatured sonicated herring sperm DNA/ml. An NPY probe was prepared with (^{32}P)-dCTP by random primer labelling of a 490 base pair rat NPY cDNA fragment (provided by Dr J.E. Dixon) to a specific activity of 1.5 x 10^9 dpm/µg. The filter was then hybridized for 24 h at 42C in the prehybridization solution to which had been added dextran sulphate (100µg/ml) and labelled probe at a concentration of 1 ng/ml. Finally the filter was washed in a solution of 2x SSC/0.2% sodium dodecyl sulphate (SDS) at 60C for 1 hour, followed by a further wash in 0.1% SSC/0.1% SDS for 1 hour.

Quantification of mRNA

Quantitative estimates of relative levels of specific peptide mRNA were made using a two-dimensional surface β-counter and computer based image capture system and image analysis software (Autograph, Positron Systems Ltd, Oxford, UK). This has a resolution of 1mm, and provides an accurate estimate of disintegrations from a defined area. Because it measures actual radioactive counts, the results produced are more linear, and obtained more rapidly than with conventional densitometry. After counting, the filter was exposed to film for 48 hrs to obtain high definition pictures of the blot. The counts obtained were then normalized to the amount of total mRNA present by stripping the filter in 0.5% SDS, 1% TE at 80C for 30 minutes and then re-probing with oligo(dT), and again counting using the Autograph software. Final results are therefore expressed as the ratio of counts of NPY mRNA and polyA+RNA/10. Between four and six replicates from each experimental group were used. These were all extracted at the same time and where possible probed on the same filter, to minimise experimental variation.

CNS cannulation and injection

Rats were anaesthetized (Hypnorm 8mg/kg i.p. Janssen Pharmaceutical Ltd, Oxford, UK. Hypnovel 4mg/kg i.p. Roche Products Ltd, Welwyn, UK.) and placed in a Kopf stereotaxic frame. Permanent 23 gauge stainless steel cannulae (Plastics One inc. Roanoke, VA, USA) were stereotactically placed 0.8mm posterior to bregma on the mid-line and implanted 6.5mm below the outer surface of the skull into the third cerebral ventricle. After surgery an obturator was inserted into each cannulae to prevent blockage. All animals were allowed a period of seven days to recover, following surgery, before being used in any study.

The injection of substances was by a stainless steel injector placed in, and projecting 0.5mm below, the tip of the cannulae. The injector was connected by polythene tubing (id 0.5mm, od 1mm) to a Hamilton (Reno, NV) syringe in a Harvard IV syringe pump (model 2681, Harvard Apparatus, Natick, MA, USA) set to dispense 10μl of solution per minute. All compounds were dissolved in 0.9% saline. The placement of cannulae were verified at the end of each study by the injection of 10μl of ink, removal of the brain, snap freezing and visual examination of coronal brain slices.

Chapter 3

Hypothalamic NPY in dietary obesity and the effects of dexfenfluramine

A. Hypothalamic NPY and Diet Induced Obesity

Introduction

The synthesis and release of any neurotransmitter which is concerned with the regulation of food intake may alter in response to a change either in diet composition, or in the amount of energy consumed. Animals fed either a varied 'cafeteria diet' or a highly palatable diet, are characterized by hyperphagia, and develop obesity, although this may be less than expected from the additional calories consumed due to a compensatory increase in diet-induced thermogenesis [145]. This is an attractive model for the study of human obesity, as it seems to reflect the effects of easy availability of highly palatable food which is the case in much of Western society. The mechanisms underlying the changes in food intake and thermogenesis are not understood, but probably involve an increased drive to eat, perhaps triggered by the high variety and palatability of the foods offered. A number of neurotransmitter systems are altered in this model of obesity including opiates [146], and serotonin [147].

This study was designed to investigate a possible role for NPY in the development of obesity in animals fed a highly palatable diet, which was of similar macronutrient composition to the control diet.

Materials and Methods

Animals

Two groups of fourteen male Wistar rats (A. Tuck and Sons Ltd, Battlesbridge, Essex, UK), initially weighing 234 ± 3g, were studied. They were kept in a temperature-controlled environment (21-23^{0}C) with a 13h light:11 hour darkness cycle. They had free access to food and water throughout the study.

Diet

One group of animals was fed a palatable diet which consisted of 33% chow, 33% Nestle condensed milk, and 7% sucrose by weight, with the remainder being water. This provided 68% energy as carbohydrate, 16% as protein and 13% as fat. This diet is a modification of a similar diet [148], and produces reliable weight gain over controls. The remaining animals had free access to standard pelleted chow (Labsure, Poole, Dorset, UK) throughout the study, which provides 66% of energy as carbohydrate, 25% as protein and 9% as fat. All animals were weighed weekly for the seven weeks of the study. Weight

changes were analysed at the start of the study and immediately before the rats were killed.

At the end of the study period, animals were killed by CO_2 inhalation and blood collected by cardiac puncture; blood was centrifuged immediately and the plasma separated and stored at -20^{0}C until the assay of glucose and insulin.

Brain microdissection

Ten animals from each group were used for measurement of NPY. Nuclei were microdissected as described in chapter 2. The nuclei sampled were the medial preoptic area (MPO), anterior hypothalamic area (AHA), lateral hypothalamic area (LHA), suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), ventromedial nucleus (VMH), arcuate nucleus (ARC) and the dorsomedial nucleus (DMN). Samples were immediately placed in 500µl acetic acid (0.5mol/l) and boiled for 10 minutes to extract the peptides. The remaining four hypothalami were dissected en bloc, snap-frozen in liquid nitrogen and stored at -70^{0}C until RNA extraction and Northern blotting for NPY mRNA.

Assay of glucose, insulin and NPY

Plasma glucose was measured in a Beckman glucose analyser. Insulin was measured by radioimmunoassay using porcine insulin standard, ^{125}I-labelled porcine insulin (Amersham International, Amersham, Buckinghamshire, UK) and guinea-pig anti-insulin serum (Wellcome, Beckenham, UK). The detection limit of this assay was 5pmol/tube (95% confidence interval). Inter- and intra-assay coefficients of variation were 6.8 and 3.5% respectively NPY was assayed in the extracts by radioimmunoassay as described earlier. Inter- and intra-assay coefficients of variation were 10.4 and 5.1% respectively. Protein was measured in all samples using the Coomassie Blue micromethod (Pierce, Rockford, USA), final peptide concentrations being expressed as fmol/µg protein.

Measurement of NPY mRNA by Northern blot analysis

The remaining four hypothalami from each group were used for Northern blot analysis of NPY mRNA as described in Chapter 2.

Statistical Analysis

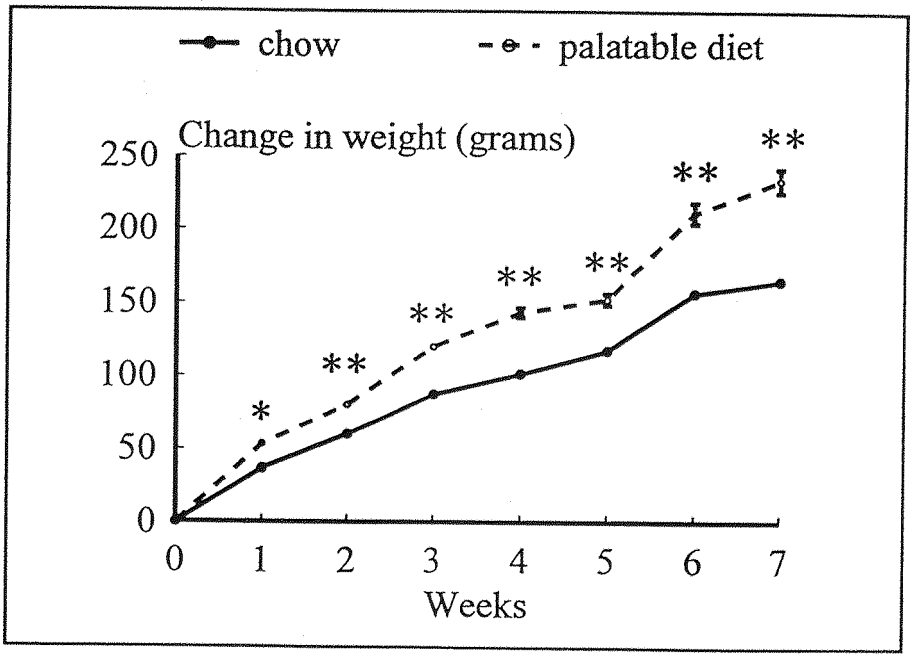
All results are given as mean ± SEM. Metabolic and weight data were compared using repeated measures analysis of variance (ANOVA), or Student's t-test, as appropriate. Comparison between the groups for the effects of diet was made by ANOVA, followed by Student's t-test to compare concentrations in individual hypothalamic nuclei. All analysis was done using the Systat (Systat, Evanston, IL, USA) package running on an IBM personal computer. Statistical significance was taken at $p<0.05$.

Results

Animals fed the palatable diet gained weight more rapidly than controls, and despite starting at similar weights were 14% heavier than controls at the end of the study (461 ± 11g vs 405 ± 5g; $p<0.001$); the pattern of weight gain can be seen in Fig 3.1. Plasma concentrations of glucose were similar in the two groups (5.4 ± 0.25mmol/l, for rats on the palatable diet vs 5.3 ± 0.26mmol/l, for the controls), but insulin concentrations were higher in animals fed the palatable diet (637 ± 63pmol/l vs 448 ± 41pmol/l; $p<0.05$). There were no significant differences between those animals used for RIA measurement and those used for mRNA measurement for any of these parameters.

Figure 3.1

Weight changes in palatable diet fed animals (broken line) compared to controls fed normal chow (solid line).



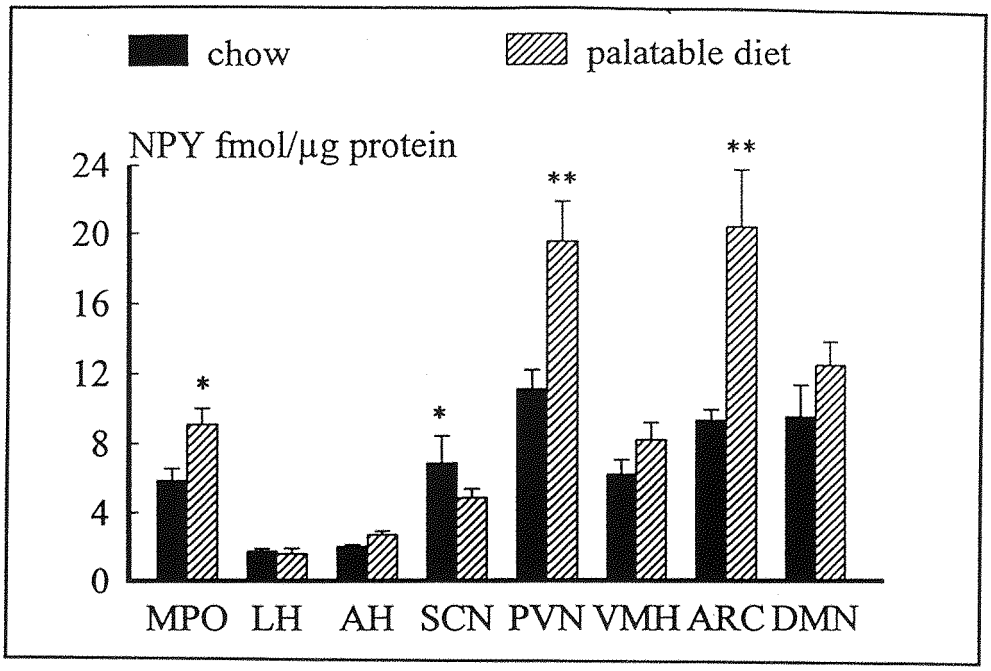
** $p<0.01$, ** $p<0.001$, Student's t-test, $n=14$*

NPY Radioimmunoassay

Protein concentrations in individual hypothalamic nuclei were similar in control and palatable diet treated groups. ANOVA revealed a significant difference between groups for NPY concentration ($p<0.001$). In animals fed the palatable diet NPY concentrations were increased in the PVN, ARC, MPO and AHA. There were no significant differences for any of the other nuclei studied (Fig 3.2).

Figure 3.2

NPY concentrations in microdissected hypothalamic nuclei in chow-fed controls (solid bars) and palatable-diet fed animals (hatched bars).



**p<0.02, **p<0.01, Students t-test, n=10.*

NPY mRNA

In contrast to the RIA data there were no differences between the groups in total hypothalamic NPY mRNA as measured by Northern Blot analysis (controls 2.4 ± 0.3; diet 2.1 ± 0.16).

Discussion

NPY appears to be a physiological mediator of feeding, and chronic administration into the PVN of the hypothalamus causes increases in carbohydrate and fat intake which result in an increase in body fat stores and obesity [106,149]. Conversely, hypothalamic concentrations of NPY are raised in starvation, diabetes and glucocorticoid administration, all catabolic states [134,150,151]. Thus peripheral metabolic status appears to regulate hypothalamic NPY concentrations, although it is not known what afferent signals regulate NPY release and gene expression in the hypothalamus. Although food intake was not measured in this study, it is recognised that animals with diet-induced obesity are hyperphagic [152]. Several possible explanations have been put forward to explain this, including changes in brain serotonin and opiates [146,147]. This study was undertaken to test the hypothesis that increased activity of NPY within specific hypothalamic nuclei

might be responsible for the hyperphagia and weight gain associated with administration of a palatable diet.

The results demonstrate that while there was no overall change in hypothalamic NPY mRNA concentrations a significant increase in NPY concentrations occurred in important appetite-regulating areas of the hypothalamus. Although regional changes in NPY synthesis are possible, the majority of hypothalamic NPY mRNA is found in the arcuate nucleus [137], and it is likely that the results represent unchanged synthesis at this site. Neuropeptide Y synthesis also occurs in the brainstem, in fibres which project to the PVN, and transection of these fibres leads to increased sensitivity to exogenous NPY [118,153]; however increases also occurred in the arcuate nucleus, suggesting that the arcuate nucleus / PVN circuit is the main circuit disturbed in this model. However the possibility that some of the increased NPY found in the PVN originated in the brainstem cannot be excluded. These results may be explained in two possible ways. First, there could be an increased rate of translation of NPY associated with increased release and higher turnover of NPY mRNA, perhaps as an effect of the increased food palatability. However in other experimental situations where NPY concentrations are raised there is a concomitant increase in NPY mRNA, suggesting that regulation is more likely to occur at the level of transcription [137,150,154]. The second and more probable explanation is that in these obese, satiated animals the NPY drive to eat is attenuated. This would result in reduced release and, if NPY synthesis were not also down-regulated, increased storage of hypothalamic NPY. It is possible that differences in diet composition are responsible for this effect, however there was little difference in the fat and carbohydrate content of the diets in our study, the main variable being the palatability of the food offered. Other workers have shown that two weeks of a high fat diet (resulting in increased weight) caused a small reduction in NPY concentration in the LHA and high carbohydrate feeding (resulting in reduced weight gain compared to controls) produced a reduction in NPY in the PVN [155]. In contrast, in the fasting state or in experimental diabetes both NPY concentrations and mRNA are increased [134,137,150,156], presumably reflecting undernutrition in these animals.

Several hormonal systems have been found to be altered in diet-induced obesity, including a reduction in basal growth hormone (GH) and growth hormone releasing-factor stimulated GH release [157], increases in serum tri-iodothyronine concentrations [60] and in female animals prolonged dioestrus [158]. NPY within the PVN has been implicated in

the control of secretion of GH [159] and thyrotrophin releasing hormone [160] and there is evidence for the involvement of hypothalamic NPY in the release of luteinizing hormone (LH)[161]. Thus changes in hypothalamic NPY, including those in the MPO, which is involved in the control of pituitary LH release, may be involved in these endocrine changes.

Paraventricular injection of NPY has been demonstrated to cause an increase in the respiratory quotient, without changing overall energy expenditure, suggesting that it may control peripheral metabolism by favouring the metabolism of carbohydrate and promoting fat synthesis from carbohydrate [127]. It has also been suggested that NPY may act to decrease thermogenesis, as the weight gain seen after chronic NPY administration is disproportionate to food intake [106]. Furthermore, NPY injected into the third ventricle or the PVN has been shown to inhibit sympathetic nerve activity to interscapular brown adipose tissue, and thermogenic activity assessed by GDP-binding [126,162]. A reduction in the activity of the NPYergic system in diet-induced obesity might contribute to increased thermogenesis, a phenomenon well recognized in animals fed cafeteria or palatable diets [60]. The apparent underactivity of the NPY system in these obese animals may thus represent an attempt to decrease fat synthesis and increase thermogenesis in response to a rise in body fat content.

The peripheral metabolic signals regulating hypothalamic NPY synthesis and release remain unknown. It has been suggested that, as NPY preferentially stimulates carbohydrate ingestion and hypothalamic NPY is increased in diabetes, peripheral insulin or glucose concentrations may play a role [137]. The animals fed the palatable diet in this study had similar glucose concentrations compared with controls, so it is unlikely that glucose alone was the signal. However levels of plasma insulin were higher, possibly reflecting a degree of insulin resistance in this model of obesity. It has been reported that there is no effect of acute or chronic peripheral insulin administration on hypothalamic NPY in individual hypothalamic nuclei [163], but more recently it has been found that central insulin administration reverses the increases in NPY mRNA seen in food deprived animals, and peripheral insulin administration prevents corticosteroid induced changes in NPY synthesis ([164] Chapter 5). Higher levels of insulin might therefore be expected to reduce NPY synthesis, this was not observed in this study, but it remains possible that insulin may regulate NPY release under some circumstances or that the ratio of glucose to insulin may be important in the control of hypothalamic NPY.

In conclusion, this data would support the hypothesis that hypothalamic NPY release reflects nutritional status and suggest that increased hypothalamic NPY activity does not mediate the hyperphagia that is a feature of diet-induced obesity. A reduction in NPY release would be expected to facilitate thermogenesis, an important effect seen in animals fed cafeteria or palatable diets. Finally, changes in hypothalamic NPY may explain some of the other neuroendocrine abnormalities seen in this model of obesity.

B. Effect of the serotonin re-uptake inhibitor, dexfenfluramine on hypothalamic NPY in diet-induced obesity

Introduction

A further study was undertaken with the aim of looking at possible changes in other hypothalamic peptides in dietary obesity and to investigate the effect of intervention with a serotoninergic drug, dexfenfluramine, which is known to be effective at attenuating obesity in this model, both via a reduction in food intake and via effects on diet-induced thermogenesis, as it prevents the reduction in thermogenesis seen when animals are pair-fed with chow controls in diet-induced obesity [148]. Furthermore, serotonin has been suggested to interact with NPY in the hypothalamus in the control of food intake [165], and it would be interesting to know if drugs which acted via the serotoninergic system influenced the synthesis of hypothalamic NPY, or other appetite regulating peptides.

The other hypothalamic neuropeptides investigated in this study were galanin, somatostatin and neurotensin. Galanin produces feeding with a preference for fat when injected into the PVN [36]. Low doses of somatostatin appear to increase food intake when administered centrally, although a reduction in food intake may occur when higher doses are used [45]. Neurotensin has the opposite effect in that local injection into the hypothalamus produces a reduction in food intake [43]. Hypothalamic neurotensin concentrations are decreased in the obese Zucker rat, a model in which NPY is increased [37].

It has been suggested that serotonin and its agonists act in part by influencing orexigenic peptide hormones such as NPY and galanin. There is some evidence that this may be the case, as dexfenfluramine inhibits NPY induced feeding in rats [116], and NPY and galanin reduce turnover of hypothalamic serotonin [166]. Another study has suggested that NPY concentrations may be reduced in some hypothalamic nuclei following acute

administration of fenfluramine [167].

The aim of this study was to assess the effects of feeding a palatable diet and dexfenfluramine treatment on body weight and hypothalamic neuropeptides important in appetite control.

Methods

Animals

Eighty-five male Wistar rats weighing 230-250g (A. Tuck and Sons Ltd, Battlesbridge, Essex, UK) were caged in groups of five with a 11/13 hour light/dark cycle and free access to food and water. The animals were kept in the animal facility for 2 weeks acclimatization before starting the study.

Diet

Animals were initially divided into two groups, sixty-five animals were fed a palatable diet which consisted of 33% chow, 33% Nestle condensed milk, 7% sucrose by weight with the remainder being water. This provided 68% energy as carbohydrate, 17% as protein and 13% as fat. The remaining animals had free access to standard pelleted chow (PRD pellets, Labsure, Poole, Dorset, UK) throughout the study, which provides 66% of energy as carbohydrate, 25% as protein and 9% as fat.

Study Design

Rats were kept on the diet for 6 weeks at which time they were randomly divided into two groups to assess the effect of 7 days and 28 days treatment with dexfenfluramine respectively. The twenty chow-fed animals were treated and analyzed with the 28 day group. All animals were weighed weekly throughout the study.

Dexfenfluramine treatment

Each group of rats was divided into two, receiving either active treatment or saline. Dexfenfluramine (Servier, Iris, Nevilly-sur-Seine, France), was given at a dose of 1.8mg/kg/day, via a mini-osmotic pump (Alza, Palo Alto, Ca.) implanted intraperitoneally under ether anaesthesia. For the 7 day study Alzet 2001 pumps were used each containing dexfenfluramine 5.7mg in 200µl 0.9% saline. For the 28 day study, starting a week later, 2ML4 pumps were used containing 22.8mg in 2mls 0.9% saline. Diet-control animals were treated with the 28 day group and received saline alone, again via mini-osmotic pumps.

Tissue Collection

At the end of the study period animals were sacrificed by CO_2 inhalation and exsanguinated by cardiac puncture: blood was taken into lithium-heparin tubes containing 200µl aprotinin, which were spun immediately and the plasma separated and stored at -20°C until the assay of glucose and insulin. The animal was then decapitated and the brain removed and placed on its ventral surface. The hypothalamus was dissected out under a binocular dissecting microscope, using coronal incisions at the optic chiasm anteriorly and the mammillary bodies posteriorly and then removing it by incisions along the perihypothalamic sulci and the anterior commissure. The hypothalamus was then divided into two halves by a further vertical cut; one half was kept at -70°C for later measurement of NPY mRNA, the other was divided into medial and lateral parts using vertical cuts through the fornix and the mammillohypothalamic bundle. A sample of frontal cortex was also taken and frozen at -70°C until measurement of brain dexfenfluramine and d-norfenfluramine levels.

Assay of glucose, insulin and neuropeptides

Plasma glucose and insulin were measured as described in section A. above. NPY, galanin, somatostatin, neurotensin were assayed by sensitive and specific radioimmunoassays described in Chapter 2. Details of the assays used are given in table 3.1. Brain dexfenfluramine and d-norfenfluramine concentrations were measured using gas chromatography by Servier Laboratories (UK) [168].

Table 3.1 - *Details of peptide radioimmunoassays*

	Antibody Specificity	Antibody Dilution	Label	Assay Sensitivity fmol/tube
NPY	COOH-terminal	1:120000	Porcine NPY	0.6
Neurotensin	COOH-terminal	1:80000	Bovine neurotensin	0.4
Galanin	NH_2-terminal	1:480000	Porcine galanin	1.0
Somatostatin	Entire	1:240000	[Tyr^{11}] somatostatin	0.4

Chromatographic Analysis

In order to confirm the identity of immunoreactivity detected by radioimmunoassay, the relevant standards and pooled hypothalamic extract from control and treated groups were subjected to fast protein liquid chromatography using a high resolution reverse phase 5/5 (Pep Rpc HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 15% (v/v) acetonitrile in water and the samples were eluted using a linear gradient of 15 to 45% acetonitrile in water over 1 hour. Fractions of 1ml were collected at 1-minute intervals and assayed for the relevant peptides as described above.

Measurement of NPY mRNA by Northern blot analysis

Hypothalamic blocks were pooled in groups of three to five for measurement of NPY mRNA. RNA extraction and Northern Blotting procedures were as described in Chapter 2.

Statistical Analysis

All results are given as mean ± SEM. Comparisons between groups for the effects of treatment and diet were made using analysis of variance (ANOVA). Individual differences were then assessed using linear contrasts. When two groups were compared Student's t-test was used. Comparisons of changes in body weight were made using repeated measures ANOVA. Statistical significance was taken at $P<0.05$. All analysis was done using Systat (Systat, Evanston, IL, USA) software on an IBM PC.

Results

Weight changes

Animals fed the palatable diet gained significantly more weight over the 6 week pre-study period than controls (145.1 ± 2.3g vs 113.4 ± 3.2g, $F(1,82)=13.05$, $p<0.001$, figure 3.3). In the 7 day study animals treated with dexfenfluramine lost weight whereas those given saline continued to gain weight (-4.0 ± 1.6g vs 11.1 ± 1.8g, $p<0.01$, figure 3.4). One animal in the 7 day study control group died following insertion of the mini-osmotic pump and has been excluded from the subsequent analysis.

In the 28 day study the dexfenfluramine group lost weight for the first week. Following this both groups continued to gain weight but the net weight gain over four weeks was much less in the group which received dexfenfluramine (33.2 ± 2.9g vs 52.5 ± 4.8g, $F(1,33)=9.86$ $p<0.01$, figure 3.4).

Figure 3.3 *Effect of palatable diet on weight gain over 6 week run in period. (Chow, n=20; Diet n=64). Difference between groups: p<0.001 by repeated measures ANOVA.*

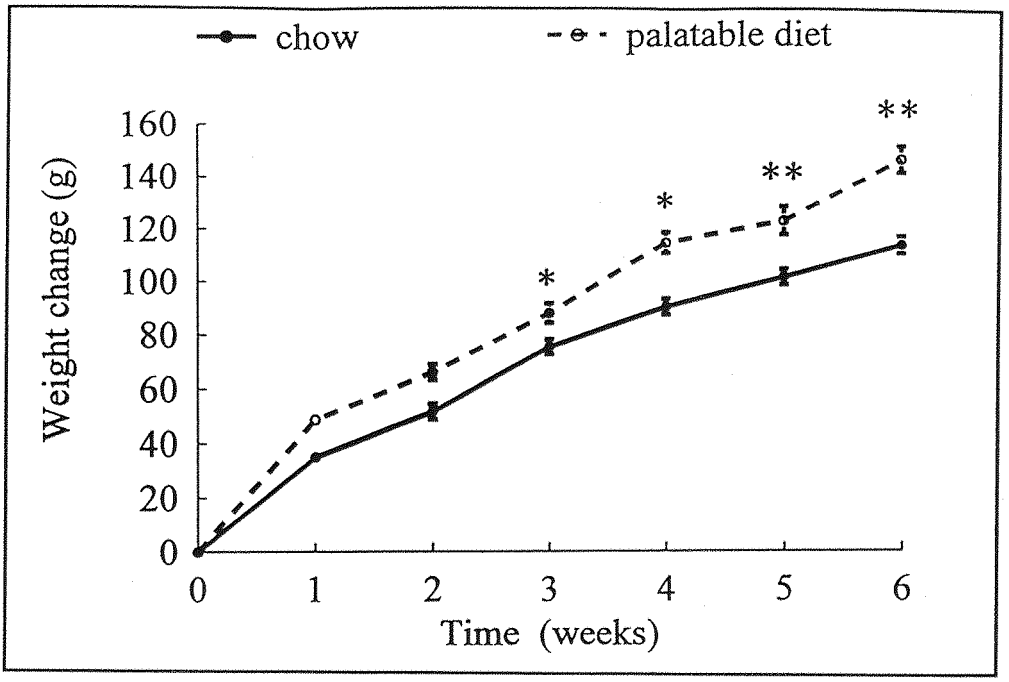
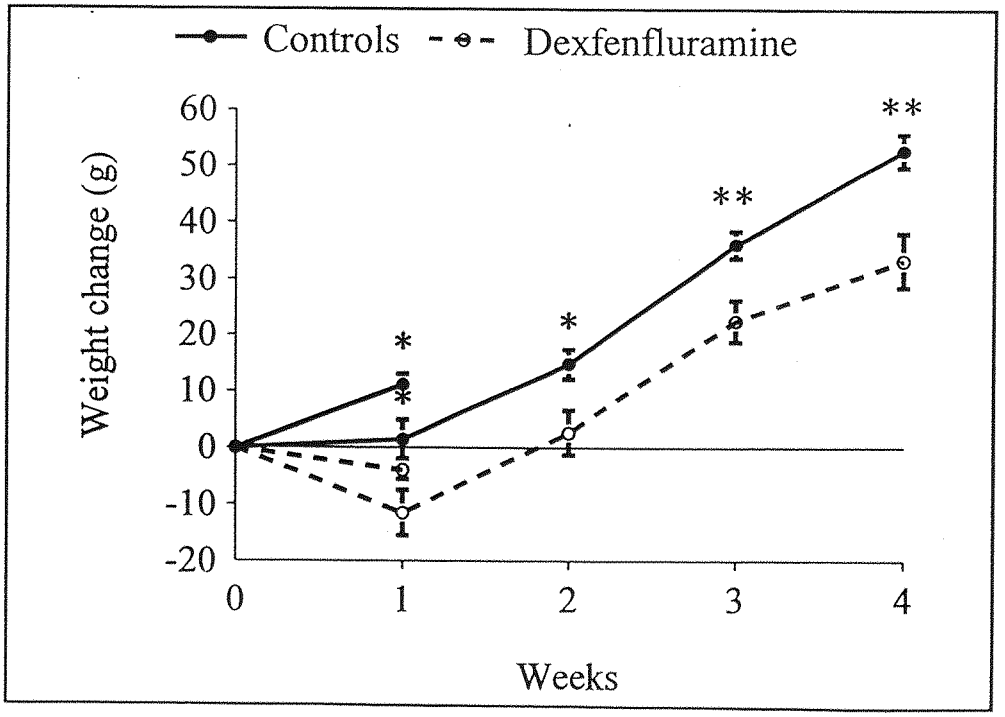


Figure 3.4 *Effect of 7 days (control n=18, dexfenfluramine n=17) and 28 days (control n=15, dexfenfluramine n=14) treatment with dexfenfluramine on weight gain in animals fed the palatable diet. All values are mean ± SEM. *p<0.05, **p<0.01*



Glucose and insulin

Plasma glucose concentrations were similar in all groups of animals in both studies. In the 28 day study insulin concentrations were higher in the animals fed the palatable diet, ANOVA: $F(2,52)=6.34$, $p<0.01$), table 3.2.

Brain dexfenfluramine levels

Animals treated with dexfenfluramine all had detectable levels of dexfenfluramine and d-norfenfluramine in the frontal cortex, which were within the expected range for the dose given (table 3.2).

Neuropeptides

Medial and lateral hypothalamic weights were consistent across all the groups examined (table 3.2).

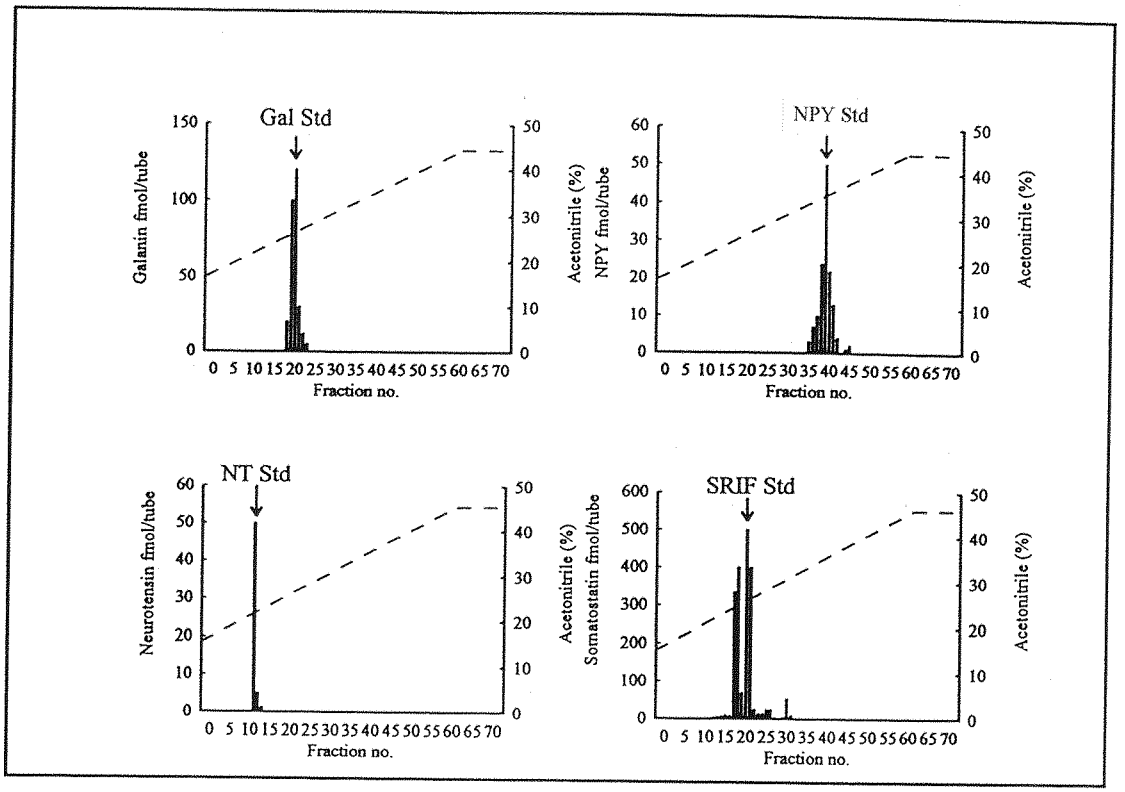
Table 3.2 - *Metabolic data, brain dexfenfluramine levels and hypothalamic weights for animals in the 28 day and 7 day studies. All values are mean ± SEM. * $p<0.01$, different from diet-control group.*

	28 Day Study			7 Day Study	
	CHOW (n=20)	DIET (n=18)	DIET+DFEN (n=17)	DIET (n=14)	DIET+DFEN (n=15)
Initial Weight (g)	308.2±2.5	316.4±3.9	316.8±5.4	305.0±3.5	304.3±4.7
Weight end run-in period (g)	421.6±5.1	457.2±6.5*	462.9±9.5*	450.7±7.2*	452±7.5*
Glucose mM	8.6 ± 0.6	9.4 ± 0.8	8.3 ± 0.6	9.9 ± 0.6	10.9 ± 1.1
Insulin pM	515 ± 53	802 ± 70*	801 ± 78*	625 ± 47	564 ± 60
Brain dexfenfluramine µg/g	ND	ND	0.77 ± 0.1	ND	0.46 ± 0.1
Brain d-norfenfluramine µg/g	ND	ND	1.57 ± 0.2	ND	2.15 ± 0.1
Medial Hypothalamic Weight mg	13.7 ± 0.9	11.9 ± 0.9	12.4 ± 0.9	12.3 ± 1.3	11.6 ± 1.0
Lateral Hypothalamic Weight mg	8.2 ± 0.7	8.0 ± 0.7	7.4 ± 0.7	8.7 ± 0.8	9.0 ± 0.8

Chromatography

FPLC confirmed that the immunoreactivity detected in hypothalamic extracts eluted in the same position as the relevant standards (Fig 3.5).

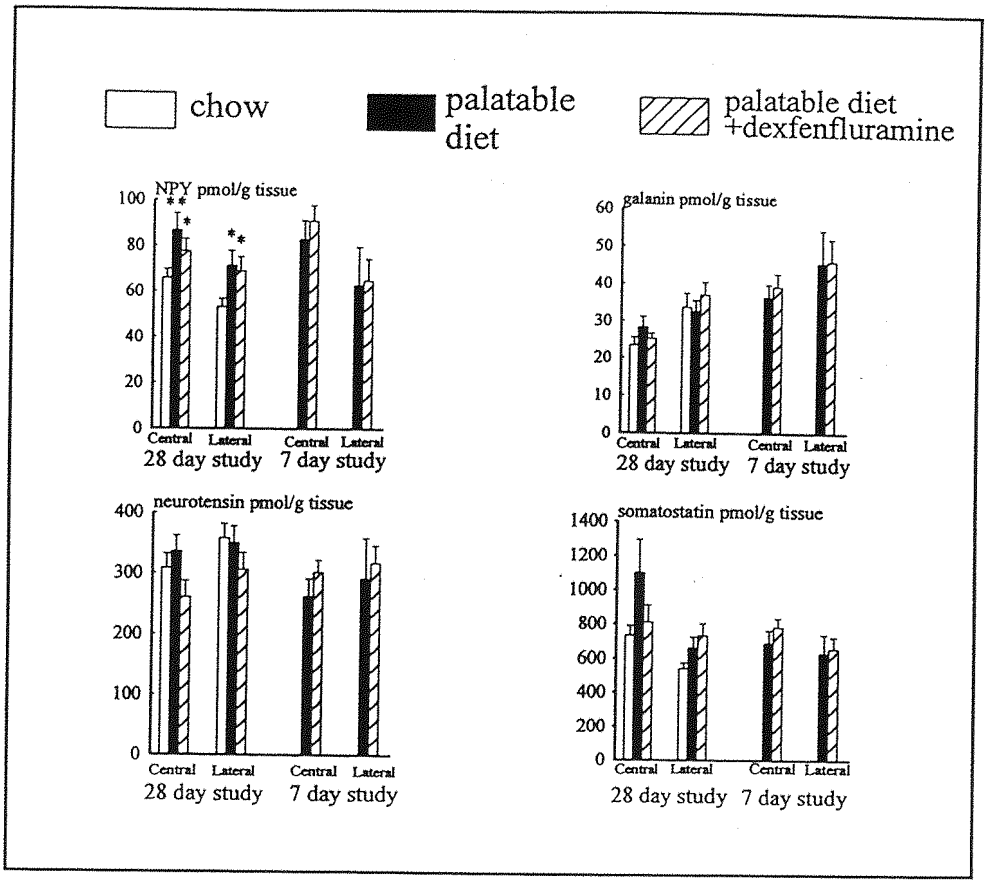
Figure 3.5



Peptide Changes

In the 28 day study there were significant differences only for NPY, 2-way ANOVA: $F(2,107)=6.51$, $P<0.01$. When individual differences were examined by linear contrasts, NPY concentration was higher in both the saline and dexfenfluramine treated palatable diet-fed animals than in the chow-fed controls in both the central ($p<0.02$ vs saline treated, $p<0.05$ vs dexfenfluramine treated) and lateral ($p<0.05$) hypothalamus. When comparing animals fed the palatable diet, there were no differences between the groups given dexfenfluramine and those given saline for any of the peptides studied in either the 28 day or the 7 day study. Graphs in figure 3.6 show hypothalamic concentrations of NPY, galanin, somatostatin and neurotensin in central and medial hypothalamic blocks in animals in the 28 day and 7 day studies.

Figure 3.6 *Concentrations of the four peptides measured in the central and lateral hypothalamus of the 28 day and 7 day study groups. All values are mean ± SEM. *p<0.05, **p<0.02*

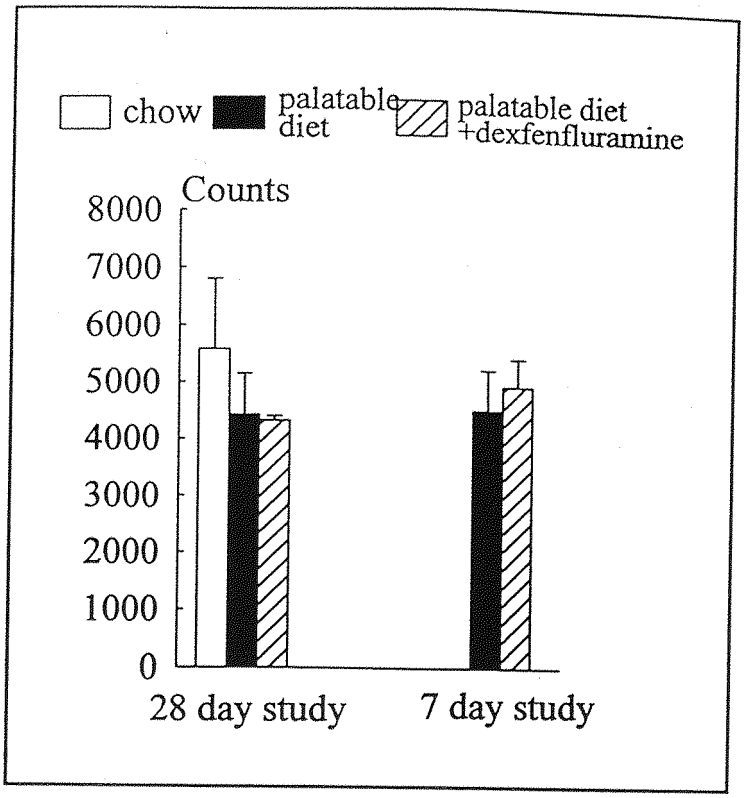


NPY mRNA

Measurement of NPY mRNA revealed no significant differences between any of the groups studied; 28 day study ANOVA: $F(2,9)=0.72$, $p>0.05$; 7 day study, $p>0.05$, in particular there was no difference between diet controls and animals fed the palatable diet where there were differences in NPY concentration detected by radioimmunoassay (figure 3.7).

Figure 3.7

Relative concentrations of total hypothalamic NPY mRNA in 28 day and 7 day study groups. All values are mean ± SEM.



Discussion

The results from this study confirm those in section A, with increases in neuropeptide Y content but not mRNA in the hypothalamus of rats made obese by feeding them a highly palatable diet. These results have been extended to show that NPY, but not galanin, neurotensin or somatostatin was altered in this model of obesity. It is possible that localised changes occurred in some hypothalamic areas, or that mRNA for these peptides was altered, but these were not measured in this study.

The anorectic drug dexfenfluramine, administered via mini-osmotic pumps in doses sufficient to produce initial weight loss and reduce long-term weight gain in rats with diet-induced obesity, does not appear to have effects on the concentrations of the hypothalamic neuropeptides NPY, galanin, somatostatin or neurotensin. In addition there were no changes in hypothalamic NPY mRNA with this treatment. These results are in contrast to those of Rogers et al who demonstrated small decreases in hypothalamic NPY in several hypothalamic areas following the administration of a single, large dose of fenfluramine (10mg/kg) [167]. There are several possible reasons for this. A lower dose of the drug was used in this study, in order to minimize non-specific (ie behavioural) effects. This dose reduced weight gain, most effectively during the first week and attenuated by a longer period of administration. Although food intake was not measured in this study, this

dose of dexfenfluramine is recognized to act mainly by reducing caloric intake [148]. The active isomer of the drug (dexfenfluramine) used in this study may have different effects from the racemic mixture used in Rogers' study. In this study, because of the large number of animals used, neuropeptide concentrations were measured in central and lateral hypothalamic blocks and it is possible that small changes in some nuclei were not detected by this technique. However the confirmation of the previous results, demonstrating increases in NPY immunoreactivity in the hypothalamus, but no change in NPY mRNA in animals with diet-induced obesity suggests that the technique of measuring peptide concentrations in the central and lateral hypothalamus is sensitive enough to detect a change in NPY concentration. If dexfenfluramine were acting in part by affecting this system, we might expect to see this change reversed.

Other workers have found that the administration of dexfenfluramine or amphetamines inhibits NPY induced feeding in rats [116]. A reduction in endogenous NPY alone as an effect of dexfenfluramine would be unlikely to produce the dramatic reduction in responsiveness to pharmacological doses of NPY seen in that study and suggests that this reduction may be occurring post-synaptically. It would be interesting to quantify the dose response of obese animals to exogenous NPY.

Connections between NPY containing and serotoninergic nerves in the hypothalamus [169], and co-localization of these neurotransmitters have also been demonstrated [170]. There is evidence to suggest that NPY affects serotonin by reducing its metabolism [171]. Thus it has been suggested that a modulatory effect of NPY on the serotoninergic feeding system may exist [165]. This study is not in conflict with this data, but would suggest that hypothalamic NPY concentrations and synthesis are not affected by serotonin.

In summary, there was no significant effects of dexfenfluramine on any of the hypothalamic neuropeptides studied in rats fed a palatable diet, although NPY content is increased in animals fed this diet. This would suggest that serotonin agonists such as dexfenfluramine may act independently of the NPYergic system, and raises the possibility that drugs designed to inhibit the action of hypothalamic NPY could have an additive effect in the treatment of obesity.

Chapter 4

Hypothalamic neuropeptides in a genetic model of obesity - the ob/ob mouse

Introduction

Abnormalities of hypothalamic peptides have been implicated in the aetiology of both acquired and genetic obesity. In the previous chapter I have demonstrated increases in NPY content, but not NPY mRNA in the hypothalamus of rats made obese by feeding a palatable diet [172]. Other workers have shown regional changes in hypothalamic NPY concentrations in rats fed high-fat or high-carbohydrate diets [155]. The obese fa/fa Zucker rat shows both increased NPY and decreased neurotensin content and mRNA, both of which could be contributing to obesity [37,154,173,174].

The ob/ob mouse has an autosomal recessive defect, features of which include hyperphagia, obesity, defective thermogenesis, insulin resistance and abnormal gonadotropin secretion. It has been suggested that the primary abnormality in this disease model is expressed as a hypothalamic defect [175]. A previous study, carried out in Professor Bloom's laboratory found decreased hypothalamic neurotensin concentration, measured by radioimmunoassay, in the genetically obese ob/ob mouse, but unchanged hypothalamic NPY, suggesting that changes in hypothalamic neurotensin, but not NPY, could be implicated in the development of obesity in this model [173]. Given that it has been proposed that hypothalamic NPY mRNA is regulated by insulin, and that conditions associated with insulin resistance may increase hypothalamic NPY expression [176], it was thought important to re-examine NPY in this insulin resistant model of obesity in a different way, by measuring mRNA levels in addition to peptide content.

The aim of this study was to extend the previous observations in the ob/ob mouse by examining both hypothalamic peptide content and mRNA for NPY, neurotensin, galanin and somatostatin in obese animals compared to lean controls.

Materials and methods

Animals

Thirty-two male obese (ob/ob) mice (Aston strain) and their lean (+/+) littermates were obtained from colonies at The Royal Veterinary College, London, UK and Aston University, Birmingham, UK. The Aston strain of ob/ob mice has been previously described [177]. In brief, C57BL/6J ob/+ breeding pairs were outcrossed with JH and CRL strains for higher litter size and faster growth rate, and the mice are maintained as a closed non-inbred colony. They were caged in groups of four to six in a temperature controlled environment (22 ± 2^{0}C), with a 12:12 h light:dark cycle and were freely fed standard pelleted chow from weaning. They were between 14 and 30 weeks of age when killed. An age related decline in hypothalamic NPY concentrations has been observed in the rat, but this only occurred when the animals were several months of age [178]. The previous work did not find differences in NPY content in the hypothalamus of the ob/ob mouse at 4, 16 or 28 weeks of age, and the reduction in neurotensin content was seen at all three ages [173]. Hence the possibility of age related changes was not addressed in this study.

Tissue and plasma collection

The mice were killed by cervical dislocation and blood obtained by cardiac puncture. They were killed in random order in the fed state between 1000 and 1300 hrs. The blood was taken into lithium heparin tubes and centrifuged immediately. An aliquot of plasma was analysed for glucose using a glucose oxidase based autoanalyzer (Beckman) and the remainder was stored at -20^0 C until assay of insulin.

The brain was rapidly removed and placed on its ventral surface. The hypothalamus was dissected *en bloc* as described in Chapter 2. For peptide estimation (8 animals in each group) the tissue was weighed and then boiled for 10 minutes in 0.5M acetic acid to extract the peptides. The extract was frozen at -20^{0}C until the peptides were assayed. For mRNA estimation the hypothalamus, (24 animals in each group) was collected into sterile polypropylene tubes and snap-frozen in liquid nitrogen. These samples were stored at -70^{0}C until RNA extraction.

Radioimmunoassays

Plasma insulin, and hypothalamic concentrations of the neuropeptides NPY, galanin, somatostatin and neurotensin were assayed in duplicate using radioimmunoassays described in chapter 2 (methods).

Chromatographic analysis

In order to confirm the identity and integrity of the NPY like immunoreactivity, hypothalamic extract from lean and obese mice was subjected separately to fast protein-liquid chromatography using a high resolution reverse phase 5/5 (Pep Rpc HR 5/5) C18 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 15% (v/v) acetonitrile in water and the samples and human/mouse NPY standard were eluted using a linear gradient of 15-45% acetonitrile in water over 70 minutes. Fractions of 0.5ml were collected at 1-min intervals, vacuum dried, reconstituted in assay buffer and assayed for NPY.

Measurement of mRNA by Northern blot analysis

Hypothalami were pooled in groups of three for measurement of mRNA. This gave samples of 41 ± 1.5mg (mean ± SEM) tissue, n=8 for each group. Total RNA was extracted as described in Chapter 2. Approximately 50μg of RNA was obtained from each sample. RNA was then size separated on 1% agarose-formaldehyde gels. Four lanes from each of the lean and obese groups were probed sequentially for NPY and neurotensin and four for somatostatin and galanin. Probes for NPY, neurotensin and somatostatin were prepared with ^{32}P-dCTP by random primer extension of the relevant rat cDNA to a specific activity of 1.5 x 10^9 dpm/μg and hybridised as described in Chapter 2 for NPY mRNA.

The galanin probe was a rat cRNA probe prepared by *in vitro* transcription using T7 RNA polymerase to a specific activity of 1.2 x 10^9 dpm/μg. Hybridization was carried out at 55°C in a solution of 50% formamide, 5x Denhardt's solution, 5x SSC, 50mmol sodium phosphate pH 6.8, 200μg/ml yeast RNA, 100μg/ml dextran sulphate and 10ng/ml RNA probe. Filters were washed twice at 65°C in a solution of 0.1x SSC/0.1%SDS. Quantitative estimates of relative levels of mRNA were made using a two-dimensional surface counter and computer-based image capture system and image-analysis software (Autograph, Positron Systems Ltd, Oxford, UK) running on an AST 386 PC. The filter was then stripped in 0.5% SDS, 1% triethanolamine (TE) at 80°C for 30 minutes and reprobed for the second mRNA.

All results were normalized to the amount of mRNA loaded by stripping the filter in 0.5% SDS, 1% TE at 80°C for 30 minutes and then re-probing with oligo dT, and again counting using the Autograph software. Final results are expressed as a ratio of specific counts for each peptide mRNA / {oligo(dT)/10}.

Statistical Analysis

All results are given as mean ± SEM. Comparisons between groups were made using Student's t-test. A p value <0.05 was taken to be statistically significant.

Results

Metabolic data

Obese mice were characteristically heavier than the lean controls. Plasma insulin concentrations were increased 20-fold. The obese mice were not hyperglycaemic relative to controls in this study. This observation has been made previously in ob/ob mice of this age from this background [173], although the glucose levels were relatively high in lean mice, this may reflect the fact that they had free access to food (Table 4.1).

Table 4.1

Characteristics of lean (+/+) and obese (ob/ob) mice used in this study

	Body weight (g)	Plasma glucose (mmol/l)	Plasma insulin (pmol/l)
Lean	37 ± 0.9	9.1 ± 0.3	207 ± 29
Obese	90 ± 2.3*	10.1 ± 1.8	5948 ± 802*

Figures are mean ± SEM, n=32 mice/group

** p<0.01 versus lean controls*

Radioimmunoassay of peptides

Hypothalamic concentrations of NPY, galanin and somatostatin were similar in lean and obese mice (table 4.2). However neurotensin was decreased by approximately 25% in the obese mice ($p<0.01$).

Table 4.2

Radioimmunoassay of peptide concentrations in the hypothalamus of lean (+/+) and obese (ob/ob) mice

	Neurotensin	NPY	Galanin	Somatostatin
Lean	220 ± 13.8	251.6 ± 15.2	39.0 ± 1.9	1529 ± 103
Obese	167 ± 9.7*	239.8 ± 29.6	38.9 ± 1.8	1647 ± 157

Figures are pmol/g tissue, mean ± SEM, n=8 mice/group

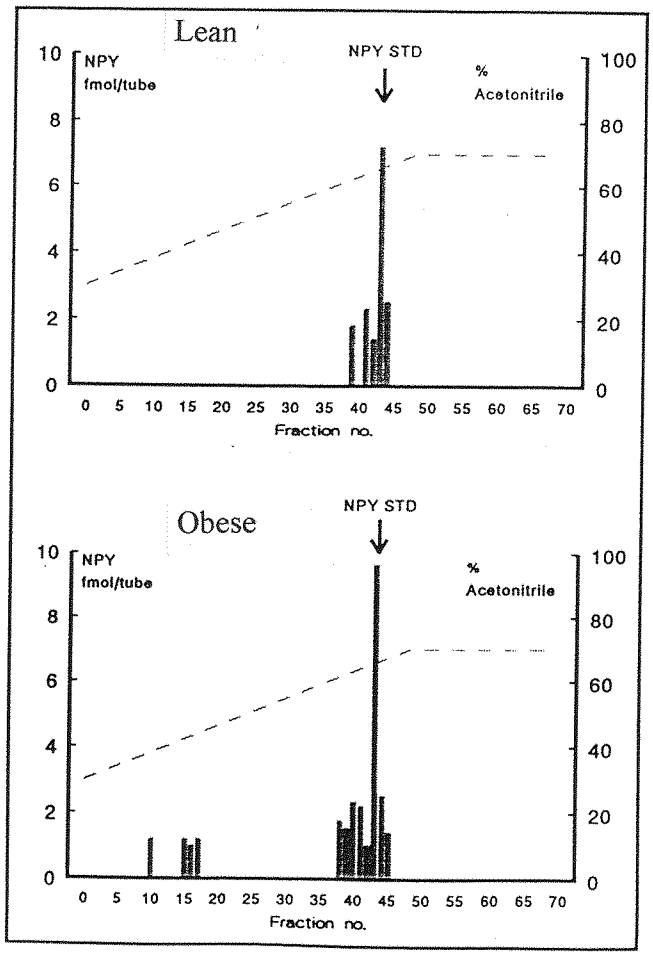
** $p<0.01$ versus lean controls*

Chromatographic analysis of NPY

FPLC confirmed that the NPY immunoreactivity eluted as a single peak in hypothalamic extracts from both lean and obese mice, and in the same position as the NPY standard, thus confirming the integrity and identity of the immunoreactivity measured (figure 4.1).

Figure 4.1

Fast protein liquid chromatography demonstrates that the immunoreactive NPY detected from lean and obese mice was intact, and eluted in the same position as the porcine NPY standard.



Northern blot analysis

There was no evidence of RNA degradation in any of the samples. There was a threefold increase in NPY mRNA in the obese mice ($p<0.01$). Neurotensin mRNA was decreased in the obese mice by 30% ($p<0.05$), in accord with the decrease in neurotensin concentration. There was no difference between the groups for either galanin or somatostatin mRNA (fig. 4.2). Northern blots for NPY and neurotensin are illustrated in figure 4.3.

Figure 4.2

Relative concentrations of mRNA for neurotensin (NT), neuropeptide Y (NPY), galanin (GAL) and somatostatin (SRIF) in the hypothalamus of lean (+/+) and obese (ob/ob) mice
** $p<0.02$; ** $p<0.01$*

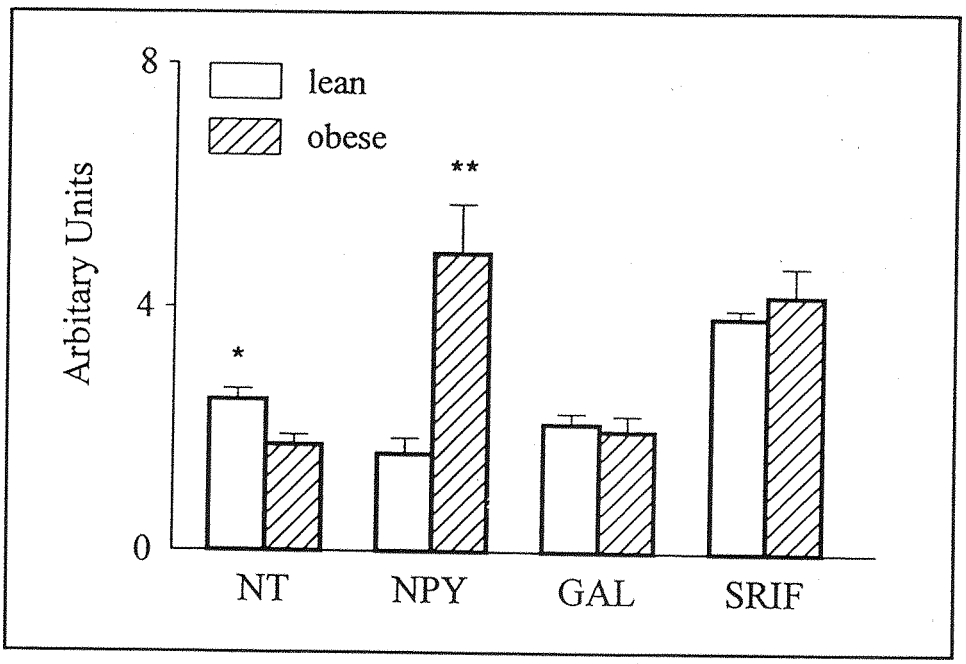
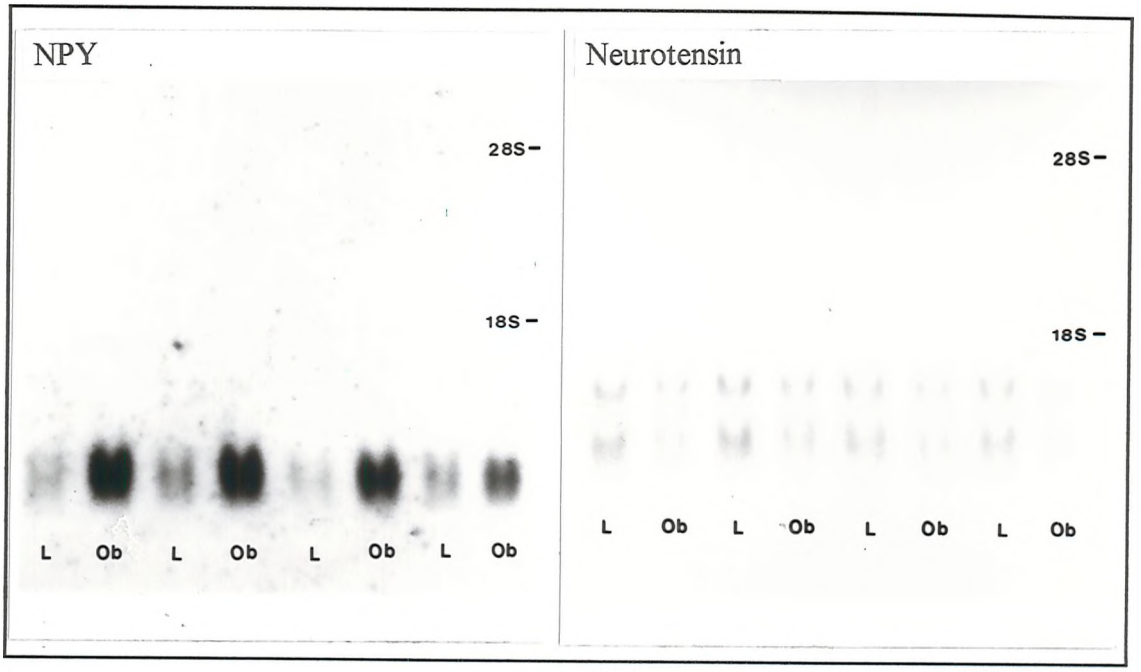


Figure 4.3

Northern blots (48h exposure) showing increased NPY mRNA and decreased neurotensin mRNA in the hypothalamus of lean (+/+) and obese (ob/ob) mice. Each lane represents RNA from three pooled hypothalami. The NPY mRNA is approx 0.8 kilobases and the neurotensin RNA is shown typically as two bands of approximately 1 and 1.5 kilobases respectively.



Discussion

The results indicate increased NPY mRNA as well as decreased neurotensin content and mRNA in the hypothalamus of the obese ob/ob mouse. It is interesting to compare these results with those reported in other genetic models of obesity and insulin resistance. In the fa/fa Zucker rat increases in NPY concentration have been found in several hypothalamic nuclei and NPY mRNA is increased in whole hypothalamus and arcuate nucleus [154,174,179]. The Zucker rat also has very similar changes in hypothalamic neurotensin to those found in the ob/ob mouse [37,180]. In contrast, NPY mRNA is increased in db/db mice when the mutation is expressed in the C57BL/KsJ strain, which exhibits a form of insulin deficient diabetes, but not when expressed on the C57BL/6J strain, which show β-cell hypertrophy and hyperinsulinaemia [181]. As the primary mutations resulting in these syndromes are not known, it is difficult to speculate

as to the reasons for the differences in NPY between ob/ob and db/db mice.

The lack of change in NPY concentration in the ob/ob mouse, despite increased mRNA, could be due to regional increases in peptide concentration which are not detected when the whole hypothalamus is examined (although changes were not detected in central and lateral hypothalamic NPY in the earlier study [173]), or factors increasing NPY degradation in the obese animals, although FPLC suggested that intact NPY was being measured in both phenotypes. Alternatively there may be a reduced NPY mRNA translation rate in the obese animals with reduced or unchanged release. However the most likely explanation is an increase in the synthesis and release of NPY but no change in net neuronal stores. Some evidence for this in relation to NPY comes from the work of Kalra et al who have, using push-pull cannulae, demonstrated increased release of hypothalamic NPY in rats deprived of food for three days, a situation known to be associated with increases in NPY mRNA [150,182]. Further studies would however be necessary to establish whether this was indeed the case in these genetically obese animals.

NPY has several important effects when administered centrally, including increased appetite [93], insulin release [125], release of CRF, ACTH and corticosterone [122,124] and modulation of LH release [183]. NPY injected into the third ventricle inhibits sympathetic activity to brown adipose tissue and thermogenic activity as assessed by GDP-binding [126,162]. Chronic administration of NPY produces weight gain and obesity [106]. It is of interest to note that the genetically obese ob/ob mouse responds to exogenously administered NPY with increased food intake in a similar way to lean controls [102]. Thus many of the features of the obesity and insulin resistance seen in the ob/ob mouse might be explained by increased hypothalamic NPYergic activity.

NPY induced feeding is dependent on corticosterone [184], and it is of interest that the ob/ob mouse has high circulating levels of corticosterone, and that obesity in the ob/ob mouse can be attenuated by adrenalectomy [185]. NPY release in vitro can be stimulated by dexamethasone and total hypothalamic NPY is increased by dexamethasone administration [151]. An increase in circulating corticosteroids is therefore one possible explanation for the increased NPY mRNA expression in the ob/ob mouse.

NPY in the hypothalamus may also be regulated by insulin. Situations in which NPY concentration and synthesis are increased are either associated with low circulating insulin levels (starvation, diabetes [136,137]) or peripheral (and, perhaps, central) insulin resistance (fa/fa Zucker rat, ob/ob mouse). Insulin administration reverses the changes in

hypothalamic NPY seen in experimental insulinopenic diabetes [186], and, in addition, intracerebroventricular insulin administration reduces NPY mRNA in the arcuate nucleus of the lean, but not the fa/fa Zucker rat following food deprivation [176], suggesting a reduced central sensitivity to insulin in the latter model. It is therefore possible that the increases in NPY mRNA in the ob/ob mouse are as a result of insulin resistance in the central nervous system.

The results of this study extend previous reports of reduced neurotensin in the brain [187] and, more specifically, the hypothalamus [173] of ob/ob mice, by indicating that these changes may be due to a reduction in neurotensin gene expression. The anorectic effect of peripherally administered neurotensin is similar in lean and obese ob/ob mice [188], suggesting that the reduction in hypothalamic neurotensin could contribute to the decreased satiety seen in the obese animals. However it remains to be seen whether these animals have normal responses to centrally administered neurotensin. Also the net effect on appetite of reduced hypothalamic neurotensin and increased hypothalamic NPY mRNA remains to be clarified. Central injection of neurotensin stimulates insulin release [189], which in the context of these findings suggests the possibility that the reduction in neurotensin may be a feedback response to increased circulating insulin. Neurotensin stimulates LH release when injected into the medial pre-optic area of the hypothalamus in rats [190], so it is possible that reductions in neurotensin may also contribute to the reduced LH release seen in the ob/ob mouse.

Neither galanin or somatostatin content or mRNA were altered in this model of obesity. These results are in accord with previous data on galanin and somatostatin content in the hypothalamus of the ob/ob mouse [173]. Galanin mRNA, in contrast to NPY, appears to be decreased in the arcuate nucleus following food restriction, and it is therefore likely to play a different role in the regulation of appetite, and to be differently regulated [156].

In summary, I have found increased neuropeptide Y gene expression and decreased neurotensin gene expression, along with decreased neurotensin content in the hypothalamus of the ob/ob mouse. It is possible that the combination of abnormal regulation of these two hypothalamic appetite regulating peptides may be of importance in the development of obesity in this model.

Chapter 5

Hormonal factors influencing hypothalamic NPY

Introduction

Identification of the peripheral metabolic and hormonal factors which regulate hypothalamic NPY may provide wider insight into the control of food intake, and the mechanisms which may lead to its alteration in disease.

Hypothalamic NPY may be regulated by insulin. NPY concentration and mRNA in the hypothalamus are increased in diabetes and starvation [136-139], both situations where there is a low circulating concentration of insulin. In diabetes the increase in NPY concentration can be reversed by insulin administration [186]. Hypothalamic NPY mRNA is increased in some animal models of obesity associated with insulin resistance, such as the ob/ob mouse [191], and the fa/fa Zucker rat [154]. Intracerebroventricular insulin reduces hypothalamic NPY mRNA in the ARC [192]. In the food-deprived Zucker rat central insulin administration reduces NPY mRNA in the ARC in lean but not obese phenotypes, suggesting hypothalamic insensitivity to insulin in the obese rat [176]. Insulin, which may act in the CNS as a long-term satiety factor [193], and has receptors in the ARC [25] may therefore reduce appetite via a reduction in NPY mRNA.

NPY induced feeding is corticosteroid dependent [117]. Adrenalectomy may reduce NPY mRNA in the ARC [194], although this has been disputed [195]. NPY content of the hypothalamus is increased by dexamethasone administration in vivo [151] and in rat fetal hypothalamic cells in culture [196]. NPY mRNA expression is increased in rat neuronal cell lines exposed to dexamethasone [197]. It is not known whether the changes in NPY content observed in vivo are associated with an increase in mRNA, or in which nuclei of the hypothalamus these changes occur.

The aim of this study was to investigate control of hypothalamic NPY by corticosteroids and insulin by measuring changes in NPY content in microdissected hypothalamic nuclei, and in total hypothalamic NPY mRNA in rats treated with dexamethasone and to establish whether this could be modulated in rats treated with exogenous insulin.

Methods

Animals

Seventy-five male Wistar rats (Tuck and Sons Ltd, Essex, UK) were used for these studies. They were caged in groups of five (except for ten animals in each group during food intake measurement, who were caged individually for three days), and housed in a

temperature controlled environment (22±2^{0}C) with a 13/11 hour light/dark cycle. They had free access to pelleted chow and water throughout the study.

Experimental design

Animals were divided into five groups of fifteen animals. Two groups were given daily subcutaneous injections of dexamethasone (0.4mg/kg/day, D. Bull Laboratories, Warwick, UK), for 28 days with one group killed on the last day of treatment and the other 5 days after treatment had stopped. Control groups were treated with saline for the same time periods. The final group received dexamethasone for 28 days in the same dose, with the addition of Ultratard insulin 60 Units/kg/day (Novo-Nordisk). The dose of insulin was chosen from a pilot study (results not shown), as the highest dose that could be given to dexamethasone treated rats without producing hypoglycemia. It was originally planned to include a group of rats treated with insulin alone, however these high doses of exogenous insulin produced severe hypoglycaemia in normal rats, and therefore this group was not included in the final study. The final group were given saline injections only. Animals were weighed at the start and end of the study. Because of the impracticalities of housing this number of animals individually food intake was accurately measured in 10 animals, randomly selected from each group for a three day period after two weeks treatment. This was done at this stage to avoid disturbing the animals at a later stage of the study. A crude measurement of food intake (total food consumed per cage / day) was made throughout the study.

At the end of the study period, blood glucose was estimated from a tail vein sample using a glucose-oxidase based reflectance meter (Glucometer 2, Ames). Animals were then killed in random order between 1000 and 1500hrs by CO_2 inhalation and exsanguinated by cardiac puncture; blood was centrifuged immediately and the plasma separated and stored at -20^{0}C until the assay of insulin.

Brain microdissection

Ten animals from each group were used for peptide extraction. Hypothalamic nuclei were microdissected as described in Chapter 2. The nuclei sampled were the medial preoptic area (MPO), anterior hypothalamic area (AHA), lateral hypothalamic area (LHA), PVN, ventromedial nucleus (VMH) and the ARC. Samples were immediately placed in 500µl acetic acid (0.5M) and boiled for 10 minutes to extract the peptides. The remaining five hypothalami were dissected en bloc, snap-frozen in liquid nitrogen and

stored at -70^{0}C until RNA extraction.

Assay of insulin and neuropeptides

Plasma insulin and NPY content in microdissected nuclei were measured as described in Chapter 2.

Measurement of NPY mRNA by Northern blot analysis

The RNA extraction and Northern blotting procedure was as described in Chapter 2.

Statistical Analysis

Comparisons between the groups was made using ANOVA, specific differences were then compared using Tukey's test. Plasma insulin data, which was not normally distributed, was log-transformed prior to statistical analysis. Statistical significance was taken at $p<0.05$.

Results

Weight, food intake, and metabolic data

All the treatment groups failed to gain weight at the same rate as controls ($F=230$, $P<0.001$), although weight gain did resume after dexamethasone was stopped ($p<0.01$), table 1. There was a significant reduction in food intake in the dexamethasone treated groups compared to controls, including the insulin treated group ($P<0.01$). Based on the cage by cage measurement of food intake, this pattern was evident throughout the study (results not shown). The insulin treated group had a tendency to eat less food than those treated with dexamethasone alone, but this failed to reach statistical significance. Blood glucose was similar in all groups ($F=2.5$, NS), but plasma insulin was 5-fold higher than controls in the dexamethasone treated animals and 10-fold higher in the group treated with insulin in addition to dexamethasone ($F=22.9$, $P<0.001$). Plasma insulin levels were similar to controls 5 days following the withdrawal of dexamethasone (table 1), suggesting that the effects of dexamethasone on insulin sensitivity were rapidly reversed.

Table 5.1 - *Metabolic data and body weight*

	28 day study			33 day study	
	CONTROL	**DEX**	**DEX + INS**	**CONTROL**	**RECOVERY**
Initial weight (g)	227±2	231±3*	221±3	227±3	222±3
Final weight (g)	372±6	232±5**	216±4**	369±5	242±4**
Mean daily food intake (g)	24.9±1.0	19.2±0.57**	17.0±1.6**	23.8±1.2	19.5±0.7**
Blood glucose mM	5.4±0.2	6.0±0.6	4.5±0.4	5.2±0.2	5.4±0.3
Plasma insulin pM	601±75	2049±243**	5440±1424+	736±212	663±176

*Initial and final body weight; mean daily food intake measured over 3 days; glucose and insulin at the end of treatment in animals treated with saline (CONTROL), dexamethasone (DEX), dexamethasone plus insulin (DEX + INS) for 28 days and in animals killed 5 days after dexamethasone treatment was stopped (RECOVERY), along with the respective control group. *p<0.05 from CONTROL, **p<0.001 from CONTROL, +p<0.01 from DEX group*

NPY content

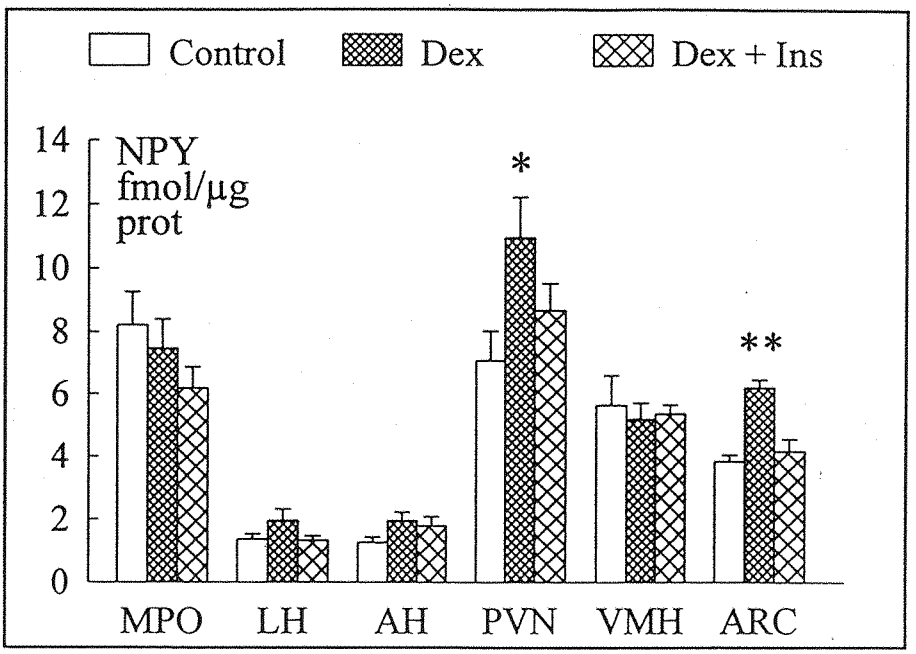
In the 28-day study analysis by ANOVA demonstrated a significant difference between groups ($F=5.4$, $p=0.006$). Tukey's test revealed significant differences between the dexamethasone treated group when compared to either controls or insulin treated animals (both $p<0.02$). When differences were examined for individual nuclei, NPY concentration was significantly increased in animals treated with dexamethasone alone in the paraventricular ($p<0.05$) and arcuate nuclei ($p<0.001$) (Figure 5.1a). In the animals given dexamethasone in whom the treatment was stopped, overall ANOVA suggested a difference between groups ($F=3.3$ $p<0.05$). ARC NPY remained increased relative to controls ($p<0.05$), whereas changes in the PVN were close to the normal value (Figure 5.1b).

Figure 5.1

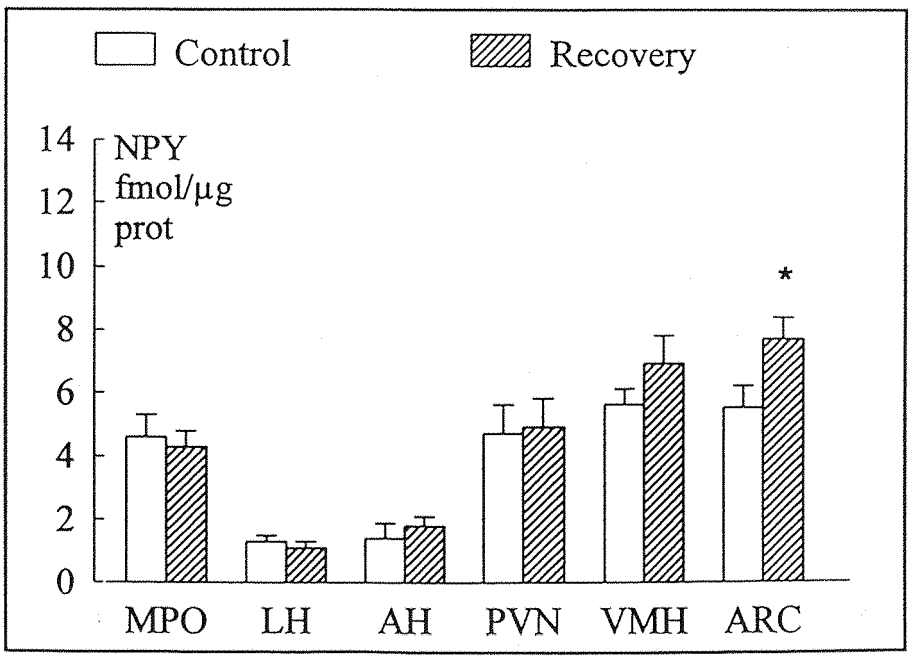
(a) NPY concentrations in microdissected hypothalamic nuclei in controls, rats treated with dexamethasone and rats treated with dexamethasone plus insulin for 28 days and (b) in rats treated for 28 days with dexamethasone, killed 5 days after treatment was stopped with corresponding controls at 33 days.

**p<0.05, **p<0.001*

(a)



(b)



NPY mRNA

Northern blot analysis revealed a single messenger RNA of approximately 700 bases as expected for NPY mRNA. The changes for total hypothalamic NPY mRNA were similar to those found for NPY peptide concentration (ANOVA, $F=4.7$, $P<0.05$); there was a 38% increase in NPY expression in dexamethasone treated animals ($p<0.05$) which was not seen in those treated concurrently with insulin (figures 5.2 and 5.3). In the group in whom treatment was stopped NPY mRNA was similar to untreated controls.

Figure 5.2

*Relative levels of hypothalamic NPY mRNA expression in control, dexamethasone treated, dexamethasone plus insulin treated and rats in whom dexamethasone was stopped for 5 days. *p<0.05*

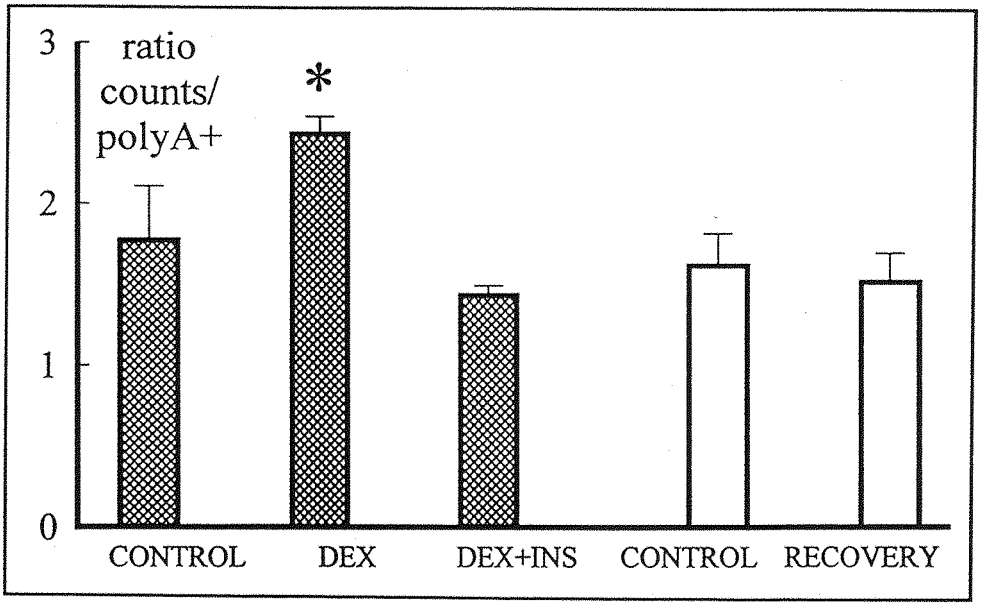
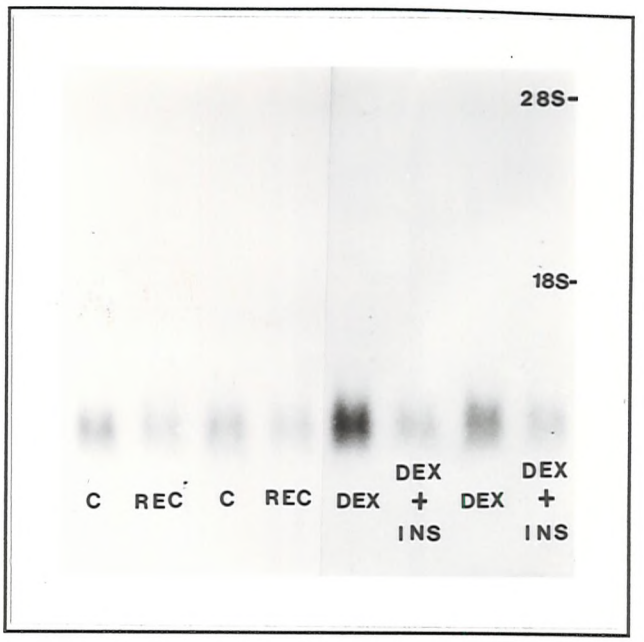


Figure 5.3

Representative Northern blots from the treatment groups. C = controls; DEX = dexamethasone treated; DEX+INS = dexaathasone plus insulin treatment; REC = 5 days following dexamethasone treatment.



Discussion

This study has extended previous findings of decreased preproNPY mRNA in the ARC following adrenalectomy [194], and increased NPY release in the presence of dexamethasone in vitro [151], by demonstrating that chronic dexamethasone treatment in vivo results in increased NPY mRNA in the hypothalamus and increased NPY content in the ARC and PVN. Although brainstem NPY concentrations and mRNA were not measured in this study, it seems likely that the increase in NPY in the PVN was largely due to increased synthesis in the ARC, as White and colleagues found no change in brainstem NPY mRNA in response to adrenalectomy [194]. Furthermore, the dexamethasone induced increase in NPY mRNA is rapidly reversed by stopping the treatment, although NPY concentrations in the ARC remained elevated, possibly due to residual stores of NPY. Finally, the effect of dexamethasone to increase hypothalamic NPY was completely prevented by high dose insulin administration.

The results of this study raise several important points in relation to the regulation of NPY by corticosteroids and insulin, with possible relevance to the appetite stimulating effect of NPY. In the dexamethasone treated animals there was an increase in hypothalamic NPY content and mRNA, despite a decrease in food intake and failure to gain weight. The mechanisms whereby dexamethasone decreases food intake are not known. This may be an indirect consequence of the peripheral catabolic effects of

corticosteroids. In addition dexamethasone may have a direct effect on the hypothalamic control of food intake by mechanisms other than those involving NPY. It is also possible that changes in macronutrient selection, which may be regulated by NPY and corticosteroids [57], did occur, but this question was not addressed in this study. There is a diurnal rhythm in the sensitivity to NPY induced feeding in rats, and endogenous NPY varies according to the time of day, with levels and sensitivity being greatest at the start of the dark phase, at the time when feeding is greatest [109,198]. These data would support the hypothesis that these changes are mediated by endogenous corticosteroids, which are at their highest level at the start of the dark phase [199].

The fact that the increased NPY mRNA was reversed rapidly following withdrawal of dexamethasone, despite only a small increase in body weight, and the prevention of the NPY changes with insulin administration, despite no significant change in food intake or body weight, suggests that the changes in NPY observed in this study were likely to be the result of specific hormonal effects, rather than due to a non-specific effect of weight loss. The increases in plasma insulin observed with dexamethasone are likely to be related to the well recognized insulin resistance produced by corticosteroids; the mechanisms underlying this are unclear, but increases in hepatic glucose output, and effects on the insulin receptor and post-receptor mechanisms have been implicated [200]. High levels of plasma insulin may therefore not affect hypothalamic NPY in this situation as they are "appropriate" to the level of insulin sensitivity.

In contrast to animals treated with dexamethasone alone, animals supplemented with insulin did not show increases in hypothalamic NPY content or mRNA over controls. In situations where the circulating insulin concentration is decreased, such as in starvation and experimental diabetes, or where there is insulin resistance, such as in genetically obese rodents, hypothalamic NPY mRNA and content are increased [136,137,150,176,201], providing indirect evidence that insulin may be involved in the regulation of hypothalamic NPY. The higher concentrations of insulin observed in these animals may therefore have directly reduced NPY mRNA and content in the hypothalamus. As already discussed, it was not possible to treat a group with insulin alone in this study, but recently published work has found an effect of intracerebroventricular insulin to modulate hypothalamic NPY mRNA [139,176]. If insulin is administered peripherally this results in hypoglycemia and subsequent hyperphagia [163], whereas centrally administered insulin reduces food intake and causes weight loss [193]. Perhaps because of the insulin resistance of the

dexamethasone treated animals, it was posssible to administer higher doses of insulin peripherally without producing hypoglycemia. If, as would be expected, the high peripheral insulin concentrations in the plasma of these animals resulted in raised CSF insulin concentrations, these might have then acted to decrease hypothalamic NPY mRNA. Insulin treatment did not significantly decrease food intake in the dexamethasone treated animals; this may be because dexamethasone has a powerful independent anorectic effect. In contrast, peripheral insulin administration producing hypoglycemia and hyperphagia has not been shown to affect hypothalamic NPY concentrations following either acute or chronic administration, although NPY mRNA was not examined in that study [163]. Acute or recurrent hypoglycemia, perhaps through direct effects on hypothalamic glucosensors, may override the more chronic mechanisms of NPY-insulin interaction in the CNS explored in this study. It therefore seems plausible that insulin is involved in the regulation of hypothalamic NPY, and that this is one possible mechanism whereby insulin may act in the CNS to regulate food intake.

In summary, hypothalamic NPY content and mRNA are increased by chronic administration of the synthetic glucocorticoid, dexamethasone, these changes are reversed following cessation of this treatment and prevented by concomitant insulin administration. This provides evidence that corticosteroids and insulin may be involved in the regulation of hypothalamic NPY, which may be of particular relevance to the possible function of insulin as a satiety factor. Reduced hypothalamic sensitivity to insulin, leading to increased corticosteroid dependent NPY gene expression could result in hyperphagia and obesity.

Chapter 6

Hypothalamic NPY in the hyperphagia of pregnancy and lactation

Introduction

Pregnancy and lactation are associated with considerable metabolic demands. These are met by a number of homeostatic mechanisms. During pregnancy in the rat food intake increases to approximately 50% more than controls. In lactation, the excess demand is even greater, and food intake may rise by as much as 400%. There is marked gut hypertrophy during lactation, presumably in response to the massive increases in ingested nutrients [202]. Changes also occur in maternal metabolism, including a marked reduction in thermogenesis during lactation [203]. Glucose metabolism is also altered, with increased insulin resistance, and raised insulin, during pregnancy, but decreased insulin concentrations and increased sensitivity to insulin, particularly in the mammary gland, during lactation [204-206]. The mechanisms involved in the regulation of food intake in lactation are poorly understood, although some satiety factors, for example CCK may play a role [207,208].

One study previously investigated NPY in lactating rats, this immunohistochemical study suggested increased NPY-like immunoreactivity in the median eminence when compared to controls [209]. No study of NPY mRNA was made. Another study suggests that NPY may be involved in the control of oxytocin release [210].

This experiment was designed to investigate the possible role of NPY in the hyperphagia and altered metabolism which occurs during pregnancy and lactation.

Methods

Animals

Timed mated pregnant and non-pregnant (virgin) female rats (B & K Universal, Hull, UK), were obtained at 15 days gestation. All the animals had free access to standard laboratory chow and water initially. They were kept under controlled temperature (21-23°C) and light (11hr light/13hr darkness). Daily food intake was measured from the day after arrival in the animal facility.

Experiment 1

Twenty-six female rats in mid-gestation, and seven virgin rats were used for this pilot study. Cohorts of five or six rats were killed in late pregnancy, in early lactation (5 days after delivery of the pups), and in late lactation (14 days after delivery). The control rats were killed at the same time as those in late pregnancy. Additional controls for the lactating rats (who had their litters adjusted to 8 by culling and fostering) were dams who

had all their young removed within 48 hours of delivery. The hypothalamus was dissected as previously described and stored at -70^{0}C until measurement of NPY RNA. Blood glucose was measured from a tail vein sample using a reflectance meter. Plasma was taken for measurement of insulin.

Experiment 2

Thirty-two pregnant and virgin female Wistar rats were used for this part of the study. Twenty-four hours after delivery of the pups, the number of pups was adjusted by culling and fostering to 8 per dam. Half the lactating females were food restricted to 80% of control lactating females intake on the previous day. Likewise control animals were either fed ad lib, or food restricted to 80% of control food intake. Each rat was paired with a control of similar weight for this purpose. All rats were killed on the 14th day of lactation for this part of the study

Tissue and plasma collection

Blood glucose was measured on a tail vein sample as described above. Plasma samples were obtained from all animals for estimation of insulin and LH. Six animals in each group were used for measurement of total hypothalamic NPY mRNA, and ten for radioimmunoassay of NPY in microdissected nuclei as described in Chapter 2.

Results

Experiment 1

Food intake was increased by 20% over controls in late pregnancy (controls: 15.6 ± 0.6g, pregnant: 19.8 ± 1.1g, $p<0.01$), and dramatically to 215% of control levels at 5 days lactation and to 355% of control levels in late lactation. There was no significant difference in randomly measured glucose levels between control, pregnant and lactating females (table 6.1). Hypothalamic NPY mRNA was unchanged in pregnancy, moderately increased after 5 days lactation (130 ± 6.2 % of control, $p<0.01$) and increased further at 14 days lactation (179 ± 14%, $p<0.001$).

Table 6.1

Blood glucose and plasma insulin levels in control, pregnant and lactating female rats (mean ± SEM). None of the differences are statistically significant.

Study group	Blood glucose (mmol/l)	Plasma insulin (pmol/l)
Control	5.9 ± 0.9	567 ± 65
Pregnant	5.6 ± 0.6	640 ± 73
Lactation stopped	6.5 ± 0.5	453 ± 45
Lactation day 5	6.5 ± 0.5	546 ± 54
Lactation day 14	6.4 ± 0.5	515 ± 43

Experiment 2

Food Intake and Body Weight

The food intake and body weight changes in control and unrestricted lactating animals were similar to those seen in the pilot study. Thus food-restricted lactating rats were still consuming over 300% of non-lactating levels of food intake. At this level of food restriction the animals lost weight, but did not harm any of the pups. Changes in body weight in the four groups are shown in table 6.2

Plasma glucose and insulin

Food restricted females had significantly lower glucose levels (3.7 ± 0.2mmol/l compared to 5.1 ± 0.2 in lactating controls, $p<0.05$). Insulin levels were similar in control and lactating females. Food restricted females had significantly lower insulin levels (49.5 ± 18.4pmol/l compared to lactating controls 215 ± 55pmol/l ($p<0.01$) Results are shown in table 6.2.

Plasma LH levels

Plasma levels of LH were decreased in lactation and in food deprived animals, however there was no additive effect of food deprivation and lactation over and above the effect seen with food deprivation alone (Table 6.2).

Table 6.2

Weight changes, blood glucose and plasma insulin and LH levels in control and lactating female rats with or without food restriction to 80% of control levels during lactation (mean ± SEM).

	Control	Control Restricted	Lactating	Lactating Restricted
Initial Weight (g)	206.7 ± 5.6	202 ± 5.4	248.6 ± 16	249.3 ± 16
Final weight (g)	217.3 ± 16	191 ± 3*	275.7 ± 15.5	240.7 ± 15#
Weight Gain (g)	+ 10.6 ± 2.7	-11 ± 2.6*	+27.1 ± 3.7	-8.6 ± 4#
Blood Glucose (mmol/l)	4.7 ± 0.2	3.9 ± 0.2*	5.1 ± 0.2	3.7 ± 0.2*
Log plasma Insulin (pmol/l)	2.5 ± 0.5	2.0 ± 0.3	2.4 ± 0.4	1.3 ± 0.4*
Plasma LH (ng/ml)	1.97 ± 0.3	1.1 ± 0.2*	0.73 ± 0.3**	0.65 ± 0.4**

** $p<0.05$, ** $p<0.01$ compared to control*

 # $p<0.01$ compared to unrestricted lactating

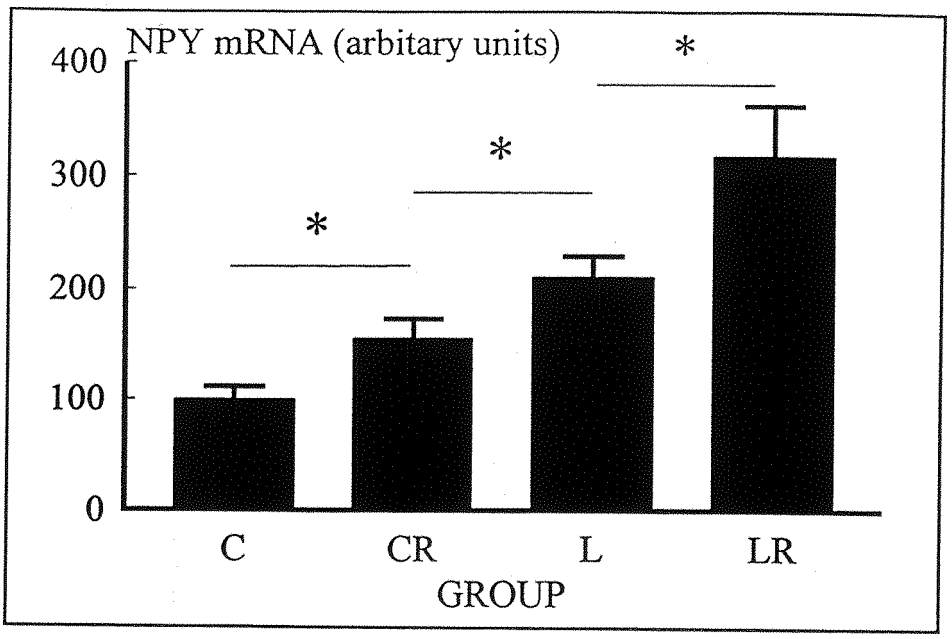
NPY mRNA

Results in the lactating animals were similar to those seen in the pilot study. Food restricted animals had the expected increase in NPY mRNA ($p<0.05$). The greatest changes occurred in the animals who were food deprived during lactation, when hypothalamic NPY mRNA levels reached 324 ± 44% ($p<0.001$) of control levels (Fig 6.1).

Figure 6.1

Hypothalamic NPY mRNA in control, food restricted and lactating rats (mean ± SEM)

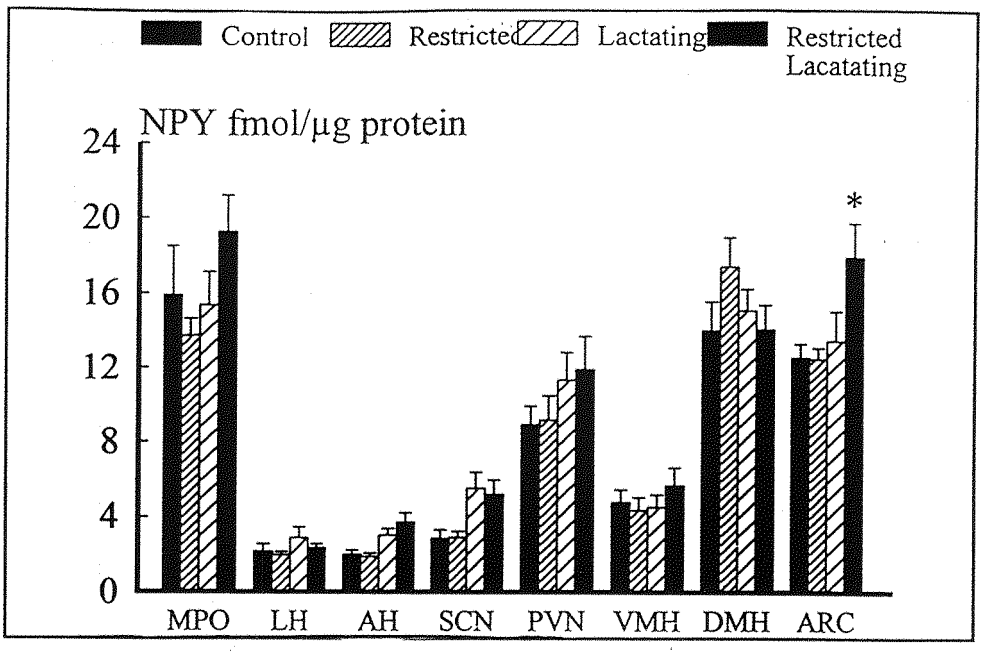
** $p<0.05$, C=control; CR=control restricted; L=lactating; LR=lactating restricted.*



NPY in microdissected hypothalamic nuclei

NPY content was significantly increased in the arcuate nucleus of food deprived lactating rats, but not in the food restricted females or in lactating females compared to controls (Fig 6.2).

Figure 6.2 *Hypothalamic NPY content in microdissected hypothalamic nuclei in control, food restricted, lactating and food restricted lactating rats.*



$p<0.05$

Discussion

NPY mRNA was increased in the hypothalamus of female rats during lactation, but not during pregnancy. The increases in lactation were most marked in the later stages, when food intake was greatest. Of particular interest is the apparent additive effect of food deprivation on these hypothalamic NPY mRNA changes in lactation. Recently published work has found a similar increase in NPY mRNA in lactation using in-situ hybridisation, and localised this change to the arcuate nucleus [206], so it is likely that the changes in total hypothalamic NPY mRNA reflect changes in this nucleus. It is not possible to tell from this study whether the changes in expression in lactation and food deprivation occur in the same subset of arcuate neurons, or whether there is recruitment of neurons from different populations which subserve the two functions. However a previous study found increased NPY immunoreactivity in the median eminence in lactation, a site not normally associated with increases in NPY during food restriction, which would support the hypothesis that different subsets of neurons are involved [209].

The increase in NPY mRNA in the hypothalamus in lactation may be concerned with the suppression of LH secretion observed in this study, either directly, or via suppression of gonadotrophin-releasing hormone secretion into the hypophyseal portal

system. It is interesting to note that food restriction also reduced plasma LH concentrations, and that the effects of lactation and food restriction were not additive in this respect. Secretion of LH is suppressed by fasting and exercise, and rapidly restored by intragastric infusion of nutrients, suggesting that specific nutritional signals are involved [211,212]. These findings would be consistent with a role for NPY in the suppression of LH secretion during fasting.

The reduction in plasma insulin levels in the food restricted animals was expected, and is in accord with previous observations suggesting an inverse relationship between insulin levels and hypothalamic NPY expression [213]. In this study however the insulin levels were unchanged during lactation, so it is difficult to ascribe the changes in NPY mRNA during lactation to alterations in insulin concentration. This could be because the changes in lactation are unrelated to the increased food intake, and are perhaps involved in other aspects of reproductive function or in the control of lactation itself. Alternatively there may be altered sensitivity to insulin in lactation; this is observed to occur in the mammary gland, which becomes very insulin sensitive during lactation, and total glucose turnover is markedly increased. Despite this the plasma glucose and insulin levels are unchanged after a meal during lactation, because of increased clearance from the mammary gland. Hence there is no evidence that total body insulin sensitivity is altered. Another possibility is that changes in steroid hormones, particularly progesterone, may be responsible for the increase in NPY mRNA which was seen in lactation.

The changes in peptide content in microdissected hypothalamic nuclei in this study were not as expected. The lack of changes in food deprivation may be due to the protocol used, which resulted in only small changes in body weight and indicates the increased sensitivity of mRNA levels over peptide content when looking for changes in activity.

In summary NPY expression in the hypothalamus is increased in lactation, and may be partly responsible for the massive hyperphagia seen in lactating rats. NPY is apparently not involved in the increased food intake seen in pregnancy. The increase in NPY mRNA in lactation and food deprivation may explain the reductions in LH secretion seen in these circumstances, although other neurotransmitter systems are also likely to be involved. The factors involved in stimulating the increase in NPY synthesis were not identified in this study, but unlike in food deprivation, insulin is less likely to be a factor in the hyperphagia of lactation. Other nutritional factors, altered gut hormone secretion or other hormones such as progesterone may all be involved.

Chapter 7

Neuropeptide Y - A physiological regulator of food intake

Introduction

Hypothalamic NPY may have an important physiological role in appetite control. The concentration of NPY, and its mRNA, are increased in the hypothalamus of rats following food deprivation and these changes are rapidly reversed by re-feeding [138,150]. Locally inserted push-pull cannulae have demonstrated that NPY is released episodically in the PVN prior to and during feeding in rats [182].

Central injection of opioid agonists stimulates feeding, while central opioid receptor blockade reduces food intake [214,215]. Stimulation of feeding in the rat occurs after activation of μ, δ or κ opioid receptors. Opioid agonists which act primarily at the κ-opioid receptor, such as dynorphin, are the most potent opioid inducers of feeding after central injection [216]. The 17-amino-acid form of dynorphin has been identified as the putative endogenous ligand for the κ-opioid receptor [217], and, therefore may be important in the regulation of feeding. Dynorphin is widely distributed in the hypothalamus and is found in relatively high concentrations in the PVN [218]. Immunoreactive dynorphin concentrations are increased in the hypothalamus of rats after food deprivation and in the dark phase [219]. Blockade of kappa receptors with the selective kappa antagonist norbinaltorphamine (norBNI) reduced deprivation induced feeding. Together these findings suggest a physiological role for the kappa opioid system in the regulation of food intake.

Noradrenaline (NA) also stimulates feeding when injected into the CNS [124], and this effect can be blocked by the α-adrenergic antagonist phentolamine [220]. NPY is co-localised with NA in neurones projecting to the PVN from the brainstem. Using microdialysis, the extracellular level of NA within the PVN of the rat has been shown to gradually increase during a period of food deprivation [221]. This pattern of release suggests that endogenous NA plays a role in the maintenance of fast-induced feeding.

Hypothalamic NPY content and mRNA are increased in animal models of diabetes [136,137]. Diabetic animals are hyperphagic and it has been suggested that this effect may be partly mediated through hypothalamic NPY [136]. The effect of exogenously administered NPY on feeding responses in diabetic animals has not been investigated. It was therefore decided to investigate the possibility that these changes may be due to changes in the sensitivity to hypothalamic neuropeptide Y.

In the absence of a high potency NPY antagonist, the aim of this study was to immunoneutralise endogenous NPY in the CNS to further examine the role of endogenous

NPY in deprivation induced feeding. The role of NPY in the regulation of feeding in experimental diabetes was also investigated. Possible interactions in the CNS with other known appetite stimulating neurotransmitters in the control of deprivation induced feeding were explored using the non-selective α-adrenoceptor antagonist phentolamine, and the kappa antagonist norbinaltorphamine (nor BNI).

Methods

Animals

Adult male Wistar rats (250g) were maintained in individual cages under controlled temperature (21-23°C) and light (11hr light, 13hr dark) with ad libitum access to food and water.

Experimental protocol

Rats were implanted with chronic indwelling cannulae in the third ventricle as described in chapter 2. All animals were allowed a period of seven days to recover from surgery before being used in the study. The placement of cannulae were verified at the end of the study by the injection of 10μl of ink, removal of the brain, snap freezing and visual examination of coronal brain slices.

The injection of substances was by a stainless steel injector, placed in, and projecting 0.5mm below the tip of the cannulae. The injector was connected by a polythene tubing (id 0.5mm, od 1mm) to a Hamilton (Reno, NV) syringe in a Harvard IV syringe pump (model 2681, Harvard Apparatus, Natick, MA, USA) set to dispense 10μl of solution per minute. All compounds were dissolved in 0.9% saline. Immediately after the ICV injections rats were placed into cages containing a single pre-weighed bowl of chow, with free access to water, and observed for two hours. At the end of the two hour period, the remaining food was carefully collected and weighed and the total food intake for each rat was calculated. All treatments were given at least 48 hours apart, between 9 am and 11 am, and were of crossover design.

Antibodies

Monoclonal antibodies were produced using the procedure described by Bidart et al [222] and their binding profiles were examined by radioimmunoassay. NPYAb showed good affinity for NPY such that 5μl diluted antibody (1:100000) incubated with

I^{125} labelled porcine NPY showed a 75% binding of labelled peptide. The antibody shows very weak cross-reactivity with PYY (IC_{50}, 2μM) [223]. A control antibody to chloroquine prepared by exactly the same method (CQAb) showed no NPY binding, even when undiluted.

Experiment 1 - antibody test in vivo

Food intake after central injection of NPY (2.4nmoles/5μl) was investigated. Satiated rats were administered ICV injections of NPYAb (5μl), saline (5μl) or CQAb ten minutes before injection of NPY (n=9). Food intake was recorded over the next two hours.

Experiment 2 - 24h food deprivation

Animals were fasted for 24 hours, following which their food intake was monitored for 2 hours. Immediately prior to feeding, animals were injected ICV with saline, 1, 3, 5, 7.5 or 10μl NPYAb or CQAb. An additional experiment was carried out using norBNI with or without the 5μl dose of NPYAb and CQAb.

Possible effects of phentolamine (180nmol in 5μl) plus CQAb or phentolamine plus 5μl NPYAb on fast-induced feeding were investigated in a separate experiment in 6 rats.

Experiment 3 - Experimental diabetes

Six rats were made diabetic by an intravenous injection of streptozotocin (55mg/kg) and their blood glucose levels measured using an Ames glucometer. At the time of the study every animal had a blood glucose greater than 16 mmol/l. Two weeks after the streptozotocin injection animals were injected centrally with either NPY, saline or, following an overnight fast, with NPYAb or CQAb. These studies were carried out in parallel with those in normal animals (blood glucose = 5.8 ± 0.6 mmol/l), so that a direct comparison between groups could be made.

Statistical Analysis

All results are given as mean ± SEM. Comparisons between groups of data for effects of NPY, fasting, antibody and phentolamine were made using ANOVA. Post-hoc comparisons were made using Tukey's test. In the case of the dose response data in normal and diabetic animals, repeat measures ANOVA was used. Statistical significance was taken at $p < 0.05$.

Results

Experiment 1 - in vivo antibody test

Injection of NPY (2.4nmol) produced an increase in food intake (5.5 ± 0.5 vs 0.9 ± 0.6g for saline treated controls). The response to NPY began approximately 20 minutes after injection and was complete within two hours. Pretreatment with CQAb (5µl) had no effect on the feeding response to NPY (6.0 ± 0.7g). Pretreatment with 5µl of the monoclonal antibody to NPY reduced the feeding response to a level which was similar to saline injected controls (1.4 ± 0.6g). Animals were viewed throughout the experiments, and the general behaviour of rats treated with NPYAb or CQAb was not observably different from that of saline treated controls.

Experiment 2 - 24 hour food deprivation

Rats exhibited a robust feeding response immediately they were introduced to food after a 24 hour fast. ICV injection of NPYAb 10 minutes before fasted animals were fed dose dependently reduced their feeding response over a two hour period compared to animals treated with CQAb (figure 7.1). The maximal reduction in feeding of 30 ± 6% {$F(1,20)=4.8$; $p<0.01$} was seen after injection of 5 µl NPYAb, and therefore this volume was used for all subsequent studies. There was no difference in the fast-induced feeding response after injection of 5 µl CQAb compared to saline. ICV injection of norBNI 10 min before fasted animals were presented with food reduced their 2-hour feeding by 34 ± 10 % {$F(1,20) =4.9$; $p<0.05$} compared to that of animals treated with saline . A significantly greater reduction in feeding of 51 ± 8 %; $p<0.05$ than observed with NPYAb alone was seen after injection of norBNI 10 min before injection of antibody (figure 7.2). Injection of phentolamine alone reduced fast-induced feeding by 39% {$F(1,20) = 5.4$; $p<0.05$}, but addition of NPYAb was not additive.

Figure 7.1 *Dose response curve for NPY Ab. Fast-induced food intake after icv injection of increasing volumes of NPYAb 10 min before presentation of food. The food eaten is expressed as a percentage of food intake after injection of the same volume of CQAb. Significant differences from CQAb are indicated: * p<0.05; **p<0.01.*

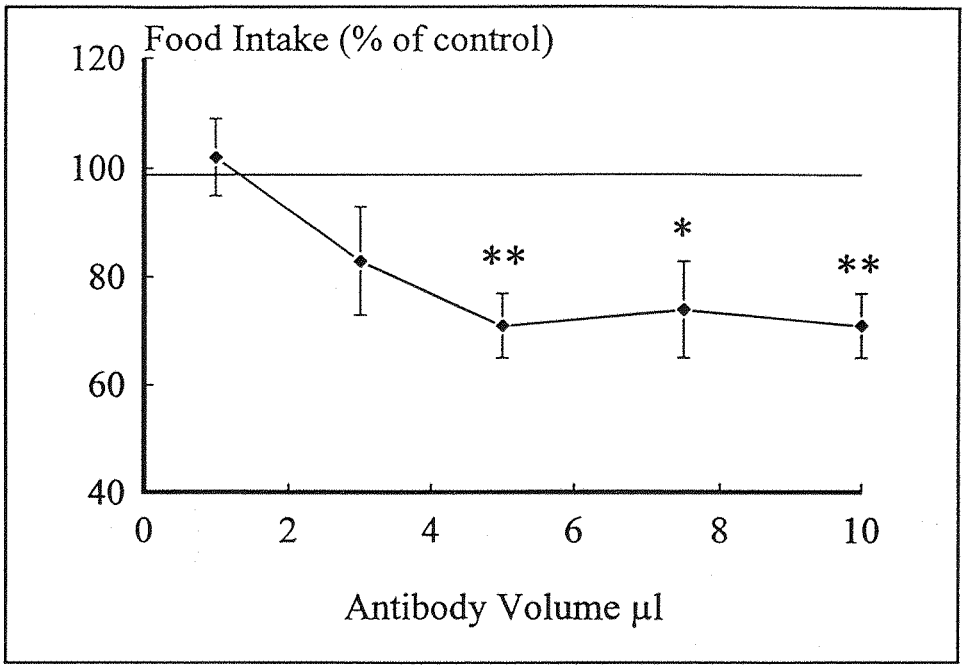
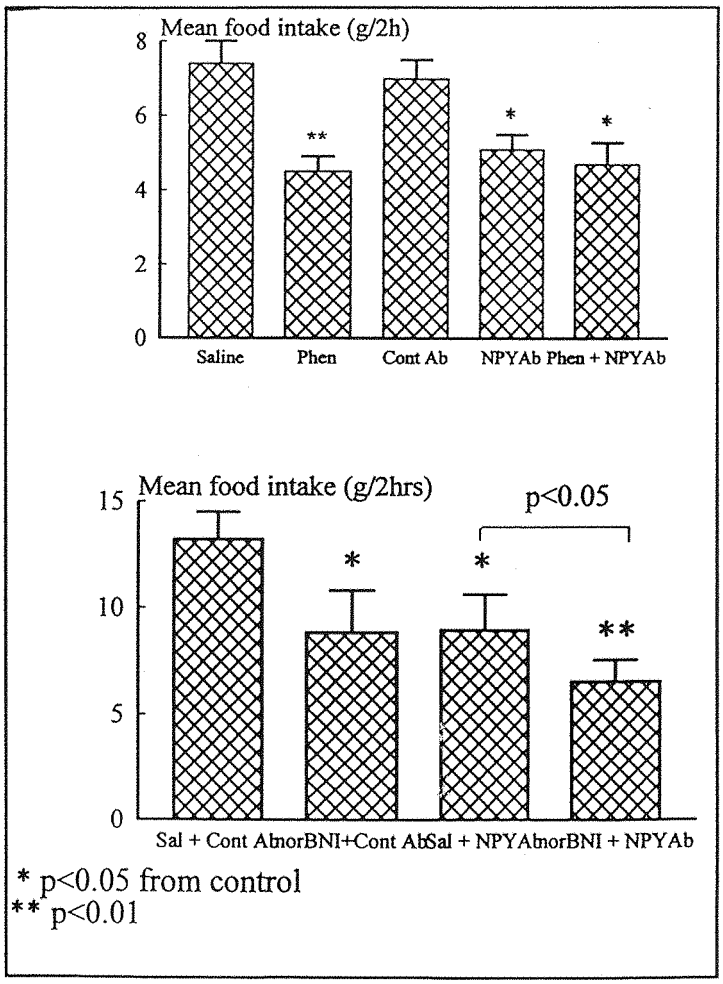


Figure 7.2
Effects of phentolamine and norBNI on fast induced feeding.
**p<0.05; **p<0.01*

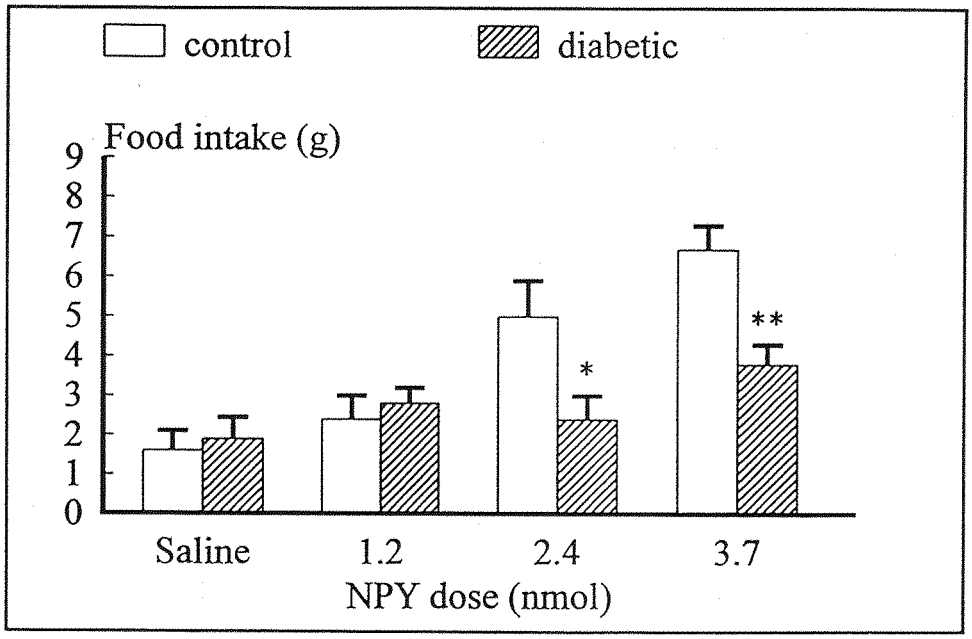


Diabetes

The expected dose dependent feeding response to NPY was seen in normal animals. There was no significant difference in the 2 hour food intake of diabetic and control animals after ICV injection of 1.2 nmoles of NPY. However, the feeding response in diabetic animals failed to increase with dose and was significantly reduced compared to normal animals at both the higher doses of NPY ($p<0.04$) (figure 7.3). Administration of NPYAb (5µl) 10 minutes before presentation of food after a 24 hour fast did not alter food intake in diabetic rats (figure 7.4).

Figure 7.3

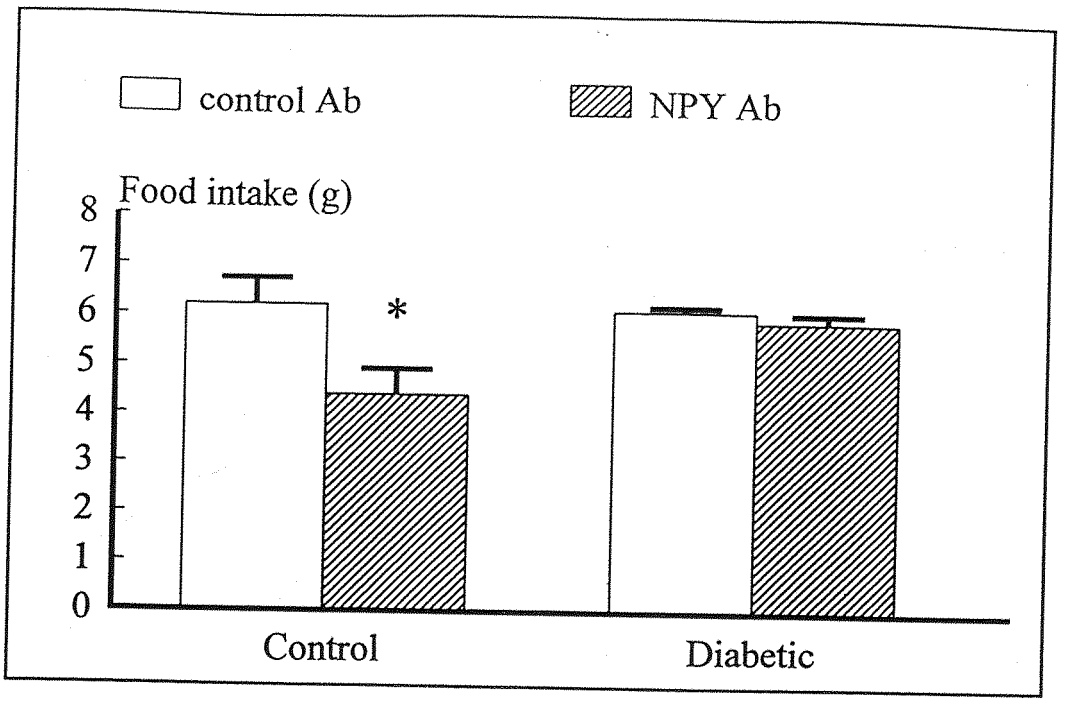
Dose response relationship between NPY dose and 2 hour feeding in control and diabetic rats.



$*p<0.05$; $**p<0.01$

Figure 7.4

Effect of NPY Ab on fast induced feeding in control and diabetic rats.



*$p<0.05$

Discussion

This study supports a physiological role for endogenous NPY in inducing food intake after a 24 hour fast. The results show that; 1) endogenous NPY in the central nervous system is required for the full feeding response to occur after food deprivation; 2) interactions exist between NPY and the noradrenergic and kappa-opioid systems in the control of feeding and 3) central sensitivity to the effect of NPY on food intake is reduced in experimental diabetes.

Immunoneutralization of endogenous NPY reduced feeding in the fasted rat by 30%. This would support the hypothesis that NPY is an important component of the neural signals involved in deprivation induced feeding. The results of this study are supported by other published studies, using either polyclonal NPY antibodies or weak NPY antagonists [131,224], although the magnitude of the effect appears to vary. One possible reason for the larger (70%) reduction in feeding in the study by Stanley et al [131] is the lack of a specific control antibody, which may have resulted in an overestimation of the inhibitory effect. Another is the fact that they used a palatable milk mash diet when testing their feeding responses, the consumption of which may have been more sensitive to

NPY inhibition.

Dynorphin also appears to have a role in the central control of food intake. This data is in agreement with other published data, showing that blockade of dynorphin with nor BNI decreases deprivation induced feeding [225]. The additive effect of norBNI and NPYAb on fast-induced feeding would suggest that the action of dynorphin may be through a separate mechanism from that of endogenous NPY. This effect (52% reduction in food intake) is not as great as would be expected from the sum of the individual reductions (64%). This could be due to experimental variation or may indicate that the action of dynorphin is partly through the same mechanism as NPY.

Central adrenergic pathways are also important mediators of food intake in the fasted rat [220], and would seem to interact with NPY. Thus phentolamine reduces 24-h fast induced feeding, and the effects of central α-adrenergic blockade and immunoneutralization of NPY on the feeding response are not additive. This finding is in accordance with a study by Morley et al [93], who found that NPY and NA did not have additive effects on food intake. Therefore, NPY and NA both appear to have a role in controlling food intake after a period of food deprivation. Although this data suggests that their actions may be mediated through a common pathway, further dose-response studies need to be carried out to confirm this.

Finally, the feeding response to central injection of NPY in diabetic rats is greatly reduced and fails to increase with dose. The same volume of NPYAb that reduced feeding in fasted control rats had no effect on fast-induced feeding responses in diabetic animals. One explanation for the lack of changes in food intake, is that central NPY levels are so elevated in diabetes that relatively small variations, produced by ICV injection of NPY or NPYAb, exert no measurable physiological effect. There is one report suggesting that NPY receptor downregulation may occur in experimental diabetes [226]. However recently completed work in Professor Bloom's laboratory, and carried out under my supervision (D. Morgan, personal communication) has been unable to reproduce this result, using either total NPY binding, or binding of any of the specific ligands for NPY receptor subtypes (NPY 13-36 for Y_2 or NPY (Leu 31, Pro 34) for Y_1), either in hypothalamic membrane preparations or by semi-quantitative autoradiography from hypothalamic slices. An alternative hypothesis is that there are changes in other neurotransmitters 'downstream' of NPY which act to reduce its effectiveness. One possibility is CRF, which inhibits NPY-induced feeding [115,227] and which may be

increased in diabetic animals, who have elevated concentrations of circulating corticosteroids [226].

These findings support a physiological role for NPY in the central control of feeding after food deprivation. There appear to be interactions between NPY and the noradrenergic and opioid systems in the control of appetite and the mechanism of action to cause fast-induced feeding may involve the action of noradrenaline and NPY through a common mechanism, whereas dynorphin, at least in part, may act independently of NPY. In diabetes, central sensitivity to the effects of NPY on food intake is markedly reduced, giving further direct evidence for changes in the central NPY system in streptozotocin-induced diabetes. Clearly these results are only the first step in fully defining the physiological role of NPY in the control of food intake. The precise sites of inhibition will have to be located, although microinjection studies suggest multiple hypothalamic and extrahypothalamic sites [105]. The clarification of the role of NPY in day to day feeding may only be made when specific longer-acting antagonists are available.

Chapter 8

Effects of centrally injected NPY on peripheral glucose metabolism

Introduction

Many appetite regulating neurotransmitters exert acute effects on glucose metabolism and energy balance when injected into various sites in the central nervous system. The main sites in the CNS mediating these effects are the paraventricular and ventromedial hypothalamic nuclei (PVN and VMH), and the nucleus of the tractus solitarius in the brainstem (NTS) [124,189,228-230]. These effects may be mediated by changes in the activity of the autonomic nervous system, which may influence glucose metabolism in a number of ways, for example by influencing secretion of islet glucoregulatory hormones, hepatic glucose production, or sensitivity to insulin in peripheral tissues.

The increased food intake produced by central injection of NPY is mediated in several hypothalamic and extrahypothalamic sites, including the perifornical hypothalamus, the PVN and other medial hypothalamic nuclei [105]. A number of studies have suggested that NPY stimulates insulin release when injected into the third cerebral ventricle or into the NTS [125,231-233]. In those studies where food was not available, and the plasma glucose concentrations were measured, it was not altered despite the rise in insulin, suggesting that NPY was acting via the nervous system to directly increase insulin secretion. The lack of change in glucose in these studies suggests that hepatic glucose output and / or peripheral insulin sensitivity are also altered by centrally-injected NPY.

The aim of this study was to investigate the effects of centrally injected NPY on glucose metabolism in more detail. Thus hepatic glucose output and peripheral insulin sensitivity were measured following central injection of NPY.

Methods

Animals

Male syngeneic Ludwig Wistar rats (250-300g) were used throughout the study. They were kept in conditions of controlled temperature and light (12h light, 12 h dark, lights off at 18.00hrs, 22^{0}C) and had free access to pelleted chow and water except where indicated. They were housed individually from the time of implantation of the icv cannulae.

ICV and peripheral venous cannulation

Rats were anaesthetized with Ketamine (45 mg/kg ip) and Xylazine (21 mg/kg ip) and permanent 23 gauge stainless steel guide cannulae (Plastics One, Roanoke, VA, USA) were stereotactically placed (Kopf) 0.8mm posterior to the bregma on the mid-line and implanted 6.5mm below the outer surface of the skull into the third cerebral ventricle. The injection of substances into the CSF was by a stainless steel injector (27 gauge) which projected 1mm below the tip of the guide cannulae. The injector was connected by a polythene tubing to a Hamilton syringe in a syringe pump (Harvard Apparatus Ltd, Edenbridge, Kent, UK) set to dispense 10µl of solution per minute. All injections were given in a volume of 5µl. Seven days after stereotactic surgery (24h before study) rats were briefly anaesthetized with ether and 19g cannulae (Bard-I-Cash, Bard International, Sunderland, UK) inserted into a femoral vein, with its tip in the inferior vena cava (for sampling of venous blood) and the superior vena cava, with its tip in the right atrium (for infusions). The cannulae were tunnelled to emerge from the back of the animal and sutured in place. Cannulae were filled with 0.154 mol/l saline and stoppered until required. All studies were undertaken in conscious, unrestrained rats taking care not to disturb the animals during icv injection or blood sampling. The femoral venous cannula was flushed with 200 µl 0.154 mol/l saline after each blood sample.

Hyperinsulinaemic-euglycaemic clamp

Rats were fasted for 10 hours before study. NPY (2.4 nmol) was injected into the third ventricle in 2 doses of 1.2 nmol each at -30 and 0 min as described above. Basal blood samples were taken at -30, -20 and -10 min for measurement of plasma insulin and blood glucose levels. At 0 min an infusion of insulin (Actrapid, Novo Industri, Bagsvaerd, Denmark) diluted in polygeline (Hoechst, Frankfurt am Main, Germany) at a rate of 100 mU/h was begun and continued for 90 min. Blood glucose was measured every 5 min using a glucose-oxidase based autoanalyser (Yellow Springs Instruments, Clandon Scientific, London, UK). An infusion of 50 % dextrose was adjusted to keep blood glucose at 4.0 mmol/l. Steady state blood glucose and glucose infusion rates were attained by 60 min. The means of the blood glucose concentrations and glucose infusion rates during the last 30 min of the clamp were used for comparison of the 2 groups. Blood samples (200 µl) were taken at 75 and 90 min for measurement of steady-state plasma insulin levels; blood was centrifuged immediately and the plasma stored at -40^{0}C until assayed.

Effects of NPY on glucose kinetics in fasted rats

Rats were prepared as before. After a 10 h fast a 2.5h primed constant infusion of 6-^{3}H-glucose (Amersham International, Amersham, UK) diluted in 0.154 mol/l saline (14.4 μCi/ml) was begun at a rate of 0.12 μCi/min. In rats steady state glucose specific activity is attained within 40 min [234]. Blood samples were therefore taken 50 and 60 min after the start of the 6-^{3}H-glucose infusion for measurement of basal plasma glucose concentration and specific activities and calculation of basal rates of glucose appearance (Ra) and disappearance (Rd). Rats were then injected (t=0 min) with a single dose of 2.4nmol of NPY as described above. Blood samples were taken at +15, +30 min and then every 10 min until +90 min for measurement of plasma glucose concentration and specific activities, and every 30 min for measurement of insulin and glucagon levels. Note that plasma, as opposed to blood glucose (as measured during the clamp) was measured for this part of the study. Blood glucose is 35% lower than plasma glucose in rats due to the low numbers of erythrocyte glucose transporters.

To determine ^{3}H glucose specific activity, 30 μl plasma was deproteinised with $Ba(OH)_2/ZnSO_4$ [235]. After centrifugation (15 min, 2000g, 4°C) an aliquot of the neutral supernatant was evaporated to remove ^3H_2O and the residue dissolved in 1.0 ml water. After adding 10 ml of scintillation fluid (Cocktail T, BDH, Poole, UK) ^{3}H dpm were counted in a PW4700 liquid scintillation counter (NV Phillips' Gloeilampenfabrieken, Lelyweg, Holland) using an external standard to correct for quenching. Quadruplicate aliquots of the infusate were processed as for the plasma samples to allow calculation of the 6-^{3}H-glucose infusion rate. Minimum recovery after deproteinisation, neutralisation and evaporation was 94%.

Calculations

Basal glucose Ra (and hence Rd) was calculated from the formula:

$$\text{Glucose turnover} = \frac{\text{tracer infused (dpm/min)}}{\text{glucose specific activity (dpm/μmol)}}$$

After NPY injection glucose Ra and glucose Rd were calculated from the 6-^{3}H-glucose data using the non-steady state equations of Steele et al [236]. A glucose

distribution volume of 0.26 l/kg was used in the calculation [237]. Glucose metabolic clearance rate (MCR) was calculated by dividing glucose Rd by the average plasma glucose concentration during each measurement interval.

Radioimmunoassays

Plasma insulin and pancreatic glucagon were measured by radioimmunoassay as described previously [191,238]. All samples were measured in duplicate in a single assay. The insulin assay used rabbit anti-insulin serum and had an intraassay coefficient of variation of 6.7%. The glucagon assay used a rabbit anti-glucagon antibody (RCS5) which did not cross react significantly with any other proglucagon derived peptides and had an intraassay coefficient of variation of 11.9% at 4.2 pmol/l and 3.8% at 21pmol/l .

Statistical analysis

Results are expressed as mean ± SEM. The areas under the glucose Ra and Rd curves were calculated using the trapezoidal rule. The significance of differences was tested using Student's unpaired or paired t-test as appropriate. Statistical significance was taken at $p<0.05$.

Results

Effect of NPY on insulin sensitivity in vivo

Body weights, basal and clamp blood glucose concentrations were similar for the control and NPY-treated groups (Table 1). Steady-state plasma insulin levels were also similar in the two groups indicating that NPY administration had no effect on insulin MCR. The amount of glucose necessary to maintain the blood glucose level at 4.0 mmol/l during the last 30 min of the clamp was significantly higher in the NPY-treated rats (227 ± 11) than in the controls (192 ± 9 μmol/min/kg) ($p<0.05$). Fasting plasma glucagon levels were similar in both groups (Table 1). During the clamp glucagon levels fell in the control rats ($P<0.05$ vs basal) but not in the NPY treated rats, and were lower at the end of the clamp in the control than in the NPY treated rats (Table 1, $p=0.06$).

Table 8.1

Baseline levels of weight, glucose and pancreatic hormones in controls and rats infused with NPY (2.4 nmol) into the third ventricle. Clamp levels are those obtained at the end of a 100 mU/hr hyperinsulinaemic euglycaemic clamp.

	Control (n=5)	NPY infused (n=7)
Weight (g)	233 ± 4	231 ± 11
Blood glucose (mmol/l)		
Basal	4.2 ± 0.3	4.1 ± 0.2
Clamp	4.0 ± 0.1	3.8 ± 0.1
Plasma insulin (pmol/l)		
Basal	38 ± 14	74 ± 19
Clamp	519 ± 62**	492 ± 35**
Plasma glucagon(pmol/l)		
Basal	5.6 ± 0.9	7.1 ± 1.1
Clamp	2.8 ± 1.2*	7.3 ± 1.2

*All data is shown as mean ± SEM; * $p<0.05$ vs basal; ** $p<0.001$ vs basal*

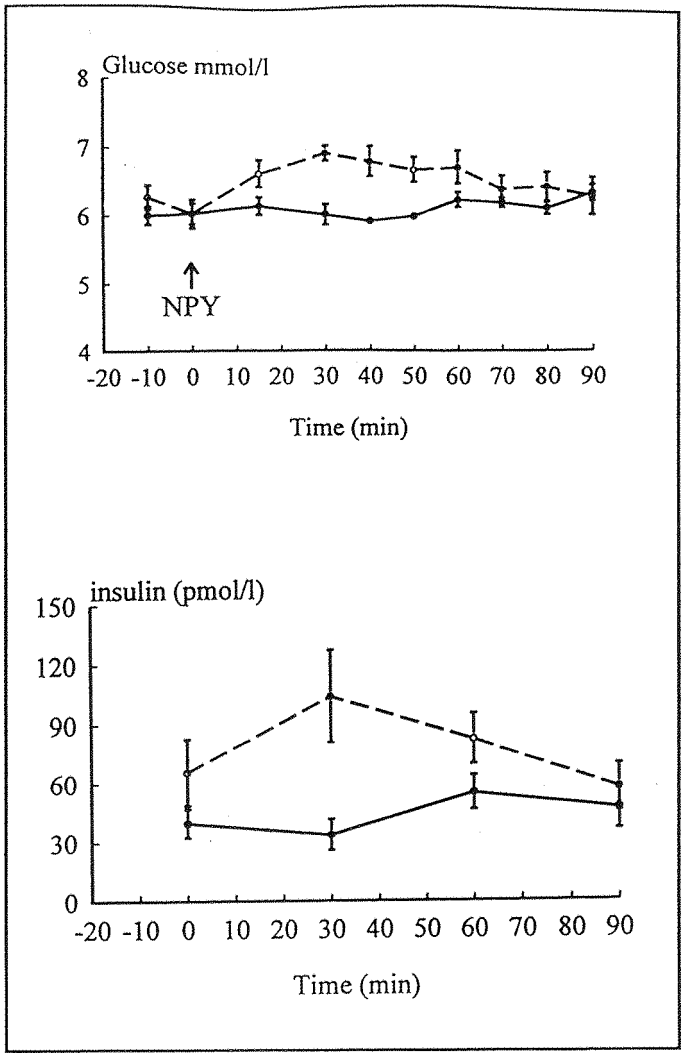
Effects of NPY on glucose kinetics and hormone levels in fasted rats

Fasting plasma glucose levels were similar in the control (6.0 ± 0.1 mmol/l) and NPY treated rats (6.2 ± 0.2 mmol/l). After NPY injection into the 3rd ventricle there was a significant rise in the plasma glucose level to a peak of 6.9 ± 0.1 mmol/l at 30 min (Figure 8.1). Injection of diluent alone into the 3rd ventricle of control rats had no effect on plasma glucose concentration. The area under the 90 min plasma glucose concentration curve was significantly higher in the NPY treated rats (9.9 ± 0.2 $mmol.l^{-1}$ h) than in the control rats (9.1 ± 0.1 $mmol.l^{-1}$ h, $p<0.01$). Basal glucose Ra and Rd were similar in control and NPY treated rats (Figure 8.1).

Figure 8.1

Effects of icv injection of NPY (open circles) and saline (closed circles) on plasma glucose and insulin levels in 10h fasted rats. In the upper panel the area under the glucose concentration curve is significantly greater following NPY treatment ($p<0.01$). Peak insulin levels are also greater ($p<0.01$).

Results are shown as mean ± SEM

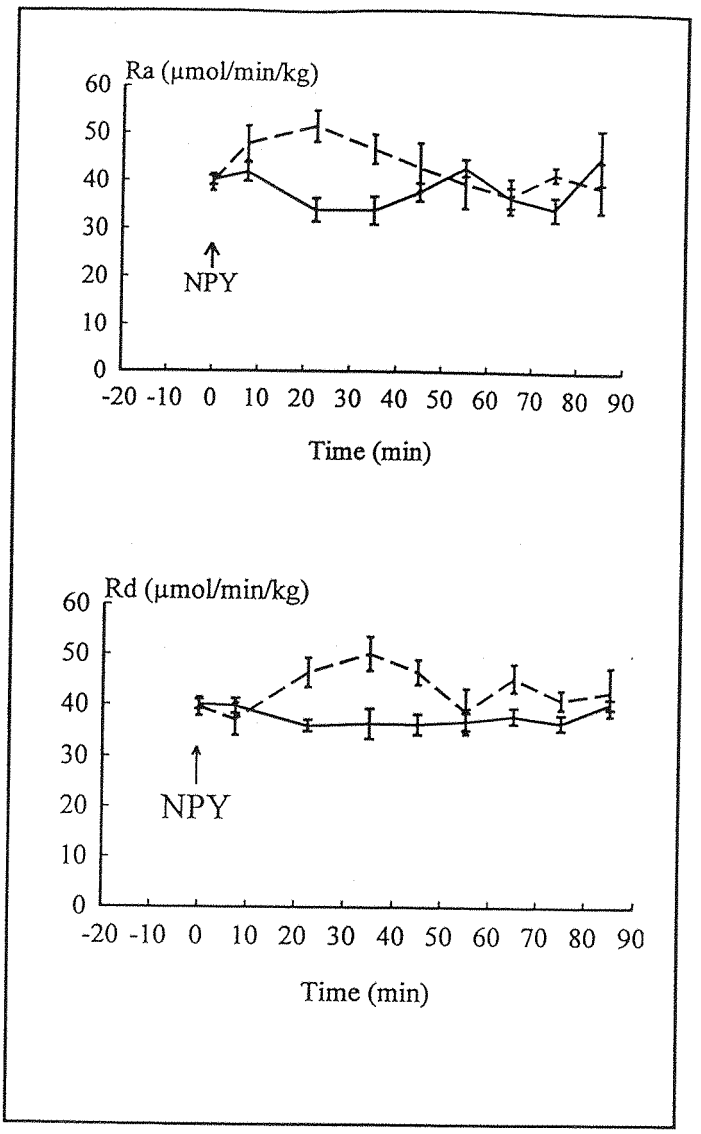


After NPY injection glucose Ra increased to a peak during the interval 20 to 30 min when it was 30% higher than in the basal state (Figure 8.2). Glucose Ra then fell gradually and was not different from that of controls between 45 and 90 min after NPY. The initial rise in glucose Ra was entirely responsible for the increase in plasma glucose levels in the NPY treated rats. During the first 30 min 381 ± 12 μmoles of glucose entered the systemic circulation in the NPY rats compared to 281 ± 15 μmoles in the control rats ($p<0.001$). Glucose Rd was unchanged during the first 15 min after NPY; it then increased to a maximum level between 30 and 40 min (Figure 8.2). In control rats glucose Rd remained unchanged throughout the 90 min. Glucose MCR showed a similar pattern to glucose Rd in the two groups of rats (data not shown). Over the entire 90 min significantly more glucose entered the systemic circulation in the NPY-treated rats (1007 ±

28μmol) than in control rats (844 ± 23μmol, $p<0.002$), and the total quantity of glucose removed was also greater (986 ± 20 vs 848 ± 17μmol, $p<0.001$).

Figure 8.2

Effects of icv injection of NPY in rats after a 10 h fast (open circles) and saline (closed circles) on glucose Ra and Rd. Results are shown as mean ± SEM. Area under the curve differences for Ra, $p<0.002$, Rd, $p<0.001$.



The increase in plasma glucose levels was accompanied by an increase in serum insulin levels; peak levels at 30 min after NPY were 3-fold higher than in control rats (Figure 8.1, $p<0.02$). Glucagon levels also rose in NPY treated rats and were significantly higher 30 minutes after NPY treatment, (basal control 3.5 ± 0.6 pmol/l vs NPY 3.1 ± 0.3 pmol /l, ns; post treatment: control 3.1 ± 0.3 vs NPY 5.5 ± 0.5 pmol/l, $p<0.05$).

Discussion

These results indicate that NPY, injected into the central nervous system of rats, influences both hepatic and peripheral tissue glucose metabolism. Hepatic glucose output was increased by 30% after NPY. This confirms previous observations that centrally

injected NPY produces a rise in plasma insulin, and shows that this is not due to a decrease in insulin MCR, confirming that the increased insulin levels are likely to be due to increased pancreatic insulin secretion. However in this study plasma glucose was also increased after central NPY injection. This rise in plasma glucose was due to increased hepatic glucose output, suggesting that at least part of the observed increase in insulin release is glucose stimulated. The increase in hepatic glucose production may be occurring as a direct result of changes in autonomic nervous system input to the liver, but the small, but significant rise in pancreatic glucagon seen in both studies (which may be greater in the portal system) may also be contributing to this. It seems likely that the increase in glucose observed after injection of NPY would be even greater, were it not for the subsequent rise in insulin. In diabetic rats glucose levels were higher one hour after NPY injection into the CNS, which would support this hypothesis [239].

Altered glucose levels in non-diabetic animals have not been previously identified in studies where increases in insulin have been observed after acute icv injection of NPY. However, in one study glucose levels were not reported [125], and in two others they were measured at a single timepoint (15 minutes [124] or 120 minutes [162]), which may have been too early or too late for the time course of the glucose changes observed. In chicks injected with NPY or saline icv insulin, but not glucose, rose to a greater extent after NPY injection; however the animals were allowed to feed, making interpretation of these data difficult [231]. In another study when NPY was injected into the nucleus of the tractus solitarius (NTS), insulin rose, but no changes in glucose were seen at 30 minutes [232]. It is therefore possible that the NTS is a site where NPY acts directly to increase insulin secretion, but the sites influencing insulin sensitivity and hepatic glucose output may be elsewhere, possibly in the PVN or perifornical areas of the hypothalamus.

Perhaps more surprising is that centrally injected NPY increased insulin sensitivity during the hyperinsulinaemic euglycaemic clamp. NPY was expected to produce insulin resistance, as suggested by other studies where the effect of centrally injected NPY on glucose and / or insulin has been measured. The observations made here would however be consistent with recent work suggesting that in animals chronically treated with icv NPY, there is a 4-fold increase in glucose uptake in white adipose tissue [240]. The mechanism of the effect on insulin sensitivity was not explored in this study. Centrally injected NPY is known to have effects on systemic blood pressure [241,242], and one possibility is an autonomic nervous system mediated change in muscle blood flow altering

insulin-mediated glucose uptake [243]. Alternatively there may be a direct neurally mediated effect on muscle sensitivity to insulin, which may occur via β-adrenergic stimulation [244].

In both studies there was a small increase in glucagon levels in NPY treated animals. The fact that glucagon secretion was not suppressed during the clamp after NPY treatment, despite hyperinsulinaemia (and without hypoglycaemia), suggests a direct effect of centrally injected NPY on glucagon secretion. Similar increases in glucagon secretion have been observed in rats following central injection of adrenaline and carbachol into the CNS, indicating that neural regulation of pancreatic glucagon secretion is not unique to NPY [245].

NPY has been shown to increase the respiratory quotient [127], and there is a well recognized increase in body fat in animals chronically treated with NPY [106,240]. Increased hepatic glucose output together with increased insulin-mediated glucose uptake and stimulation of insulin release would be consistent with the hypothesis that NPY in the central nervous system has a role in the metabolism of carbohydrate and the storage of energy as fat. Hypothalamic NPY is increased in a number of situations, including food restriction, exercise, insulin deficient diabetes and in genetically obese, insulin resistant animals [136,138,191,246]. The changes in glucose metabolism observed after NPY in this study would be consistent with NPY mediating some of the physiological changes in glucose metabolism seen during short-term food deprivation and after exercise, specifically mobilisation of hepatic glucose stores and utilisation of glucose as a metabolic fuel.

In conclusion, central injection of NPY has a number of effects on glucose metabolism, including the release of insulin and glucagon from the endocrine pancreas, an increase in hepatic glucose output and improved peripheral insulin sensitivity. These changes are consistent with the proposed role for hypothalamic NPY as an important integrator of energy balance and carbohydrate metabolism.

Chapter 9

General Discussion

Overview

The studies described in this thesis have explored the relationships between hypothalamic neuropeptide Y (and to a lesser extent some other hypothalamic peptides such as neurotensin and galanin), food intake and peripheral metabolism. Neuropeptide Y is recognized to be the most potent appetite stimulant known. I have demonstrated its importance in the normal control of food intake by using immunoneutralising antibodies to block the effect of the endogenous peptide in vivo, and used this technique to study its relationship with other neurotransmitters concerned with the regulation of food intake and peripheral metabolism. The finding of different changes in hypothalamic NPY in two models of obesity, one genetic, the other environmental, may explain some of the features of these conditions. The regulation of hypothalamic NPY expression by peripheral hormone levels and the modulation of hormone levels and glucose metabolism by NPY illustrates the presence of feedback signals between the CNS and the periphery, which may become disturbed in disease.

The physiological role of hypothalamic NPY

The normal physiological role of NPY in the hypothalamus appears to be as a signal to search for food, particularly carbohydrate containing food. Background evidence for this comes from studies which have shown that increases in NPY mRNA, peptide content and release in the hypothalamus are increased after food deprivation [138,139,182,247]. This has recently been confirmed in continuing studies in Professor Bloom's laboratory, where increased NPY release from the PVN has been found in rats deprived of food for 48h, using a novel microdialysis procedure (Lambert, Wilding unpublished data). Furthermore, in animals on a scheduled feeding regimen, NPY release from the PVN declines as a meal progresses suggesting a rapid switch off, perhaps mediated by satiety factors [182]. In food deprived animals the increased levels of NPY mRNA, and the content of NPY in the ARC and PVN decline more slowly, but return to normal after 72 hours refeeding [138]. In the food deprived situation I have demonstrated the physiological importance of NPY in mediating food intake, with the observation that the amount of food consumed is reduced by 30% after administration of NPY into the third ventricle. Other workers have suggested that this effect may also be observed after infusion of NPY antisera directly into the PVN, however I have not been able to reproduce this result (unpublished observations), and given the multiple sites at which NPY has been observed to act to stimulate food intake [105], it seems unlikely that immunoneutralisation

at one site would be effective.

NPY interacts in a complex way with the many other neurotransmitters involved in the control of food intake; as mentioned in the introduction, this complicates the study of such physiological systems, because of the multiple redundant pathways. The fact that blocking NPY can acutely decrease food intake in response to a strong stimulus (starvation) is further evidence of its physiological importance. I have made some observations regarding the interactions of NPY with other neurotransmitters, suggesting that the serotonin agonist dexfenfluramine does not act by altering NPY synthesis (although local effects on NPY release and effects of NPY on serotonin were not studied). NPY induced feeding is closely related to the feeding produced by noradrenaline, and the ability of the α-adrenergic blocker phentolamine to inhibit NPY-induced feeding is further evidence for this. Interactions with opioid peptide systems are also of interest, as the results of current studies suggest the presence of opioid dependent and independent pathways for NPY-induced feeding.

As well as stimulating food intake NPY also has a number of metabolic effects, including inhibition of thermogenesis, stimulation of lipoprotein lipase, and the increase of insulin release [124,125,162,240]. The observed effects on the respiratory quotient suggest a shift towards the metabolism of carbohydrate under most circumstances, although the dose and time relationships are complex [127]. My own studies have concentrated on the effects on glucose metabolism and have found that NPY has stimulatory effects on hepatic glucose output and increases peripheral insulin sensitivity. Whilst these findings are consistent with other observations on insulin release and respiratory quotient, it is difficult to reconcile these findings with the fact that NPY release is increased during food deprivation and with the well known metabolic consequences of starvation. One possibility is that NPY may have different effects depending on the metabolic state of the animal. In animals who are able to feed regularly (as was the case in the animals for the metabolic studies), NPY may have a purely anabolic effect. During food deprivation, the effects of NPY may be predominantly to increase food seeking behaviour, with little or no metabolic effects. The effect of NPY may vary according to the dose used, possibly due to interactions with different receptors; for example NPY decreases the respiratory quotient at low doses, but increases it at higher doses, with an intermediate dose having no effect. Furthermore the stimulatory effects appeared to increase in latency depending on the dose used.

I have studied some of the factors which may regulate hypothalamic NPY expression, in particular corticosteroids and insulin. The findings that dexamethasone increased hypothalamic NPY levels and expression are largely in accordance with other published work [151,196]. The observation that the dexamethasone induced increase in NPY could be prevented by concomitant insulin administration supports the concept that these two hormones regulate NPY in a reciprocal fashion. Defective regulation of NPY by insulin and corticosteroids may be a factor in the development of obesity in genetically obese rodents (see below).

The role of NPY in decreasing LH secretion, which has been observed in a number of studies [100,161,248], was highlighted in my study of NPY in the hyperphagia of lactation, and the demonstration that plasma LH levels varied inversely with hypothalamic NPY mRNA. It is well recognised that gonadal activity and thus the ability to reproduce are decreased in food deprived states, and increased levels of hypothalamic NPY release may be an important mediator of these effects. This may partially explain reduced fertility during lactation. This may have clinical relevance in disorders such as anorexia nervosa and bulimia, where CSF NPY levels have been found to be increased.

Hypothalamic NPY in disease

This work has established the alterations in hypothalamic NPY in two models of obesity, and these changes, taken together with the known physiology of NPY as summarised above may explain some of the features of these obese models. In genetically obese rodents, NPY expression and content are increased. Although in vivo release has not been measured using any of these models, it seems likely that this will also be increased, as release seems to be closely allied to synthesis and mRNA levels. Increased release of NPY will increase food intake, and the reduction in thermogenesis, the increase in hepatic glucose output and insulin release, along with direct effects on the activity of lipoprotein lipase will favour fat deposition. Genetically obese rodents are markedly insulin resistant and have high levels of corticosterone, and it seems possible that these two factors are contributing to the increased NPY synthesis in the hypothalamus in these animals. Studies using chronic infusions of NPY into the hypothalamus of normal rats have demonstrated that this mimics many of the metabolic features seen in obese rodents. Another recent study has shown that the fa/fa Zucker rat is insensitive to the satiety effects of insulin infused into the CNS, and furthermore that whilst the increase in NPY observed in food deprivation can be reversed by central insulin administration in lean Zucker rats,

insulin has no effect on the central NPY mRNA level in obese Zucker rats, suggesting that their insensitivity to insulin extends to the CNS, and may, via NPY, be a fundamental factor in their development of obesity [176].

Acquired obesity in otherwise normal animals produced by feeding a cafeteria diet, also results in obesity, but NPY release may be decreased in this situation, as is suggested by increases in peptide levels in the PVN and ARC, but lack of change in mRNA. A reduction in NPY release may explain some of the metabolic alterations seen in this situation, such as increases in thermogenesis and alterations in pituitary hormone secretion. It is interesting to note that the sensitivity of animals to diet-induced obesity varies greatly from strain to strain, and even from animal to animal, suggesting genetic and other factors may play a role in the development of obesity in this model [249]. This is perhaps the closest model to the development of obesity in humans, where the role of NPY is currently unclear, and must await the development of specific antagonists to the receptor(s) in the hypothalamus that are involved in the regulation of food intake.

Future studies

My work on hypothalamic NPY in the control of food intake and metabolism is continuing and a number of future studies are planned or ongoing. These include the use of the immediate early gene c-fos as a marker of neuronal activation to precisely identify those neurons which are activated in conditions of altered food intake and following the central injection of NPY and other neurotransmitters. Provisional data produced in collaboration with Dr J. Herbert (Dept of Anatomy, Cambridge University) has identified a population of neurones within the PVN which are activated following icv injection of neuropeptide Y. Other collaborative work in Cambridge is with Professor C.N. Hales, investigating the role of NPY in the abnormalities seen in rats whose mothers are fed low protein diets. This was prompted by epidemiological studies indicating a high risk of obesity, hypertension and diabetes mellitus in people with low birth weight. It is possible that hypothalamic NPY is 'programmed' to a higher set point in the offspring of mothers fed low protein diets, and could be responsible for some of the metabolic abnormalities observed. I am extending the observations made in genetically obese mice by looking at the effects of pair feeding and adrenalectomy. The measurement of in-vivo release of NPY is now possible using microdialysis combined with a sensitive radioimmunoassay, and using an electrochemical detection system, monoamine neurotransmitters can be

measured in the same samples, thus enabling the study of neurotransmitter release in different physiological and disease states. Studies investigating the physiological role of other neurotransmitters are also envisaged, using newly acquired antagonists to neurotensin, bombesin and CRF.

References

1. Bernard C: Chiens rendus diabetiques. *C R Soc Biol* 1:60:1849

2. Hetherington AW, Ranson SW: Hypothalamic lesions and adiposity in the rat. *Anat Rec* 78:149-172, 1940

3. Brobeck JR: Hypothalamic control of food intake in rats and cats. *Yale J Biol Med* 24:123, 1951

4. Contreras RJ, Kosten T, Bird E: Area postrema: part of the autonomic circuitry of caloric homeostasis. *Fed Proc* 43:2966-2968, 1984

5. Morgane PJ: The function of the limbic and rhinic forebrain-limbic midbrain systems and reticular formation in the regulation of food and water intake. *Ann N Y Acad Sci* 157:806, 1969

6. Gold RM, Jones AP, Sawchenko PE, Kapatos G: Paraventricular area: Critical focus of a longitudinal circuitry mediating food intake. *Physiol Behav* 18:1111-1119, 1977

7. Tokunaga K, Fukushima M, Kemnitz JW, Bray GA: Comparison of ventromedial and paraventricular lesions in rats that become obese. *Am J Physiol* 251:R1221-R1227, 1986

8. Brobeck JR: Mechanism of development of obesity in animals with hypothalamic lesions. *Physiological Reviews* 26:5411946

9. Hoebel BG, Teitelbaum P: Weight regulation in normal and hypothalamic hyperphagic rats. *J Comp Physiol Psychol* 619:1891966

10. Kissileff HR: Free feeding in normal and "recovered lateral" rats monitored by a pellet-detecting eatometer. *Physiol Behav* 5:1631970

11. Liddle RA, Goldfine ID, Williams JA: Bioassay of plasma cholecystokinin in rats: effects of food, trypsin inhibitor, and alcohol. *Gastroenterology* 87:542-550, 1984

12. Gibbs J, Young RC, Smith GP: Cholecystokinin decreases food intake in rats. *J Comp Physiol Psychol* 84:488-495, 1973

13. Gibbs J, Fauser DJ, Rowe EA, Rolls BJ, Rolls ET, Maddison SP: Bombesin suppresses feeding in rats. *Nature* 282:208-210, 1979

14. Crawley JN, Kiss JZ: Paraventricular nucleus lesions abolish the inhibition of feeding induced by systemic cholecystokinin. *Peptides* 6:927-935, 1985

15. Dourish CT, Rycroft W, Iversen SD: Postponement of satiety by blockade of cholecystokinin (CCK-B) receptors. *Science* 245:1509-1511, 1989

16. Corwin RL, Gibbs J, Smith GP: Increased food intake after type A but not type B cholecystokinin receptor blockade. *Physiology and Behaviour* 50:255-258, 1991

17. Stuckey JA, Gibbs J, Smith GP: Neural disconnection of gut from brain blocks bombesin induced satiety. *Peptides* 6:1249-1252, 1985

18. Geary N: Pancreatic glucagon signals postprandial satiety. *Neurosci Biobehav Rev* 14:323-338, 1190

19. Oomura Y, Ono T, Ooyama H, Wayner MJ: Glucose and osmosenstive neurons of the rat hypothalamus. *Nature* 222:282-284, 1969

20. Leung PMB, Rogers QR: Effect of amino acid imbalance and deficiency on dietary choice patterns of rats. *Physiol Behav* 37:747-758, 1986

21. Shimizu N, Oomura Y, Sakata T: Modulation of feeding by endogenous sugar acids acting as hunger or satiety factors. *Am J Physiol* 246:R542-R550, 1984

22. Knoll J: Satietin: a 50,000 dalton glycoprotein in human serum with potent, long-lasting and selective anorectic activity. *J Neural Transm* 59:1631984

23. Larue-Achagiotis C, Le-Magnen J: Effect of long term insulin on body weight and food intake: intravenous versus intraperitoneal routes. *Appetite* 6:319-329, 1985

24. Corp ES, Woods SC, Porte D, Jr., Dorsa DM, Figlewicz DP, Baskin DG: Localization of 125I-insulin binding sites in the rat hypothalamus by quantitative autoradiography. *Neurosci Lett* 70:17-22, 1986

25. Marks JL, Porte DJ, Stahl WL, Baskin DG: Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology* 127:3234-3236, 1990

26. Young WS: Periventricular hypothalamic cells in the rat brain contain insulin mRNA [published erratum appears in Neuropeptides 1986 Nov-Dec;8(4):401]. *Neuropeptides* 8:93-97, 1986

27. Schwartz MW, Sipols AJ, Kahn SE, Latteman DP, Taborsky JG, Bergman RN, Woods SC, Porte JD: Kinetics and specificity of insulin uptake from plasma into cerebrospinal fluid. *Am J Physiol* 259:E378-E383, 1990

28. Woods SC, Stein LJ, McKay LD, Porte JD: Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* 282:503-505, 1979

29. McGowan MK, Andrews KM, Grossman SP: Chronic intrahypothalamic infusions of insulin or insulin antibodies alter body weight and food intake in the rat. *Physiol Behav* 51:753-766, 1992

30. Tempel DL, Shor-Posner D, Dwyer D, Leibowitz SF: *Am J Physiol* 256:R541-R548, 1989

31. Grossman SP: Direct adrenergic and cholinergic stimulation of hypothalamic mechanisms. *Am J Physiol* 202:872-882, 1962

32. Leibowitz SF, Hammer NJ, Chang K: Feeding behaviour induced by central nor-epinephrine injection is attenuated by discrete lesions in the hypothalamic paraventricular nucleus. *Pharmacol Biochem Behav* 19:945-950, 1983

33. Morley JE: The neuroendocrine control of appetite. The role of endogenous opiates, cholecystokinin, TRH, gamma-amino butyric acid and the diazepam receptor. *Life Sci* 27:355-368, 1980

34. Gosnell BA, Morley JE, Levine AS: Opioid-induced feeding: localization of sensitive brain sites. *Brain Res* 369:177-184, 1986

35. Stanley BG, Leibowitz SF: Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. *Proc Natl Acad Sci U S A* 82:3940-3943, 1985

36. Kyrkouli SE, Stanley BG, Leibowitz SF: Galanin: stimulation of feeding induced by medial hypothalmic injection of this novel peptide. *Eur J Pharmacol* 122:159-160, 1986

37. Beck B, Burlet A, Nicolas JP, Burlet C: Neurotensin in microdissected brain nuclei and in the pituitary of the lean and obese Zucker rats. *Neuropeptides* 13:1-7, 1989

38. Curzon G: Serotonin and appetite. *Ann N Y Acad Sci* 600:521-530, 1990

39. Smith GP, Schneider LH: Relationships between mesolimbic dopamine function and eating behavior. *Ann N Y Acad Sci* 537:254-261, 1988

40. Kyrkouli SE, Stanley BG, Leibowitz SF: Bombesin-induced anorexia: sites of action in the rat brain. *Peptides* 8:237-241, 1987

41. Krahn DD, Gosnell BA, Levine AS, Morley JE: Behavioral effects of corticotropin-releasing factor: localization and characterization of central effects. *Brain Res* 443:63-69, 1988

42. de-Beaurepaire R, Suaudeau C: Anorectic effect of calcitonin, neurotensin and bombesin infused in the area of the rostral part of the nucleus of the tractus solitarius in the rat. *Peptides*

9:729-733, 1988

43. Hawkins MF: Central nervous system neurotensin and feeding. *Physiol Behav* 36:1-8, 1986

44. Lin MT, Chen JJ, Ho LT: Hypothalamic involvement in the hyperglycemia and satiety actions of somatostatin in rats. *Neuroendocrinology* 45:62-67, 1987

45. Feifel D, Vaccarino FJ: Central somatostatin: a re-examination of its effects on feeding. *Brain Res* 535:189-194, 1990

46. de-Beaurepaire R, Freed WJ: Anatomical mapping of the rat hypothalamus for calcitonin-induced anorexia. *Pharmacol Biochem Behav* 27:177-182, 1987

47. Chance WT, Balasubramaniam A, Zhang FS, Wimalawansa SJ, Fischer JE: Anorexia following the intrahypothalamic administration of amylin. *Brain Res* 539:352-354, 1991

48. Inokuchi A, Oomura Y, Nishimura H: Effect of intracerebroventricularly infused glucagon on feeding behavior. *Physiol Behav* 33:397-400, 1984

49. Lambert PD, Wilding JPH, Ghatei MA, Bloom SR: A role for GLP-1(7-36) amide in the central control of feeding behaviour. *Digestion* 360-361, 1994

50. Levine AS, Morley JE: The effects of prostaglandins (PGE_2 and PGF_2 alpha) on food intake in rats. *Pharmacol Biochem Behav* 19:231981

51. McCarthy DO, Kluger MJ, Vander AJ: Effect of centrally administered interleukin-1 and endotoxin on food intake of fasted rats. *Physiol Behav* 36:745-749, 1986

52. Morley JE, Horowitz M, Morley PM, Flood JF: Pituitary adenylate cyclase activating polypeptide (PACAP) reduces food intake in mice. *Peptides* 13:1133-1135, 1992

53. King BM, Smith RL: Hypothalamic obesity after hypohysectomy or adrenalectomy: Dependence on corticosterone. *Am J Physiol* 249:R522-R526, 1985

54. Shimomura Y, Bray GA, Lee M: Adrenalectomy and steroid treatment in obese (ob/ob) and diabetic (db/db) mice. *Horm Metab Res* 19:295-299, 1987

55. Castonguay TW, Dallman MF, Stern JS: Some metabolic and behavioural effects of adrenalectomy on obese Zucker rats. *Am J Physiol* 251:R923-R933, 1986

56. Ceccatelli S, Cintra A, Hokfelt T, Wikstrom AC, Gustafsson JA: Coexistence of glucocorticoid receptor-like immunoreactivity with neuropeptides in the hypothalamic paraventricular nucleus. *Exp Brain Res* 78:33-42, 1989

57. Tempel DL, Leibowitz SF: PVN steroid implants: effect on feeding patterns and macronutrient selection. *Brain Research Bulletin* 23:553-560, 1989

58. Plotsky PM: Pathways to the secretion of adrenocorticotropin: a view from the portal. *J Neuroendocrinol* 3:1-9, 1991

59. Swanson LW, Sawchenko PE, Rivier J, Vale WW: Organization of ovine corticotropin releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology* 36:165-186, 1983

60. Rothwell NJ, Stock MJ: A role for brown adipose tissue in diet induced thermogenesis. *Nature* 281:31-32, 1979

61. Himms-Hagen J: Defective brown adipose tissue thermogenesis in obese mice. *Int J Obes* 9 Suppl 2:17-24, 1985

62. Vander-Tuig JG, Knehans AW, Romsos DR: Reduced sympathetic nervous system activity in rats with ventromedial hypothalamic lesions. *Life Sci* 30:913-920, 1982

63. Holt SJ, Wheal HV, York DA: Hypothalamic control of brown adispose tissue in Zucker lean and obese rats. Effect of electrical stimulation of the ventromedial nucleus and other hypothalamic centers. *Brain Res* 405:227-233, 1987

64. Sakaguchi T, Bray GA, Eddlestone G: Sympathetic activity following paraventricular or ventromedial hypothalamic lesions in rats. *Brain Res Bull* 20:461-465, 1988

65. Lupien JR, Tokunaga K, Kemnitz JW, Groos E, Bray GA: Lateral hypothalamic lesions and fenfluramine increase thermogenesis in brown adipose tissue. *Physiol Behav* 38:15-20, 1986

66. Tatemoto K, Carlquist M, Mutt V: Neuropeptide Y - A novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296:659-660, 1982

67. Larhammar D, Ericsson A, Persson H: Structure and expression of the rat neuropeptide Y gene. *Proc Natl Acad Sci U S A* 84:2068-2072, 1987

68. Blomqvist AG, Soderberg C, Lundell I, Milner RJ, Larhammar D: Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and Torpedo marmorata DNA clones. *Proc Natl Acad Sci U S A* 89:2350-2354, 1992

69. Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, Polak JM: Neuropeptide Y distribution in the rat brain. *Science* 221:877-879, 1983

70. Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rosser MN, Roberts GW, Crow TJ,

Tatemoto K, Polak JM: Neuropeptide Y distribution in human brain. *Nature* 308:5841983

71. Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR: Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* 89:1070-1077, 1985

72. O'Donohue TL, Chronwall BM, Pruss RM, Mezey E, Kiss JZ, Eiden LE, Massari VJ, Tessel RE, Pickel VM, DiMaggio DA, Hotchkiss AJ, Crowley WR, Zukowska-Grojek Z: Neuropeptide Y and Peptide YY Neuronal and Endocrine Systems. *Peptides* 6:755-768, 1985

73. Lundberg JM, Terenius L, Hokfelt T, Tatemoto K: Comparative immunohistochemical and biochemical analysis of pancreatic polypeptide-like peptides with special reference to presence of neuropeptide Y in central and peripheral neurons. *J Neurosci* 4:2376-2386, 1984

74. Jamal H, Jones PM, Byrne J, Suda K, Ghatei MA, Kanse SM, Bloom SR: Peptide contents of neuropeptide Y, vasoactive intestinal polypeptide, and beta-calcitonin gene-related peptide and their messenger ribonucleic acids after dexamethasone treatment in the isolated rat islets of Langerhans. *Endocrinology* 129:3372-3380, 1991

75. Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA, O'Donohue TL: The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience* 15:1159-1181, 1985

76. Mikkelsen JD, O'Hare MMT: An immunohistochemical and chromatographic analysis of the distribution and processing of proneuropeptide Y in the rat suprachiasmatic nucleus. *Peptides* 12:177-185, 1991

77. Chronwall BM, Chase TN, O'Donohue TL: Co-existence of neuropeptide Y and somatostatin in human cortical and rat hypothalamic neurons. *Neurosci Lett* 52:213-217, 1984

78. Chronwall BM, Knight RM, O'Donohue TL: Colocalization and segregation of peptides in the hypothalamic arcuate nucleus of the rat. *Neurosci Abstr* 10:1551985

79. Kagotani Y, Hisano S, Tsuruo Y, Daikoku S, Okimura Y, Chihara K: Intragranular co-storage of neuropeptide Y and arginine vasopressin in the paraventricular magnocellular neurons of the rat hypothalamus. *Cell Tissue Res* 262:47-52, 1990

80. Takyashi K, Mouri T, Murakami O, Itoi K, Sone M, Ohneda M, Nozuki M, Yoshinaga K: Increases of neuropeptide Y-like immunoreactivity in plasma during insulin induced hypoglycaemia in man. *Peptides* 9:433-435, 1988

81. Miller MA, Sagnella GA, Markandu ND, MacGregor GA: Radioimmunoassay for plasma neuropeptide-Y in physiological and physiopathological states and response to sympathetic activation. *Clinica Chimica Acta* 192:47-54, 1990

82. Wahlestedt C, Hakanson R: Effects of neuropeptide Y (NPY) at the sympathetic neuroeffector junction. Can pre- and postjunctional receptors be distinguished?. *Med Biol* 64:85-88, 1986

83. Allen JM, Yeats JC, Causon R, Brown MJ, Bloom SR: Neuropeptide Y and its flanking peptide in human endocrine tumours and plasma. *J Clin Endocrinol Metab* 64:1199-1204, 1987

84. Grouzmann E, Comoy E, Bohoun C: Plasma neuropeptide Y concentrations in patients with neuroendocrine tumours. *J Clin Endocrinol Metab* 68:808-813, 1989

85. Skoglund G, Gross R, Bertrand GR, Ahren B, Loubatieres-Mariani MM: Comparison of the effects of neuropeptide Y and norepinephrine on insulin secretion and vascular resistance in perfused rat pancreas. *Diabetes* 40:660-665, 1991

86. Westfall TC, Martin J, Chen XL, Ciarleglio A, Carpentier S, Henderson K, Knuepfer M, Beinfeld M, Naes L: Cardiovascular effects and modulation of noradrenergic neurotransmission following central and peripheral administration of neuropeptide Y. *Synapse* 2:299-307, 1988

87. Vallejo M, Lightman SL: Pressor effect of centrally administered neuropeptide Y in rats: role of sympathetic nervous system and vasopressin. *Life Sci* 38:1859-1866, 1986

88. Westfall TC, Martin J, Chen XL, Ciarleglio A, Carpentier S, Henderson K, Knuepfer M, Beinfeld M, Naes L: Cardiovascular effects and modulation of noradrenergic neurotransmission following central and peripheral administration of neuropeptide Y. *Synapse* 2:299-307, 1988

89. Martin JR, Knuepfer MM, Westfall TC: Hemodynamic effects of posterior hypothalamic injection of neuropeptide Y in awake rats. *Am J Physiol* 261:H814-H824, 1991

90. Tsuda K, Tsuda S, Goldstein M, Masuyama Y: Effects of neuropeptide Y on norepinephrine release in hypothalamic slices of spontaneously hypertensive rats. *Eur J Pharmacol* 182:175-179, 1990

91. Maccarrone C, Jarrott B, Conway EL: Comparison of neuropeptide Y immunoreactivity in hypothalamic and brainstem nuclei of young and mature spontaneously hypertensive and

normotensive Wistar-Kyoto rats. *Neurosci Lett* 68:232-238, 1986

92. Jarrott B, Lewis SJ, Louis WJ, Maccarrone C, Shulkes A: Regional brain concentrations of several putative peptide neurotransmitters in normotensive and spontaneously hypertensive rats: effects of continuous (10-day) clonidine infusion. *J Cardiovasc Pharmacol* 10 Suppl 12:S14-S21, 1987

93. Morley JE, Levine AS, Gosnell BA, Kneip J, Grace M: Effect of neuropeptide Y on ingestive behaviors in the rat. *Am J Physiol* 252:R599-R609, 1987

94. Flood JF, Hernandez EN, Morley JE: Modulation of memory processing by neuropeptide Y. *Brain Res* 421:280-290, 1987

95. Widdowson PS, Halaris AE: Chronic desipramine treatment reduces regional neuropeptide Y binding to Y2-type receptors in rat brain. *Brain Res* 539:196-202, 1991

96. Heilig M, Wahlestedt C, Ekman R, Widerlov E: Antidepressant drugs increase the concentration of neuropeptide Y (NPY)-like immunoreactivity in the rat brain. *Eur J Pharmacol* 147:465-467, 1988

97. Widdowson PS, Ordway GA, Halaris AE: Reduced neuropeptide Y concentrations in suicide brain. *J Neurochem* 59:73-80, 1992

98. Kerkerian L, Guy J, Lefevre G, Pelletier G: Effects of neuropeptide Y (NPY) on the release of anterior pituitary hormones in the rat. *Peptides* 6:1201-1204, 1985

99. Kalra SP, Crowley WR: Nor-epinephrine like effects of neuropeptide Y on LH release in the rat. *Life Sci* 35:1173-1176, 1984

100. McDonald JK, Lumpkin MD, Samson WK, McCann SM: Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. *Proc Natl Acad Sci U S A* 82:561-564, 1985

101. Clark JT, Kalra PS, Crowley WR, Kalra SP: Neuropeptide Y and pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology* 115:427-429, 1984

102. Morley JE, Hernandez EN, Flood JF: Neuropeptide Y increases food intake in mice. *Am J Physiol* 253:R516-R522, 1987

103. Miner JL, Della-Fera MA, Paterson JA, Baile CA: Lateral cerebroventricular injection of neuropeptide Y stimulates feeding in sheep. *Am J Physiol* 257:R383-R387, 1989

104. Pau MC, Pau KF, Spies HG: Characterization of central actions of neuropeptide Y on food and water intake in rabbits. *Physiol Behav* 44:797-802, 1988

105. Stanley BG, Magdalin W, Seirafi A, Thomas WJ, Leibowitz SF: The perifornical area: The major focus of a patchily distributed hypothalamic neuropeptide Y sensitive feeding system(s). *Brain Res* 604:304-317, 1993

106. Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF: Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides* 7:1189-1192, 1986

107. Stanley BG, Daniel DR, Chin AS, Leibowitz SF: Paraventricular nucleus injections of peptide YY and neuropeptide Y preferentally enhance carbohydrate ingestion.. *Peptides* 6:1205-1211, 1985

108. Stanley BG, Anderson KC, Grayson MH, Leibowitz SF: Repeated hypothalamic stimulation with neuropeptide Y increases daily carbohydrate and fat intake and body weight gain in female rats. *Physiol Behav* 46:173-177, 1989

109. Tempel DL, Leibowitz SF: Diurnal variations in the feeding responses to norepinephrine, neuropeptide Y and galanin in the PVN. *Brain Research Bulletin* 25:821-825, 1990

110. Levine AS, Morley JE: Neuropeptide Y: a potent inducer of consummatory behaviour in rats. *Peptides* 5:1025-1030, 1984

111. Leibowitz SF: Hypothalamic neuropeptide Y in relation to energy balance. *Ann N Y Acad Sci* 611:284-301, 1990

112. Morley JE, Levine AS, Gosnell BA, Kneip J, Grace M: Effect of neuropeptide Y on ingestive behaviors in the rat. *Am J Physiol* 252:R599-R609, 1987

113. Schick RR, Schusdziarra V, Nussbaumer C, Classen M: Neuropeptide Y and food intake in fasted rats: effect of naloxone and site of action. *Brain Res* 552:232-239, 1991

114. Rowland NE: Peripheral and central satiety factors in neuropeptide Y-induced feeding in rats. *Peptides* 9:898-992, 1988

115. Heinrichs SC, Cole BJ, Pich EM, Menzaghi F, Koob GF, Hauger RL: Endogenous corticotropin-releasing factor modulates feeding induced by neuropeptide Y or a tail pinch stressor. *Peptides* 13:879-884, 1992

116. Bendotti C, Garattini S, Samanin R: Eating caused by neuropeptide-Y injection in the paraventricular hypothalamus: response to (+)-fenfluramine and (+)-amphetamine in rats. *J Pharm Pharmacol* 39:900-903, 1987

117. Stanley BG, Lanthier D, Chin AS, Leibowitz SF: Suppression of neuropeptide Y-elicited

eating by adrenalectomy or hypophysectomy: reversal with corticosterone. *Brain Res* 501:32-36, 1989

118. Sahu A, Dube MG, Kalra SP, Kalra PS: Bilateral neural transections at the level of mesencephalon increase food intake and reduce latency to onset of feeding in response to neuropeptide Y. *Peptides* 9:1269-1273, 1988

119. Meister B, Ceccatelli S, Hokfelt T, Anden NE, Anden M, Theodorsson E: Neurotransmitters, neuropeptides and binding sites in the rat mediobasal hypothalamus: effects of monosodium glutamate (MSG) lesions. *Exp Brain Res* 76:343-368, 1989

120. Abe M, Saito M, Shimazu T: Neuropeptide Y in the specific hypothalamic nuclei of rats treated neonatally with monosodium glutamate. *Brain Res Bull* 24:289-291, 1990

121. Haas DA, George SR: Neuropeptide Y administration acutely increases hypothalamic corticotropin-releasing factor immunoreactivity: lack of effect in other rat brain regions. *Life Sci* 41:2725-2731, 1987

122. Wahlestedt C, Skakerberg G, Ekman R, Heilig M, Sundler F, Hakanson R: Neuropeptide Y (NPY) in the area of the hypothalamic paraventricular nucleus activates the pituitary-adrenocortical axis in the rat. *Brain Research* 417:33-38, 1987

123. Inoue T, Inui A, Okita M, Sakatani N, Oya M, Morioka H, Mizuno N, Oimomi M, Baba S: Effect of neuropeptide Y on the hypothalamic-pituitary-adrenal axis in the dog. *Life Sci* 44:1043-1051, 1989

124. Leibowitz SF, Sladek C, Spencer L, Tempel D: Neuropeptide Y, epinephrine and nor-epinephrine in the paraventricular nucleus:stimulation of feeding and the release of corticosterone, vasopressin and glucose. *Brain Research Bulletin* 21:905-912, 1988

125. Moltz JM, McDonald JK: Neuropeptide Y: direct and indirect action on insulin secretion in the rat.. *Peptides* 6:1155-1159, 1985

126. Egawa M, Yoshimatsu H, Bray GH: Neuropeptide Y suppresses sympathetic activity to interscapular brown adipose tissue in rats. *Am J Physiol* 260(2 Pt 2):R328-R234, 1991

127. Menendez JA, McGregor IS, Healey PA, Atrens DM, Leibowitz SF: Metabolic effects of neuropeptide Y injections into the paraventricular nucleus of the hypothalamus. *Brain Res* 516:8-14, 1990

128. Rimland J, Xin W, Sweetnam P, Saijoh K, Nestler EJ, Duman RS: Sequence and expression of a neuropeptide Y receptor cDNA. *Mol Pharmacol* 40:869-875, 1991

129. Herzog H, Hort YJ, Shine J, Selbie LA: Molecular cloning, characterization and localization of the human homolog to the reported bovine NPY Y3 receptor: lack of NPY binding and activation. *DNA and Cell Biology* 12:465-472, 1993

130. Dumont Y, Fournier A, St Pierre S, Schwartz TW, Quirion R: Differential distribution of neuropeptide Y1 and Y2 receptors in the rat brain. *Eur J Pharmacol* 191:501-503, 1990

131. Stanley BG, Magdalin W, Seirafi A, Nguyen MM, Leibowitz SF: Evidence for neuropeptide Y mediation of eating produced by food deprivation and for a varient of the Y1 receptor mediating this peptide's effect. *Peptides* 13:581-587, 1992

132. Chance WT, Sheriff S, Foley-Nelson T, Fischer JE, Balasubramaniam A: Pertussis toxin inhibits neuropeptide Y-induced feeding in rats. *Peptides* 10:1283-1286, 1989

133. Herzog H, Hort YJ, Ball HJ, Hayes G, Shine J, Selbie L: Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proc Natl Acad Sci USA* 89:5794-5798, 1992

134. Williams G, Steel JH, Cardoso H, Ghatei MA, Lee YC, Gill JS, Burrin JM, Polak JM, Bloom SR: Increased hypothalamic neuropeptide Y concentrations in diabetic rat. *Diabetes* 37:763-772, 1988

135. Williams G, Lee YC, Ghatei MA, Cardoso HM, Ball JA, Bone AJ, Baird JD, Bloom SR: Elevated neuropeptide Y concentrations in the central hypothalamus of the spontaneously diabetic BB/E Wistar rat. *Diabetic Med* 6:601-607, 1989

136. Williams G, Gill JS, Lee YC, Cardoso HM, Okpere BE, Bloom SR: Increased neuropeptide Y concentrations in specific hypothalamic regions of streptozocin-induced diabetic rats. *Diabetes* 38:321-327, 1989

137. White JD, Olchovsky D, Kershaw M, Berelowitz M: Increased hypothalamic content of preproneuropeptide-Y messenger ribonucleic acid in streptozotocin-diabetic rats. *Endocrinology* 126:765-772, 1990

138. Sahu A, Kalra PS, Kalra SP: Food deprivation and ingestion induce reciprocal changes in neuropeptide Y concentrations in the paraventricular nucleus.. *Peptides* 9:83-86, 1988

139. White JD, Kershaw M: Increased hypothalamic neuropeptide Y expression following food deprivation. *Mol Cell Neurosci* 1:41-48, 1989

140. Paxinos GWatson C *The rat brain in stereotaxic co-ordinates*. Academic Press, Sydney.1982

141. Jacobowitz DM: Removal of discrete fresh regions of the rat brain. *Brain Research* 80:111-115, 1974

142. Berson SA, Yalow RS: Isotopic tracers in the study of diabetes. *Adv Biol Med Phys* 6:349-420, 1958

143. Allen JM, Yeats JC, Adrian TE, Bloom SR: Radioimmunoassay of neuropeptide Y. *Reg Peptides* 8:61-70, 1984

144. Chomczynski P, Sacchi N: Single-Step method of RNA isolation by acid guanidinium thiocyante-phenol-chloroform extraction. *Anal Biochem* 162:156-159, 1987

145. Rothwell NJ, Stock MJ: The development of obesity in animals: the role of dietary factors. *Clin Endocrinol Metab* 13:437-449, 1984

146. Cooper SJ, Jackson A, Morgan R, Carter R: Evidence for opiate receptor involvement in the consumption of a high palatability diet in overfed rats. *Neuropeptides* 5:345-348, 1985

147. Blundell JE: Serotonin and Appetite. *Neuropharmacol* 23:1537-1551, 1984

148. McCormack JG, Dean H, Jennings GJ, Blundell JE: Effects of chronic low doses of d-fenfluramine on weight gain and calorie intake, brown adipose tissue thermogenic parameters and brain neurotransmitter content in rats fed chow or palatable diets. *Int J Obesity* 13:625-633, 1989

149. Stanley BG, Anderson KC, Grayson MH, Leibowitz SF: Repeated hypothalamic stimulation with neuropeptide Y increases daily carbohydrate and fat intake and body weight gain in female rats. *Physiology and Behaviour* 46:173-177, 1989

150. O'Shea RD, Gundlach AL: Preproneuropeptide Y messenger ribonucleic acid in the hypothalamic arcuate nucleus is increased by food deprivation or dehydration. *J Neuroendocrinol* 3:11-14, 1991

151. Corder R, Pralong F, Turnill D, Saudan P, Muller AF, Gaillard RC: Dexamethasone treatment increases neuropeptide Y levels in rat hypothalamic neurones. *Life Sci* 43:1879-1886, 1988

152. Sclafani A, Springer D: Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes. *Physiology and Behaviour* 17:461-471, 1976

153. Sahu A, Kalra SP, Crowley WR, Kalra PS: Evidence that NPY-containing neurons in the brainstem project into selected hypothalamic nuclei: implication in feeding behavior. *Brain Res* 457:376-378, 1988

154. Sanacora G, Kershaw M, Finkelstein JA, White JD: Increased hypothalamic content of preproneuropeptide Y messenger ribonucleic acid in genetically obese Zucker rats and its regulation by food deprivation. *Endocrinology* 127:730-737, 1990

155. Beck B, Stricker-Kronrad A, Burlet A, Nicholas JP, Burlet C: Influence of diet composition on food intake and hypothalamic neuropeptide Y (NPY) in the rat. *Neuropeptides* 17(4):197-203, 1991

156. Brady LS, Smith MA, Gold PW, Herkenham M: Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food deprived rats. *Neuroendocrinology* 52:441-447, 1990

157. Renier G, Gaudreau P, Hajjad H, Deslauriers N, Houde-Nadeau M, Brazeau P: Decreased pituitary growth hormone response to growth hormone-releasing factor in cafeteria fed rats: dietary and obesity effects. *Neuroendocrinology* 52:284-290, 1990

158. Glick Z, Yamini S, Lupien J, Sod-Moriah U: Estrous cycle irregularities in overfed rats. *Physiology and Behaviour* 47:307-310, 1990

159. Rettori V, Milenkovic L, Aguila MC, McCann SM: Physiologically significant effect of neuropeptide Y to suppress growth hormone release by stimulating somatostatin discharge. *Endocrinology* 126:2296-2301, 1990

160. Toni R, Jackson IM, Lechan RM: Neuropeptide Y immunoreactive innervation of thyrotrophin releasing hormone synthesising neurons in the rat hypothalmic paraventricular nucleus. *Endocrinology* 126:2444-2453, 1990

161. Crowley WR, Kalra SP: Regulation of luteinizing hormone secretion by neuropeptide Y in rats: hypothalamic and pituitary actions. *Synapse* 2:276-281, 1988

162. Billington CJ, Briggs JE, Grace M, Levine AS: Effects of intracerebroventricular injection of neuropeptide-Y on energy metabolism. *Am J Physiol* 260(2 pt 2):R321-R327, 1991

163. Corrin SE, McCarthy HD, McKibbin PE, Williams G: Unchanged hypothalamic neuropeptide Y concentrations in hyperphagic, hypoglycaemic rats: evidence for specific metabolic regulation of hypothalamic NPY. *Peptides* 12:425-430, 1991

164. Wilding JPH, Gilbey SG, Lambert PD, Ghatei MA, Bloom SR: Increases in neuropeptide Y content and gene expression in the hypothalamus of rats treated with dexamethasone are prevented by insulin. *Neuroendocrinology* 57:581-587, 1993

165. Leibowitz SF, Shor-Posner G: Brain serotonin and eating behavior. *Appetite* 7 Suppl:1-14, 1986

166. Vallejo M, Carter DA, Biswas S, Lightman SL: Neuropeptide Y alters monoamine turnover in the rat brain. *Neurosci Lett* 73:155-160, 1987

167. Rogers P, McKibbin PE, Willaims G: Acute fenfluramine administration reduces neuropeptide Y concentrations in specific hypothalmic regions of the rat: possible implications for the anorectic effect of fenfluramine. *Peptides* 12:251-255, 1991

168. Richards RP, Gordon BH, Ings RMJ, Campbell DB, King LJ: THe measurement of d-fenfluramine and its metabolite, d-norfenfluramine in plasma and urine with an application of the method to pharmacokinetic studies. *Xenobiotica* 19:547-553, 1989

169. Guy J, Pelletier G, Bosler O: Serotonin innervation of neuropeptide Y-containing neurons in the rat arcuate nucleus. *Neuroscience Letters* 85:9-13, 1988

170. Blessing WW, Howe PRC, Joh TH, Oliver JR, Willoughby JO: Distribution of tyrosine hydroxylase and neuropeptide Y-like immunoreactive neurons in rabbit medulla oblongata, with attention to colocalization studies, presumptive adrenaline-synthesizing perikarya, and vagal preganglionic cells. *J Comp Neurol* 248:285-300, 1986

171. Vallejo M, Carter DA, Biswas S, Lightman SL: Neuropeptide Y alters monoamine turnover in the rat brain. *Neuroscience Letters* 73:155-160, 1987

172. Wilding JPH, Gilbey SG, Mannan M, Aslam N, Ghatei MA, Bloom SR: Increased neuropeptide Y content in individual hypothalamic nuclei, but not neuropeptide Y mRNA, in diet induced obesity in rats. *J Endocrinol* 132:299-304, 1992

173. Williams G, Cardoso H, Lee YC, Ghatei MA, Flatt PR, Bailey CJ, Bloom SR: Reduced hypothalamic neurotensin concentrations in the genetically obese diabetic (ob/ob) mouse: possible relationship to obesity.. *Metabolism* 40:1112-1116, 1991

174. McKibbin PE, Cotton SJ, McMillan S, Holloway B, Mayers R, McCarthy D, Williams G: Altered neuropeptide Y concentrations in specific hypothalmic regions of obese (fa/fa) Zucker rats - possible relationship to obesity and neuroendocrine disturbances. *Diabetes* 40:1423-1429, 1991

175. Bray GA, York DA: Hypothalamic and genetic obesity in animals: an autonomic and endocrine hypothesis. *Physiological Reviews* 59:719-809, 1979

176. Schwartz MW, Marks JL, Sipols AJ, Baskin DG, Woods SC, Kahn SE, Porte D: Central

insulin administration reduces neuropeptide-Y messenger RNA expression in the arcuate nucleus of food-deprived lean (FA/FA) but not obese (fa/fa) Zucker rats. *Endocrinology* 128:2645-2647, 1991

177. Flatt PR, Bailey CJ: Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia* 20:573-577, 1981

178. Sahu A, Kalra PS, Crowley WR, Kalra SP: Evidence that hypothalamic neuropeptide Y secretion decreases in aged male rats: implications for reproductive aging. *Endocrinology* 122:2199-2203, 1988

179. Beck B, Burlet A, Nicolas JP, Burlet C: Hypothalamic neuropeptide Y (NPY) in obese Zucker rats: implications in feeding and sexual behaviors. *Physiol Behav* 47:449-453, 1990

180. Beck B, Burlet A, Nicolas JP, Burlet C: Hyperphagia in obesity is associated with a central peptidergic dysregulation in rats. *J Nutr* 120:806-811, 1990

181. Chua SC, Brown AJ, Hennessey KL, Leibel RL, Hirsch J: Food deprivation and hypothalamic gene expression: effects of strain background and diabetes mutation. *Molecular Brain Research* 11:291-299, 1991

182. Kalra SP, Dube MG, Sahu A, Phelps CP: Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. *Proc Natl Acad Sci USA* 88:10931-10935, 1991

183. Kalra SP, Allen LG, Sahu A, Kalra PS, Crowley WR: Gonadal steroids and neuropeptide Y-opioid-LHRH axis: interactions and diversities. *J Steroid Biochem* 30:185-193, 1988

184. Kalra SP, Dube MG, Kalra PS: Continuous Intraventricular infusion of neuropeptide Y evokes episodic food intake in satiated female rats: effects of adrenalectomy and cholecystokinin.. *Peptides* 9:723-728, 1988

185. Bailey CJ, Day C, Bray GA, Lipson LG, Flatt PR: Role of adrenal glands in the development of abnormal glucose and insulin homeostasis in genetically obese (ob/ob) mice. *Horm Metab Res* 18:357-360, 1986

186. Sahu A, Sninsky CA, Kalra PS, Kalra SP: Neuropeptide-Y concentration in microdissected hypothalamic regions and in vitro release from the medial basal hypothalamus-preoptic area of streptozotocin-diabetic rats with and without insulin substitution therapy. *Endocrinology* 126:192-198, 1990

187. Sheppard MC, Bailey CJ, Flatt PR, Swanston-Flatt SK, Shennan KI: Immunoreactive

neurotensin in spontaneous syndromes of obesity and diabetes in mice. *Acta Endocrinol Copenh* 108:532-536, 1985

188. Bailey CJ, Flatt PR: Anorectic effect of fenfluramine, cholecystokinin and neurotensin in genetically obese (ob/ob) mice. *Comp Biochem Physiol A* 84:451-454, 1986

189. Brown MR: Neuropeptides: central nervous system effects on nutrient metabolism. *Diabetologia* 20:299-304, 1981

190. Ferris CF, Pan JX, Singer EA, Boyd ND, Carraway RE, Leeman SE: Stimulation of luteinizing hormone release after stereotaxic microinjection of neurotensin into the medial preoptic area of rats. *Neuroendocrinology* 38:145-151, 1984

191. Wilding JPH, Gilbey SG, Bailey CJ, Batt RAL, Williams G, Ghatei MA, Bloom SR: Increased neuropeptide Y messenger RNA and decreased neurotensin messenger RNA in the hypothalamus of the obese (ob/ob) mouse. *Endocrinology* 132:1939-1944, 1993

192. Schwartz MW, Sipols AJ, Marks JL, Sanacora G, White JD, Scheurink A, Kahn SE, Baskin DG, Woods SC, Figlewicz DP, Porte D: Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology* 130:3608-3616, 1992

193. Porte D, Woods SC: Regulation of food intake and body weight by insulin. *Diabetologia* 20:274-280, 1981

194. White BD, Dean RG, Martin RJ: Adrenalectomy decreases neuropeptide Y mRNA levels in the arcuate nucleus. *Brain Res Bull* 25:711-715, 1990

195. Ponsalle P, Uht R, Srivasta L, White JD: Glucocorticoid regulation of hypothalamic NPY. *THe Endocrine Meeting* 75-(Abstract), 1991

196. Barnea A, Cho G, Hajibeigi A, Aguila MC, Magni P: Dexamethasone-induced accumulation of neuropeptide-Y by aggregating fetal brain cells in culture: a process dependent on the developmental age of the aggregates. *Endocrinology* 129:931-938, 1991

197. Higuchi H, Yang HY, Sabol SL: Rat neuropeptide Y precursor gene expression. mRNA structure, tissue distribution, and regulation by glucocorticoids, cyclic AMP, and phorbol ester. *J Biol Chem* 263:6288-6295, 1988

198. Jhanwar Uniyal M, Beck B, Burlet C, Leibowitz SF: Diurnal rhythm of neuropeptide Y-like immunoreactivity in the suprachiasmatic, arcuate and paraventricular nuclei and other hypothalamic sites. *Brain Res* 536:331-334, 1990

199. Krieger DT, Hauser H: Comparison of synchronization of circadian corticosteroid

rythyms of photoperiod and food. *Proc Natl Acad Sci USA* 75:1577-1581, 1978

200. Stojanovska L, Rosella G, Proietto J: Evolution of dexamethasone-induced insulin resistance in rats. *Am J Physiol* 258:E748-E756, 1990

201. Beck B, Jhanwar Uniyal M, Burlet A, Chapleur Chateau M, Leibowitz SF, Burlet C: Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status. *Brain Res* 528:245-249, 1990

202. Cripps AW, Williams VJ: The effect of pregnancy and lactation on food intake, gastrointestinal anatomy and the absorbtive capacity of the small intestine in the albino rat. *British Journal of Nutrition* 33:17-32, 1975

203. Isler D, Trayhurn P, Lunn PG: Brown adipose tissue metabolism in lactating rats: the effect of litter size. *Ann Nutr Metab* 28:101-109, 1984

204. Lecturque A, Fere P, Satabin P, et al: In vivo insulin resistance during pregnancy in the rat. *Diabetologia* 19:521-528, 1980

205. Koiter TR, Poelstra K, Scheringa M, Van Der Schaaf-Verdonk GCJ, Steffens AB, Schuiling GA: Glucose and insulin responses during mixed meals or infusion of glucose in pregnant and lactating rats. *Physiol Behav* 46:881-887, 1989

206. Burnol AF, Leturque A, Ferre P, Girard J: Glucose metabolism during lactation in the rat: quantitative and regulatory aspects. *Am J Physiol* 245:E351-E358, 1983

207. McLaughlin CL, Baile CL, Peikin SR: Hyperphagia during lactation: satiety response to CCK and growth of pancreas. *Am J Physiol* 244:E61-E65, 1983

208. Wager-Srdar SA, Morley JE, Levine AS: The effect of cholecystokinin, bombesin and calcitonin on food intake in virgin, lactating and postweaning female rats. *Peptides* 7:729-734, 1986

209. Ciofi P, Fallon JH, Croix D, Polak JM, Tramu G: Expression of neuropeptide Y precursor-immunoreactivity in the hypothalamic dopaminergic tubero-infundibular system during lactation in rodents. *Endocrinology* 128:823-834, 1991

210. Parker SL, Crowley WR: Central stimulation of oxytocin release in the lactating rat: interaction of neuropeptide Y with alpha-1-adrenergic mechanisms. *Endocrinology* 132:658-666, 1993

211. Schreihofer DA, Parfitt DB, Cameron JL: Suppression of luteinizing hormone secretion during short-term fasting in male rhesus monkeys: the role of metabolic versus stress signals.

Endocrinology 132:1881-1889, 1993

212. Schreihofer DA, Amico JA, Cameron JL: Reversal of fasting-induced suppression of luteinizing hormone (LH) secretion on male rhesus monkeys by intragastric nutrient infusion: evidence for rapid stimulation of LH by nutritional signals. *Endocrinology* 132:1890-1897, 1993

213. McKibbin PE, McCarthy HD, Shaw P, Williams G: Insulin deficiency is a specific stimulus to hypothalamic neuropeptide Y: a comparison of the effects of insulin replacement and food restriction in streptozocin-diabetic rats. *Peptides* 13:721-727, 1992

214. Mann PE, Pasternak GW, Hahn EF, Curreri G, Lubin E, Bodnar RJ: Comparison of the effects of chronic administration of naloxone and naloxonazine upon food intake and maintenance of body weight in rats. *Neuropharmacology* 27:349-355, 1988

215. Levine AS, Grace M, Billington CJ: The effect of centrally administered naloxone on deprivation and drug induced feeding. *Pharmacol Biochem Behav* 36:409-412, 1990

216. Gosnell BA, Levine AS, Morley JE: The stimulation of food intake by selective agonists of mu, kappa and delta. *Life Sci* 38:1081-1088, 1986

217. Chavkin C: Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* 215:413-415, 1982

218. Molineaux CJ, Feuerstein G, Faden AL, Cox BM: Distribution of immunoreactive dynorphin in discrete brain nuclei; comparison with vasopressin. *Neurosci Lett* 33:179-184, 1982

219. Przewlocki W, Lason W, Konecka AM, Herz A, Reid LD: The opioid peptide dynorphin, circadian rythyms, and starvation. *Science* 219:71-73, 1983

220. Leibowitz SF: Reciprocal hunger regulating circuits involving alpha- and beta-adrenergic receptors located, respectively, in the ventromedial and lateral hypothalamus. *Proc Natl Acad Sci USA* 67:1063-1070, 1970

221. Stanley BG, Schwartz DH, Hernandez L, Hoebel BG, Leibowitz SF: Patterns of extracellular norepinephrine in the paraventricular hypothalamus: relationship to circadian rhythm and deprivation-induced eating behavior. *Life Sci* 45:275-282, 1989

222. Bidart JM, Ozturk M, Bellet DH, Jolivet M, Gras-Masse H, Troalen F, Bohuon CJ, Wands JR: Identification of epitopes associated with hCG and teh beta hCG carboxyl terminus by monoclonal antibodies produced against a synthetic peptide. *J Immunol*

134:457-464, 1985

223. Grouzemann E, Comoy E, Walker P, Burnier M, Bohuon C, Waeber B, Brunner H: Production and characterization of four anti-neuropeptide Y monoclonal antibodies. *Hybridoma* 11:409-424, 1993

224. Leibowitz SF, Xuereb M, Kim T: Blockade of natural and neuropeptide Y-induced carbohydrate feeding by a receptor antagonist PYX-2. *Neuroreport* 3:1023-1026, 1992

225. Levine AS, Grace M, Billington CJ, Portoghese PS: Norbinaltorphamine decreases deprivation and opioid induced feeding. *Brain Res* 534:60-64, 1990

226. Frankish HM, McCarthy HD, Dryden S, Kilpatrick A, Williams G: Neuropeptide Y receptor numbers are reduced in the hypothalamus of streptozotocin-diabetic and food deprived rats: further evidence of increased activity of hypothalamic NPY-containing pathways. *Peptides* 14:941-948, 1993

227. Heinrichs SC, Menghazi F, Merlo Pich E, Hauger RL, Koob GF: Corticotropin-releasing factor in the paraventricular nucleus modulates feeding induced by neuropeptide Y. *Brain Res* 611:18-24, 1993

228. Steffens AB, Scheurink AJW, Luiten PGM, Bohus B: Hypothalamic food intake regulating areas are involved in the homeostasis of blood glucose and plasma FFA levels. *Physiology and Behaviour* 44:581-589, 1988

229. Bartolome JV, Bartolome MB, Harris EB, Pauk JS, Schanberg SM: Regulation of insulin and glucose plasma levels by central nervous system beta-endorphin in preweanling rats. *Endocrinology* 124:2153-2158, 1989

230. Fisher DA, Brown MR: Somatostatin analog: plasma catecholamine suppression mediated by the central nervous system. *Endocrinology* 107:714-718, 1980

231. Kuenzel WJ, McMurtry J: Neuropeptide Y: brain localization and central effects on plasma insulin levels in chicks. *Physiol Behav* 44:669-678, 1988

232. Dunbar JC, Ergene E, Barraco RA: Neuropeptide Y stimulation of insulin secretion is mediated via the nucleus tractus solitarius. *Horm Metab Res* 24:103-105, 1992

233. Walker HC, Romsos DR: Similar effects of NPY on energy metabolism and on plasma insulin in adrenalectomized ob/ob and lean mice. *Am J Physiol* 264:E226-E230, 1993

234. Kruszynska YT, McCormack JG: Effect of nutritional status on insulin sensitivity in vivo and tissue enzyme activities in the rat. *Biochem J* 258:699-707, 1989

235. Somogyi MJ: Determination of blood sugar. *J Biol Chem* 160:69-73, 1945

236. Steele R: Influences of glucsoe loading and of injected insulin on hepatic glucose output. *Annals of the New York Academy of Sciences* 82:420-430, 1959

237. Wolfe RR *Tracers in metabolic research: radioisotope and stable isotope spectometry methods*. Alan R Liss inc., New York.1984

238. Bloom SRLong RG *Radioimmunoassay of gut regulatory peptides*. Saunders, London.1982

239. Marks J & Davies L Effects of icv neuropeptide Y on serum insulin and glucose suggest a possible modulation of peripheral insulin sensitivity. (Abstract) *Endocrine Society of America Absr*, 75:5151993

240. Zarjevski N, Cusin I, Rohner-Jeanrenaud F, Jeanrenaud B: Chronic intracerebroventricular neuropeptide Y administration to normal rats mimics hormonal and metabolic changes of obesity. *Endocrinology* 133:1753-1758, 1993

241. Chen X, Henderson K, Beinfeld MC, Westfall TC: Alterations in blood pressure of normotensive and hypertensive rats following intrathecal injections of neuropeptide Y. *J Cardiovasc Pharmacol* 12:473-478, 1988

242. Martin JR, Beinfeld MC, Westfall TC: Blood pressure increases after injection of neuropeptide Y into posterior hypothalamic nucleus. *Am J Physiol* 254:H879-H888, 1988

243. Baron AD, Laakso M, Brechtel G, Edelman SV: Mechanism of insulin resistance in insulin-dependent diabetes mellitus: a major role for reduced skeletal muscle blood flow. *Journal of Clinical Endocrinology & Metabolism* 73:637-643, 1991

244. Scheidegger K, Robbins DC, Danforth EJ: Effects of chronic beta receptor stimulation on glucose metabolsim. *Diabetes* 33:1144-1149, 1984

245. Iguchi A, Matsunaga H, Gotoh M, Nomura T, Yatomi A, Sakamoto N: Central hyperglycaemic effect of adrenaline and carbachol. *Acta Endocrinol Copenh* 109:440-445, 1985

246. Lewis DE, Shellard L, Koeslag DG, Boer DE, McCarthy D, McKibbin PE, Russell JC, Williams G: Intense exersise and food restriction cause similar hypothalamic neuropeptide Y increases in rats. *Am J Physiol* 264:E279-E284, 1993

247. Lambert PD, Wilding JPH, Gilbey SG, Ghatei MA, Bloom SR: Microdialysis sampling from the paraventricular nucleus of the hypothalamus reveals increased neuropeptide Y

release in response to diabetes and food deprivation. *Diabetic Medicine* Abstract:1993

248. Kalra SP, Fuentes M, Fournier A, Parker SL, Crowley WR: Involvement of the Y-1 receptor subtype in the regulation of luteinizing hormone secretion by neuropeptide Y in rats. *Endocrinology* 130:3323-3330, 1992

249. Rothwell NJ, Saville ME, Stock MJ: The effects of feeding a "cafeteria" diet on energy balance and diet-induced thermogenesis in four strains of rat.. *J Nutr* 112:1515-1524, 1982

Publications Arising From This Work

Papers

1. Wilding, J.P.H., Gilbey, S.G., Mannan, M.M., Aslam, N., Ghatei, M.A., Bloom, S.R. Increased neuropeptide Y content in individual hypothalmic nuclei, but not hypothalamic NPY messenger RNA in diet induced obesity in rats. Journal of Endocrinology (1992) 132 299-304

2. Wilding, J.P.H., Gilbey, S.G.,Jones,P.M., Mannan, M.M., Aslam, N., Ghatei, M.A., Bloom, S.R. Dexfenfluramine treatment and hypothalamic NPY in diet induced obesity in rats. Peptides 1992 13 557-563

3. Wilding, J.P.H., Gilbey, S.G., Bailey, C.J., Batt, R.A.L., Williams, G., Ghatei, M.A. and Bloom, S.R. Increased neuropeptide Y messenger RNA and decreased neurotensin messenger RNA in the hypothalamus of the obese (ob/ob) mouse. Endocrinology 1993 132 1939-1944

4. Lambert, P.D., Wilding, J.P.H., Bohuon,C., Comoy, E.,Gilbey,S.G.,and Bloom, S.R. A role for neuropeptide-Y, dynorphin, and nor-adrenaline in the central control of food intake after food deprivation Endocrinology 1993 133 29-324.

5. Wilding, J.P.H. , Gilbey, S.G., Lambert, P.D., Ghatei, M.A. and Bloom, S.R. Increases in neuropeptide Y content and gene expression in the hypothalamus of rats treated with dexamethasone are prevented by insulin. Neuroendocrinology 1993 57 581-587

6. Lambert, P.D., Wilding, J.P.H., Al-Dokhayel, A.A.M., Gilbey, S.G. and Bloom S.R. The effect of central blockade of kappa-opioid and alpha-adrenergic receptors on neuropeptide Y-induced in the rat. Brain Research 1993 629 146-148

7. Wilding, J.P.H., Lambert ,P.D., Al-Dokhayel,A.A.M., Ghatei,M.A. and Bloom , S.R. Effect of peripheral naloxone treatment on regional hypothalamic NPY concentrations and hypothalamic NPY messenger RNA Peptides (in press)

Manuscript submitted for publication

1. Wilding, J.P.H., Kruszynska, Y.T., Lambert, P.D., Bloom, S.R. Effects of central neuropeptide Y injection on peripheral insulin sensitivity and glucose metabolism in fasted rats (Diabetes)